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Human galectin-2: expression profiling by RT-PCR/immunohistochemistry and its introduction as a histochemical tool for ligand localization

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Summary. Sugar-encoded information of glycoconjugates is translated into cellular responses by endogenous lectins. Galectins stand out against other lectin families due to their wide range of functions including cell adhesion, tissue invasion or growth regulation exerted at extracellular, membrane, cytoplasmic and nuclear sites. This remarkable versatility warrants close scrutiny of their emerging network, in this study with focus on homodimeric human galectin-2. We first detected presence of specific mRNA in various tissue types by processing *post mortem* and surgical specimens by RT-PCR protocols. Overlap of gene expression was noted with proto-type galectins-1 and -7 and also family members from the other two subgroups. To monitor expression on the level of protein a polyclonal anti-galectin-2 antibody was raised. Immunopositivity was semi-quantitatively assessed in sections of 209 human samples establishing an array both of normal tissues and samples with inflammation or benign/malignant growth. In general, positivity was predominantly epithelial without restriction of staining to certain tissue types, as fittingly indicated by our RT-PCR analysis. Staining was not limited to the cytoplasm but also included nuclear sites. To examine the suitability of the labeled lectin as a histochemical probe we biotinylated galectin-2 under activity-preserving conditions and introduced it to tissue profiling. Specific cytoplasmic staining proved the validity of the concept. Our results encourage systematic histopathologic studies by immuno- and lectin histochemistry, especially by adding galectin-2 as study object to galectin fingerprinting which has already yielded prognostic information on galectins-1, -3, -4 and -8 and hereby contributed to define functional overlap/divergence in this lectin family.

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Introduction

Lectin histochemistry is a popular approach for detecting changes in glycosylation (glycomic profile) (Caselitz, 1987; Spicer and Schulte, 1992; Danguy et al., 1994; Brockhausen et al., 1998). The results of such studies intimate an inherent functionality of carbohydrate epitopes. This notion is flanked by the following recent insights regarding glycosylation: a) highest frequency of occurrence among known post-translational protein modifications; b) intriguing structural complexity of the chains; c) occurrence of embryonic defects and diseases caused by deregulated glycosylation; d) unsurpassed coding capacity in oligomers, making it possible to endow structural units of minimal size with high-density informational properties; and e) sophisticated enzymatic machinery for glycan assembly, substitution and remodeling (Brockhausen and Schachter, 1997; Laine, 1997; Pavelka, 1997; Sharon and Lis, 1997; Reuter and Gabius, 1999; Gabius et al., 2002, 2004; Spiro, 2002; Haltiwanger and Lowe, 2004; Hirabayashi, 2004; Nomura, 2004).

The implied concept of the sugar code calls for availability of receptors, which translate the carbohydrate signals into biochemical effects. Indeed, different families of mammalian lectins are present to complete a functional protein-carbohydrate interaction system (Gabius, 1997a, 2000; Kaltner and Stierstorfer, 1998). The thermodynamics of the binding process benefits from two remarkably favorable factors of oligosaccharides as ligands: a) their limited flexibility; and b) the key-like selection of energetically preferred conformations by lectins (Gabius, 1998, 2001; Gabius et al., 2004). When predicting likely sites for binding of

glycan chains, the spatially accessible determinants at branch ends should be primary contact points. Because spatial accessibility is also a prerequisite for introducing substitutions by glycosyltransferases, terminal ß-galactosides have attained a central role as core for the generation of a wide panel of signals. Matching the complexity of structural glycan modifications at these sites, diverse members of different lectin families such as galectins, siglecs and C-type lectins can read the distinct sugar-encoded messages. Fittingly, structural modifications at the branch ends of a glycan can modulate the meaning of the message. For example, α2,6-sialylation can act as switch-off or switch-on signal (Gabius, 1997a; Angata and Brinkman-Van der Linden, 2002; Crocker, 2004). This interrelationship between glycan synthesis/remodeling and lectin binding signifies that changes in the glycomic profile can have the functional meaning implied above.

To add further support to this notion the approach to extend the current status of analysis of expression of endogenous lectins is imperative. Like enzymes in glycan assembly/remodeling lectin families are diversified. They often form a group of homologous members, as documented for galectins by data base mining and cloning studies (Cooper, 2002; Lahm et al., 2004). Once this intrafamily diversification had been unveiled its presence immediately raised questions about the functions of each protein and extent of functional overlap/divergence in comparison to the related lectins. Following the detection of a lectin gene the monitoring of its profile of tissue expression at the levels of the mRNA and protein is a salient step en route to defining its physiological relevance. We herein address this issue for human galectin-2, a homodimeric (proto-type) ß-galactoside-binding protein.

The existence of this galectin had first been noted by screening a human HepG2 hepatoma cDNA library, where the relative abundance of its message reached only 0.00003 % of all clones (Gitt and Barondes, 1986, 1991; Gitt et al., 1992). Due to its high level of sequence identity to galectin-1 at 43%, its homodimeric quaternary structure in solution and also its overall similarity in folding and binding-site architecture to human galectin-1, its initial designation as galectin-2 is still appropriate (Gitt et al., 1992; He et al., 2003; Lobsanov et al., 1993; López-Lucendo et al., 2004). Regarding cell and tissue expression RT-PCR analysis of human tumor lines detected the presence of galectin-2-specific mRNA in five of 21 colon cancers but not in the ten tested lung and the nine tested breast carcinoma lines (Lahm et al., 2001). Due to the enormous sensitivity of this method, which can even detect minimal amounts of message down to the level of so-called illegitimate expression (Solmi et al., 2004), tissue profiling should advisably include immunohistochemistry. This type of monitoring revealed a restriction to gastrointestinal expression with presence in epithelial cells lining the gastric luminal surface in rat after obtaining galectin-2 cDNA from clone S147 of a rat stomach library (Oka et al., 1999). On the biochemical level, rat galectin-2 was analyzed for monovalent sugar binding by frontal affinity chromatography. Within the panel of 41 pyridylaminated oligosaccharides the comparison to human galectin-1 singled out several differences (Hirabayashi et al., 2002). When directly comparing human galectins-1 and -2, they shared binding to the glycoprotein $ß_1$-integrin and to ganglioside GM_1 on blots and cell surfaces (Sturm et al., 2004; André et al., 2005a). Significant disparity between these two prototype galectins showed up when activated T cells were tested: CD3 and CD7 were only reactive with galectin-1, caspase involvement in apoptosis onset was different and only galectin-1 reduced the percentage of cyclin-$ß_1$-positive cells (Sturm et al., 2004). Both lectins were detected in cell extracts of the non-transformed intestinal epithelial line IEC-6, whereas galectin-2 was absent in stimulated T cells (Sturm et al., 2004). Whether and how the differences in the proximal promoter regions of human genes for galectins-1 and -2 are reflected on the level of the expression pattern has yet to be clarified (Gitt et al., 1992; Sturm et al., 2004).

When listing the current status of knowledge on ligands for galectin-2, it is pertinent to add its reactivity with peptide motifs in proteins, a feature also known from galectins-1 and -3 (Liu et al., 2002; Arnusch et al., 2004; Liu, 2004; André et al., 2005b). By searching candidate genes for susceptibility to myocardial infarction, two lymphotoxin-α gene polymorphisms were described, a result which is in line with an effect of this cytokine on the size of atherosclerotic lesions in animal studies (Ozaki et al., 2002; Schreyer et al., 2002). Two-hybrid screening and immunocytochemistry inferred the interaction of galectin-2, present in macrophages and smooth muscle cells, with lymphotoxin-α and also α- and ß-tubulins, implicating the lectin in regulating cytokine secretion relevant for the pathogenesis of myocardial infarction (Ozaki et al., 2004). This connection to clinically relevant (glyco)proteins as galectin-2 ligands highlights the potential to consider tissue lectins themselves as promising probes for the histochemical detection of functional markers. In fact, galectins-1 and -3 have already proven their value as histochemical reagents in differential diagnosis and prognostic evaluations of tumors (André et al., 1999; François et al., 1999; Schwarz et al., 1999; Delorge et al., 2000; Kayser et al., 2003; Hancq et al., 2004; Plzák et al., 2004). Likewise, the detection of these two lectins in tissues had been the starting point for immunohistochemical galectin fingerprinting (Gabius et al., 1986a; Gabius, 1997b; Camby et al., 2001; Sheikholeslam-Zadeh et al., 2001; Danguy et al., 2002; Wollina et al., 2002; Nagy et al., 2003; Lefranc et al., 2005; Saussez et al., 2005). To pursue the attractive perspective of adding detection of galectin-2 and its binding sites to this monitoring we thus initiated systematic profiling of galectin-2 expression first by RT-PCR using tissue samples and then by immunohistochemistry. The following questions

were addressed: a) what is the expression profile measured by RT-PCR analysis in different organs and in comparison to other human galectins?; b) what is the expression profile measured by immunohistochemistry in human tissues when analyzing a total of 209 individual specimens?; c) are there differences between disease-free tissue samples and inflammatory/malignant specimens?; d) is ligand detection *in situ* possible with labeled galectin-2?

Materials and methods

RT-PCR detection of mRNA for galectins

Post mortem tissue samples were obtained by routine autopsy (12 h maximum), shock-frozen in liquid nitrogen and stored at -80°C. Pieces of 20-30 mg weight were cut from each specimen on ice with a scalpel and extracted using the RNeasy RNA extraction kit (Qiagen, Hilden, Germany) according to the protocol supplied by the manufacturer. RNA quality and quantity were assessed spectrophotometrically and by agarose gel electrophoresis combined with ethidium bromide staining, respectively. Only preparations which showed sufficient yields and no significant degradation of the prominent rRNA bands were used for the following steps. Samples with about 5 µg total RNA per aliquot were brought to a total volume of 17 µl with diethyl-pyrocarbonate (Sigma-Aldrich, Steinheim, Germany)-treated water in a 200 µl reaction tube, to which 2 µl of 10x DNase buffer (10 mM Tris/HCl, pH 7.4; 10 mM $MgCl_2$) and 1 µl of DNase I-containing solution (10 U/µl; Roche Diagnostics, Mannheim, Germany) had been added. DNase-mediated digestion was performed for 30 min at 37°C in a thermocycler (master cycler gradient; Eppendorf, Hamburg, Germany), followed by abolishing the DNase activity at 75°C for 10 min. Reverse transcription of the purified samples was performed using the SuperScript reverse transcription system from Invitrogen (Karlsruhe, Germany) according to the protocol supplied by the manufacturer. Quality and quantity of the resulting cDNA samples were monitored by amplifying the sequence for ß-actin (using the primer pair ß-actin sense: 5'-GGCATCGTGAT GGACTCCG-3' and ß-actin antisense: 5'-GCTGGA AGGTGGACAGCGA-3', resulting in a predicted size of 613 bp for the amplification product) via a standard PCR protocol: 20 µl total reaction volume containing 1 µl of sample, 1 µl of each primer-containing solution (diluted to 10 µM), 2 µl of 10x reaction buffer (Qiagen, Hilden, Germany), 4 µl 5x commercial Q-solution (Qiagen, Hilden, Germany), 0.4 µl of 10 mM dNTP mix, 0.1 µl of Taq-DNA-polymerase-containing solution (5 U/µl; Qiagen, Hilden, Germany) and 10.5 µl sterile water; initial denaturation of the samples was performed for 4 min at 94°C, followed by 29 repeats of a cycle including a 1 min denaturation step at 94°C, a 1 min primer annealing step at 55°C and a 1 min primer elongation step at 72°C. A final elongation step was carried out for 10 min at 72°C. The resulting PCR products were examined by agarose gel electrophoresis and ethidium bromide staining. cDNA samples that yielded an appropriate PCR product for ß-actin were further used for detection of cDNAs for galectins. RT-PCR experiments for this purpose were again performed in 20 µl reaction aliquots, containing the same amounts of components as in the case of the ß-actin test. Used primer pairs were: Gal-1 sense: 5'-AACCTGGAGAG TGCCTTCGA-3', Gal-1 antisense: 5'-GTAGTTGATGG CCTCCAGGT-3' (predicted size for Gal-1 amplification product: 323 bp), Gal-2 sense: 5'-ATGACGGGGGA ACTTGAGGTT-3', Gal-2 antisense: 5'-TTACGCTCAG GTAGCTCAGGT-3' (predicted size for Gal-2 amplification product: 358 bp), Gal-3 sense: 5'-GTGCCTCGCATGCTGATAAC-3', Gal-3 antisense: 5'-CATTGAGTTTTTTAACCCGATGATTG-3' (predicted size for Gal-3 amplification product: 309 bp), Gal-4 sense: 5'-TGGTAAATGGAAATCCCTTCTATG-3', Gal-4 antisense: 5'-GAGCTGTGAGCCCTCCTT-3' (predicted size for Gal-4 amplification product: 270 bp), Gal-7 sense: 5'-ATGTCCAACGTCCCCCACA-3', Gal-7 antisense: 5'-TGACGCGATGATGAGCACC-3' (predicted size for Gal-7 amplification product: 282 bp), Gal-8 sense: 5'-TCTGGGCATTTATGGCAAAGTG-3', Gal-8 antisense: 5'-CATGGGGGTGTTCAACCTTG-3' (predicted size for Gal-8 amplification products: 175 bp or 301 bp, respectively, for the two isoforms) and Gal-9 sense: 5'-ACCTCTGCTTCCTGGTGCA-3', Gal-9 antisense: 5'-GCACTGTGTGGATGACTGTC-3' (predicted size for Gal-9 amplification products: 207 bp or 303 bp, respectively, for the two isoforms). Primer design was guided to include intron sequences, thereby minimizing interference by residual genomic DNA, and to reach similar melting temperatures, as described (Lahm et al., 2001, 2003). The standard PCR protocol used for all galectin primer pairs differed from that for the ß-actin-specific PCR by raising the annealing temperature to 57°C and repeating the amplifying cycle for 35 times. PCR products were routinely analyzed using agarose gel electrophoresis and ethidium bromide staining. These profiles and the intensity of the bands were independently evaluated semiquantitatively by three investigators using a scoring system given in detail in the footnotes of the respective tables.

Specimens of human tissues obtained by surgical resection were immediately frozen in liquid nitrogen and stored at -80°C. A total of 30 human specimens were examined: six cases of stomach tissue (three normal cases and three adenocarcinomas), six cases of colon tissue (three normal cases and three adenocarcinomas), nine cases of lung tissue (three normal cases, three adenocarcinomas and three epidermoid carcinomas), six cases of thyroid tissue (three normal cases and three papillary carcinomas), six cases of kidney tissue (three normal cases and three Gravitz' tumors) and six cases of bladder tissue (three normal cases and three urothelial carcinomas). For the following processing we employed a modified protocol: Total RNA was prepared with the

Tripure Isolation Reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's technical recommendations. RNA quality was assessed by analysis with the Agilent 2100 bioanalyzer. The first-strand cDNA synthesis used the SuperScript reverse transcriptase enzyme (NV Invitrogen SA, Merelbeke, Belgium). First, 1 µl RNA-containing solution (1 µg/µl) was mixed with 1 µl oligo-$dT_{(12-18)}$ primers (0.5 µg/µl) and water to a volume of 12 µl and then heated at 70°C for 10 minutes to disrupt any secondary structures in the RNA. In the following step, 1 µl enzyme-containing solution (200 U/µl), 1 µl dNTPs mix (10 mM each), 0.2 µl RNase-inhibitor-containing solution (40 U/µl), 2 µl DTT (0.1 M) and 4 µl first-strand buffer (5x concentrated) were added to yield a total reaction volume of 20 µl. The reverse transcriptase reaction was performed by incubation at 42°C for 50 minutes, followed by the inactivation step at 70°C for 15 minutes after which the solution was quickly chilled on ice. Three separate samples of cDNA were prepared for each RNA preparation. They were mixed, purified on a column with the High Pure PCR Fragment Purification Kit (Roche Diagnostics, Mannheim, Germany), and the cDNA was finally eluted in a total volume of 50 µl elution buffer. The concentration of each cDNA was measured in a spectrophotometer and adjusted to a final concentration of 10 ng/µl. These cDNA preparations could then be used as a template for amplification in PCR. cDNA integrity was checked by ß-actin-specific PCR analysis. The PCR reactions were performed in a total volume of 25 µl containing 0.125 µl Taq DNA polymerase (NV Invitrogen SA, Merelbeke, Belgium; 5 U/µl), 1 µl dNTPs mix (10 mM each), 2.5 µl PCR buffer (10x concentrated), 1.25 µl of each primer-containing solution (10 µM) and a variable concentration of $MgCl_2$ depending on the type of cDNA to be amplified. The mix comprised 2 µl cDNA-containing solution (= 20 ng) and water. The reaction tubes were incubated in a thermal cycler at 95°C for 10 minutes to completely denature the template. Forty cycles of PCR amplification (25 cycles for ß-actin) were performed as follows: denaturation at 95°C for 45 seconds, annealing at a variable temperature (see below) for 45 seconds and primer extension at 72°C for 45 seconds (1 minute and 15 seconds for ß-actin-specific cDNA). The tubes were incubated for an additional 10 minutes at 72°C, and the reaction mixture was rapidly chilled at 4°C thereafter. Sequences and distinct reaction conditions of each primer pair used and the calculated lengths of the expected products are as follows: ß-actin sense: 5'-AAATCGTGCGTGACATTAAGG-3', ß-actin antisense: 5'-CTAAGTCATAGTCCGCCTAG-3' (3 mM $MgCl_2$ in the PCR reaction, annealing temperature 62°C, predicted size for the amplification product: 525 bp), Gal-1 sense: 5'-CCATCGTGTGCAACAGC-3', Gal-1 antisense: 5'-GCTTGACGGTCAGGTTG-3' (2 mM $MgCl_2$ in the PCR reaction, annealing temperature 60°C, predicted size for the amplification product: 129 bp), Gal-2 sense: 5'-GAACTTGAGGTTAAGAACATGG-3', Gal-2 antisense: 5'-GGTGATCTTCCCGTTGT-3' (5 mM $MgCl_2$ in the PCR reaction, annealing temperature 61°C, predicted size for the amplification product: 211 bp) and Gal-7 sense: 5'-ATGTCCAACGTCCCCCACAAG-3', Gal-7 antisense: 5'-TGACGCGATGATGAGCACCT-3' (1.5 mM $MgCl_2$ in the PCR reaction, annealing temperature 60°C, predicted size for the amplification product: 282 bp). The amplification products were routinely analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Semiquantitative evaluation was carried out as described above. A cDNA preparation obtained from a colon tumor sample was routinely used as positive control.

Antibody production, processing and quality controls

Polyclonal antibodies against human galectin-2 were raised in a rabbit as described previously (Sturm et al., 2004), and the immunglobulin G (IgG) fraction was purified from the resulting serum by protein A-Sepharose 4B (Amersham Biosciences, Freiburg i. Br., Germany) chromatography as described (Kaltner et al., 1997). Quality controls to probe for cross-reactivity to other members of the galectin family, i.e. human galectins-1, -3, -4, -7 and -8, which had been isolated after recombinant expression by an optimized protocol using fractionation over lactosylated Sepharose 4B as key step (Gabius, 1990; André et al., 2001; Nagy et al., 2002; Wu et al., 2002; Kopitz et al., 2003), were performed separately by Western blotting and ELISA assays (Gabius et al., 1986b; Kaltner et al., 2002; Nagy et al., 2003; Manning et al., 2004). Affinity depletion of the IgG fraction was performed using Sepharose 4B-immobilized galectins-1 and -3, which had been coupled separately to the resin after its activation by divinyl sulfone, at a density of 5-7 mg/ml resin (Gabius et al., 1991). Further quality controls included probing for proper antigen detection in cell and tissue extracts. For this purpose, extracts of cells of the colon adenocarcinoma line HCT-15 and its transfectants engineered to express galectin-2, which were produced using the pcDNA™3.1 vector (Invitrogen, Karlsruhe, Germany) as a genetic platform to insert the galectin-2 cDNA as described previously (André et al., 2004a), were processed as a test model. In detail, cells were washed off from the surface of the culture flask with phosphate-buffered saline (PBS), pH 7.2, containing 2 mM EDTA, then washed twice in PBS to remove serum constituents and traces of EDTA, suspended in lysis buffer (20 mM PBS pH 7.2, 2 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 50 mM lactose, 2 mM dithiothreitol, 0.3 µM aprotinin, 12 µM leupeptin and 1 mM pefabloc reagent) and lyzed by sonication for 20 sec on ice. After centrifugation (5 min at 14,000 rpm, 4°C) the protein content of the supernatant was determined by a modified Bradford assay (BioRad, München, Germany), and the samples were stored at -20°C prior to analysis.

Proteins were then separated using discontinuous

SDS polyacrylamide gel electrophoresis (4% polyacrylamide in the stacking gel, 15 % polyacrylamide in the running gel) and blotted onto a nitrocellulose membrane (0.2 µm pore size; Schleicher & Schuell, Dassel, Germany) using continuous tank blotting (Bio-Rad, München, Germany). The membrane was briefly dried and then treated with Ponceau S (Sigma-Aldrich, Steinheim, Germany) staining solution (0.1% in 1% acetic acid) to check extent of electrophoretic transfer. Positions of molecular weight markers and of protein-containing lanes were marked with a ball pen. Protein-binding sites on the membrane were then blocked by incubation for 1 h in a 5% solution of milk powder in TBS/Tween buffer (50 mM Tris/HCl, pH 7.5, containing 150 mM NaCl and 0.05% Tween-20) at room temperature. Following saturating the protein-binding capacity the membranes were incubated overnight in a 1 µg/ml antibody-containing solution in blocking buffer at 4 °C. After three 10 min washing steps to remove the first-step antibodies from the solution in TBS/Tween buffer, the procedure for IgG visualization was started by an incubation step with a 0.5 µg/ml solution of horseradish-peroxidase-labeled goat anti-rabbit IgG (Sigma-Aldrich, Steinheim, Germany) in blocking buffer for 2 h at room temperature. Again, the membrane was then washed three times for 10 min in TBS/Tween buffer to remove the second-step reagent and immediately subjected to enhanced chemiluminescence detection by using reagents and protocol of the commercial supplier (Amersham Biosciences, Freiburg i. Br., Germany). Signal detection was performed by covering the membrane with cellophane and exposing it to an X-ray film (Hyperfilm ECL, Amersham Life Science, Little Chalfont, UK) for 5 min. Films were developed and fixed using standard Kodak reagents (developer D-19 and fixer from Sigma-Aldrich, Steinheim, Germany).

Immuno- and lectin histochemical detection of galectin-2 and its ligands in tissue sections

The studied panel consisted of 209 cases which had entered the files at the Laboratory of Pathology of the Erasmus Hospital (Brussels, Belgium) between 1992 and 2003. It was chosen in order to establish a representative sampling of tissue types and pathologies after thorough histological case-by-case examination based on hematoxylin-eosin-stained slides. This series contained 73% surgical specimens (n =153) and 27% routine biopsy specimens (n=56) covering a wide panel of normal (n=57), inflammatory (n=41), benign (n=24) and malignant human tissue types (n=87), as listed in Table 1. When possible, tissue specimens of normal, inflammatory, benign and malignant origin for each tissue type were processed (including two to four cases of each origin) and analyzed in parallel for each condition (see Table 1 for details). In addition, 18 cases were also analyzed regarding their galectin-2-binding sites. The galectin was biotinylated for this purpose under activity-preserving conditions (Sturm et al., 2004) and checked for extent of labeling and lack of a negative influence by labeling on quaternary structure and activity using hemagglutination, cell staining and solid-phase assays as described (André et al., 2003, 2004b, 2005c; Purkrábková et al., 2003). Case selection was performed considering the immunohistochemical results, and the resulting panel consisted of nine cases of normal and malignant tissue, respectively: three specimens each of normal biliary tract, normal colon, normal bladder, and of the corresponding malignancies, i.e. cholangiocarcinomas, colon adenocarcinomas and urothelial bladder tumors. Galectin- and immunohistochemical processing followed a routine protocol, as described previously (Nagy et al., 2000, 2003). In detail, clinical samples were fixed for 24 hours in buffered formalin (4%), dehydrated and embedded in paraffin wax. Histochemical analysis was performed on 5-µm-thick sections from each clinical tissue block using a commercialized automated system (Mark 5, DPC) with an improved standardization protocol. Antigen presence was visualized by streptavidin-biotin-peroxidase complex kit reagents (Immunologic Laboratories, AD Duiven, The Netherlands) with diaminobenzidine/hydrogen peroxide (Biogenex Laboratories, San Ramon, CA) as chromogenic substrates. To ascertain antigen/lectin-ligand-specific staining control tissue sections were processed without the incubation step with the first-step antibody or the biotinylated probe to exclude antigen/lectin-independent staining, e.g. by binding of kit reagents such as the sugar chains of peroxidase to mannose-binding sites (Kuchler et al., 1990). The use of a control serum instead of the antibody fraction excluded staining by non-specific mechanisms such as Fc-dependent binding. Counterstaining with hematoxylin concluded the processing. The antibody and the biotinylated probe were used at 1:100 and 1:2000 dilutions, respectively. A semi-quantitative evaluation of the intensity of histochemical staining was carried out. Both epithelial and stromal compartments were analyzed including a detailed monitoring within the epithelial compartment of distinct cellular subgroups characteristic of a tissue type. For each case under study the entire slide area was examined. To avoid intra-observer bias, two independent observers evaluated the sections in parallel.

The semi-quantitative staining assessment for the epithelial compartment included scoring of the following parameters: a) staining intensity with cytoplasmic intensity divided into three subgroups as negative (0), weak to medium (1) and strong (2); b) staining pattern characterized by the presence of a focal or diffuse cytoplasmic staining pattern and scored as 0 for the negative cases, 1 for a focal distribution and 2 for a diffuse cytoplasmic pattern; c) subcellular staining distribution divided into four groups as negative (0), completely cytoplasmic (C), mixed nuclear and cytoplasmic (N+C) and completely nuclear (N) staining. We also added the category of the rarely seen subcellular staining pattern which underlined the cellular membrane

Table 1. Summary of the panel of normal, inflammatory, benign and malignant human tissue types evaluated immunohistochemically for galectin-2 presence.[a]

TISSUE TYPE	NORMAL No. OF CASES ANALYZED	INFLAMMATORY TISSUE TYPE	No. OF CASES ANALYZED	BENIGN TISSUE TYPE	No. OF CASES ANALYZED	MALIGNANT TISSUE TYPE	TOTAL No. OF CASES ANALYZED	NUMBER OF CASES FOR ANALYZED FOR EACH TISSUE TYPE
Epithelial origin								
Endocrine system								
Pituitary gland	-	-	-	Adenoma	3	-	-	3
Parathyroid gland	-	-	-	Adenoma	3	-	-	3
Thyroid gland	-	Thyroiditis	3	Multinodular goiter	3	Papillary carcinoma	3	
				Microfollicular adenoma	3	Follicular carcinoma	3	
						Anaplastic carcinoma	3	18
Pulmonary system								
Lung	3	Interstitial pneumopathies	3	-	-	Adenocarcinoma	3	
						Epidermoid carcinoma	3	
						Large cell carcinoma	3	15
Pleura	3	-	-	-	-	Mesothelioma	3	6
Digestive tract								
Salivary gland (mixed)	3	Sialadenitis	3	Pleomorphic adenoma	3	-	-	9
Gut								
Esophagus	-	Intestinal metaplasia	3	-	-	Barrett with dysplasia	3	
						Adenocarcinoma	3	9
Fundus	3	Gastritis	3	-	-	Adenocarcinoma	3	9
Antrum	3	-	-	-	-	-	-	3
Duodenum	3	Non-specific duodenitis	3	-	-	Carcinoma of ampulla of Vater	3	
		Coeliac sprue	2					11
Ileum	3	Crohn's ileitis	3	-	-	-	-	6
Colon	3	Crohn's colitis	3	-	-	Dysplastic adenoma	3	
						Adenocarcinoma	3	12
Liver	3	Hepatic rejections	3	-	-	Hepatocarcinoma	2	
						Hepatoblastoma	3	11
Biliary tract	3	Primary biliary cirrhosis	3	-	-	Cholangio-carcinoma	2	8
Pancreas	3	Chronic pancreatitis	3	-	-	Adenocarcinoma	3	9
Urogenital system (incl. Breast)								
Kidney	4	-	-	-	-	Grawitz tumors	3	7
Bladder	3	-	-	-	-	Urothelial carcinoma	3	6
Ovary	2	-	-	-	-	Serous adenocarcinoma	2	4
Prostate	-	Prostatitis	3	Hyperplasias	3	Prostatic intraepithelial neoplasia	3	
						Adenocarcinoma	3	12
Testis	3	-	-	-	-	Seminoma	3	6
Breast	3	-	-	Fibroadenoma	3	Canalicular adenocarcinoma	3	
						Lobular adenocarcinoma	3	12
Skin								
	3	Lichen planus	3	Melanocytic: compound naevi	3	Melanocytic: melanoma	3	
						non-melanocytic: Spinocellular carcinoma	3	
						Basocellular carcinoma	3	18
Non-epithelial origin								
Brain	3	-	-	-	-	glial tumors:		
						Astrocytoma grade I	1	
						Astrocytoma grade II	1	
						Astrocytoma grade III	1	
						Glioblastoma	1	7
Lymph Node	3	-	-	-	-	Lymphoma:		
						Hodgkin's lymphoma	1	
						Follicular lymphoma	1	5
TOTAL CASES	57		41		24		87	209

[a]: the studied cases were invariably positive (for further details on staining characteristics, see Table 4 and Table 5).

(cm). In the case of the stromal compartment we similarly divided the staining intensity into three subgroups as negative (0), weak to medium (1) and strong (2), and the focal or diffuse distribution of the staining pattern was characterized as 0 for negative cases, 1 for a focal cytoplasmic distribution and 2 for a diffuse cytoplasmic distribution. This scoring system is used in Tables 4-6. It should be noted that all these parameters were considered as significant only if at least 10% of the tissue presented them.

Results

Tissue profiling by RT-PCR

In our first set of experiments to start answering the questions given at the end of the introduction we prepared RNA from a panel of tissue types from three patients *post mortem*. Further processing including reverse transcription proceeded only with fractions which passed the quality controls by spectrophotometry and gel electrophoresis. Furthermore, we used the signal for the cDNA of the house-keeping gene ß-actin as internal quality and loading control. Primer design exploited the presence of introns in galectin genes so as to minimize the risk of amplifying any residual genomic DNA in RNA preparations. The rather high number of cycles was deliberately chosen to enable detection of signals also from low-abundance transcripts. As shown in Fig. 1, a non-uniform tissue profile for presence of galectin-2-specific cDNA was observed. Liver, kidney and skin gave clear indications for expression (Table 2). Compared to the situation with galectin-2, cDNA for the chimera-type galectin-3 was uniformly present in the illustrated test panel (Fig. 1). Although different susceptibility to degradation cannot unequivocally be

Table 2. Determination of gene expression for galectins in human tissues (*post mortem* samples of three patients) by RT-PCR.[a]

PATIENT NR.	ORGAN	ß-ACTIN	Gal-2	Gal-1	Gal-4	Gal-7	Gal-8	Gal-9
A	Brain	+	-/(+)	++	+	++	+	(+)
B	Heart	++	(+)	++	-	-	+	++
A	Trachea	++	-	++	+	++	++	+
A	Lung	++	(+)	++	+	++	++	++
C	Thyroid gland	++	-/(+)	++	++	++	++	+
C	Esophagus	++	-	++	++	++	+	+
A	Stomach	++	-/(+)	++	(+)	+	+	++
B	Colon	++	(+)	++	++	-	++	++
C	Liver	++	++	++	++	++	++	+
A	Kidney	++	++	++	++	++	++	++
C	Prostate	++	-/(+)	++	++	++	++	+
C	Skin	++	+	++	++	++	++	+
C	Lymph node	++	(+)/+	++	++	+	++	+

[a]: galectin gene expression was determined by RT-PCR analysis and semiquantitatively evaluated by three independent investigators as described in Materials and methods. Signal presence was first classified as "+" (positive) and "-" (negative). Signal intensity was then further subclassified as "++" (very strong) or as "(+)" (weak but consistently significant). To account for variation of signal presence and/or intensity in separate experiments individual scoring is presented as "-/(+)" and "(+)/+".

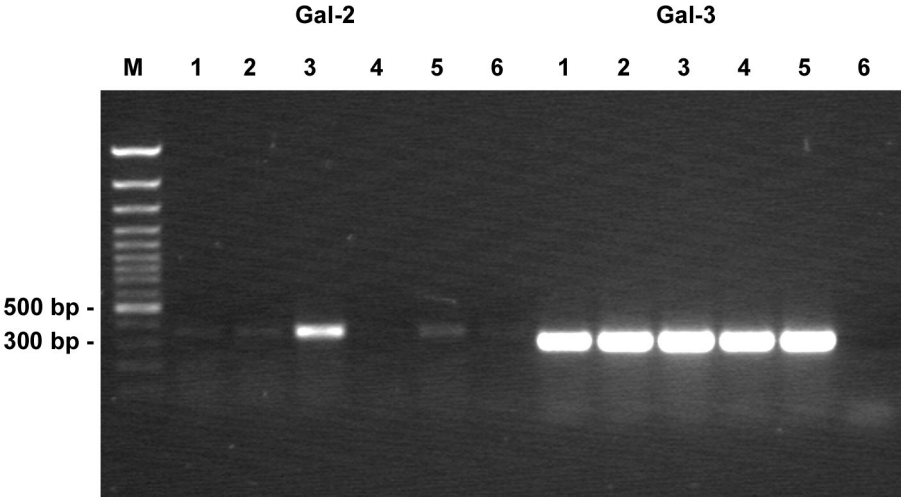

Fig. 1. Determination of gene expression for galectins-2 and -3 in a panel of human *post mortem* tissue types by RT-PCR. 1% agarose running gel, stained with ethidium bromide. M: molecular weight marker (100 bp ladder, New England Biolabs, Frankfurt a. M., Germany), lanes 1-6 define the tissue origin of the template cDNA used in the corresponding PCR experiment as follows: 1, heart; 2, lung; 3, kidney; 4, stomach; 5, colon; 6, negative control (H_2O). For each PCR experiment the complete reaction volume of 20 µl was loaded onto the gel.

excluded, this illustration adds to the controls to reduce risk of false-negative results. When examining cDNA presence for two further proto-type galectins (galectins-1 and -7) and the tandem-repeat-type galectins-4, -8 and -9, a rather uniform pattern of positivity was detectable (Table 2). The results thus indicated a rather widespread occurrence of at least minute amounts of mRNAs for galectins including galectin-2 in tissue samples obtained by autopsy.

To further address the pertinent issue of RNA degradation we next performed the RT-PCR analysis using surgical specimens as starting material. We processed samples from three different patients for each tissue type and could confirm signal presence of galectin-2 in any tested preparation (Table 3). No interindividual variation of signal presence or intensity was seen for galectin-2. Of note, malignancy was also associated with galectin-2 gene expression. In comparison to the most closely related galectin-1 the expression profile for galectin-2 showed quantitative variations within the tested panel of samples, in this respect resembling the situation encountered in the case of galectin-7 (Table 3). Having herewith demonstrated tissue presence of galectin-2-specific cDNA also in surgical specimens less prone to suffer from degradation, the prerequisite was fulfilled to embark on immunohistochemical monitoring. For this purpose, we used human galectin-2 from recombinant production as

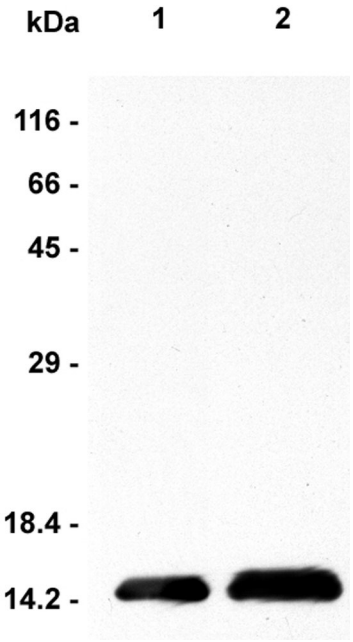

Fig. 2. Western blot analysis of antibody reactivity. Lane 1 of the nitrocellulose membrane was loaded with 50 ng of human galectin-2 from recombinant production as positive control; lane 2 contains 50 µg extract of HCT-15 colon adenocarcinoma cells engineered to express galectin-2. The set of molecular weight markers contains the following components: ß-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), α-lactoglobulin (18.4 kDa) and lysozyme (14.2 kDa). Incubation with the first- and second-step antibodies was performed with a 1:1000 dilution of the primary antibody (incubation overnight at 4 °C) and a 1:2000 dilution of the secondary antibody (2 h incubation at room temperature).

Table 3. Determination of gene expression for proto-type galectins in human tissues (surgical specimen of three patients per tissue type) by RT-PCR.[a]

TISSUE TYPE	Gal-2	Gal-1	Gal-7
Normal stomach	++	++	(+)
Gastric adenocarcinoma	++	++	+
Normal colon	++	++	-/(+)
Colon adenocarcinoma	+	++	+
Normal lung	++	++	+
Pulmonary adenocarcinoma	++	++	+
Pulmonary epidermoid carcinoma	++	++	++
Normal thyroid	(+)	++	+
Papillar carcinoma of the thyroid	++	++	++
Normal kidney	++	++	-/+
Grawitz tumor	++	++	-/++
Normal bladder	++	++	++
Urothelial carcinoma of the bladder	++	++	++

[a]: galectin gene expression was determined by RT-PCR analysis and semiquantitatively evaluated by three independent investigators as described in Materials and methods. For scoring system, please see footnote to Table 2.

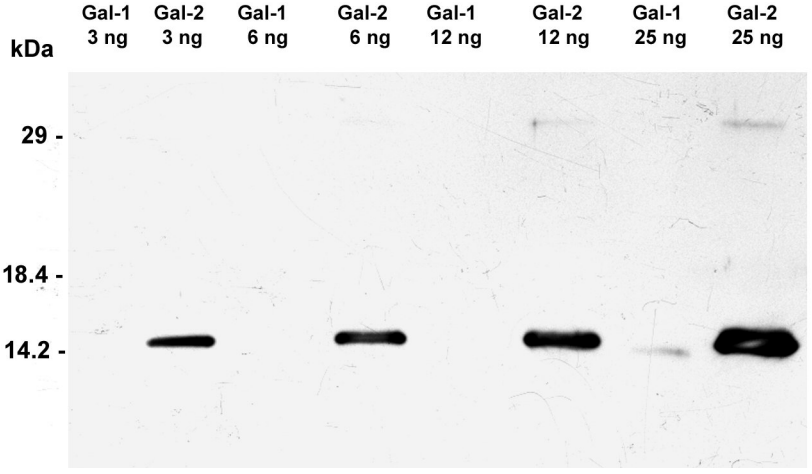

Fig. 3. Assessment of cross-reactivity against galectin-1 found in the polyclonal anti-galectin-2 antibody preparation. Western blot analysis of purified galectins-1 and -2 in concentrations increasing from 3 to 25 ng from left to right was performed under conditions given in detail in Methods and the legend to Fig. 2.

an antigen to raise polyclonal antibodies.

Tissue profiling by immunohistochemistry

Prior to starting monitoring we subjected the antibody preparation to quality controls. High-avidity binding to human galectin-2 was observed in Western blots, and cell extracts were negative except for presence of endogenous galectin-2, as shown exemplarily in Fig. 2. Because the score of sequence identity among members of this lectin family with the galectin-2 sequence as reference reaches 43 % for galectin-1 and 20-28 % for the other galectins and because a previous report had noted weak cross-reactivity of an anti-galectin-2 preparation against galectin-3 (Oka et al., 1999), we took special care to test a series of family members, i.e. galectins-1, -3, -4, -7, and -8, for cross-reactivity. Using ELISAs with 100 ng protein as a probe only galectins-1 and -3 gave a signal with about 1.7% of staining intensity above background level relative to galectin-2. Fig. 3 documents the Western blot analysis of galectin-1 relative to the protein used as antigen, for which a weak band of the dimer is also visible. The antibody preparation is thus a suitable tool for the monitoring of tissues without highly abundant expression of galectins-1 and -3. As a refinement of the preparation, removal of cross-reactive material was accomplished by affinity chromatography using purified galectins-1 and -3 separately as ligand, ascertained by ELISAs and Western blotting (not shown). To confirm the notion that this antibody preparation will be useful for immunohistochemical analysis Fig. 4 presents the differential patterns of localization of galectin-2 compared to galectin-1.

Positivity of the reaction was controlled by demonstrating its strict antigen dependence during the immunohistochemical monitoring, a representative example of a colon tumor section shown in Fig. 5A. While our RT-PCR data had indicated the presence of at least minute amounts of galectin-2-specific mRNA, this profiling clearly revealed expression of this proto-type galectin in human tissues on the level of the protein. As summarized in Table 4 and exemplarily illustrated in Fig. 5B-H, epithelial cells and also neuropile, neuron bodies and blood vessel walls in normal cortex were positive, with staining being present in cytoplasm and nuclei. Differences in cellular localization between the closely related galectins-1 and -2 were readily detectable in the biliary tract. Whereas strong epithelial staining was observed for galectin-2 detection (Figs. 4A, 5B), these cells were devoid of any reactivity with the galectin-1-specific antibody, which in turn led to staining of connective tissue and smooth muscle cells (Fig. 4B). A caveat in this respect concerns Sertoli cells, where the strong galectin-1 presence, shown previously (Wollina et al., 1999), might yield a false-positive result. Moving to tissue inflammation, no major alteration of the characterisics of expression was observed (Table 4). As could be expected from the RT-PCR data of tumor specimen presented in Table 3, we also found immunoreactivity in the tested tumors which we illustrate together with sections of normal tissue (Table 5, Fig. 6A-F). On average, staining in malignancy appeared more homogeneous and diffuse than in the disease-free tissue counterparts. The intracellular staining pattern showed a remarkable disparity. A loss of nuclear presence was consistently seen in cholangiocarcinomas, hepatocarcinomas as well as mammary and gastric adenocarcinomas, whereas the processed thyroid carcinomas were characterized by nuclear galectin-2 presence (Tables 4, 5). In addition to tissues and tumors of epithelial origin we tested sections of lymph nodes and lymphomas, melanocytic tumors and brain/glial tumors, as listed in Table 1. Galectin-2 expression was consistently seen in plasmocytes (cytoplasmic staining) but not paracortical or medullary lymphocytes. Melanocytic tumors revealed cytoplasmic and nuclear reactivity. Intensity was strong with diffuse pattern in the melanoma cases. The fairly weak indication for presence of galectin-2-specific mRNA in

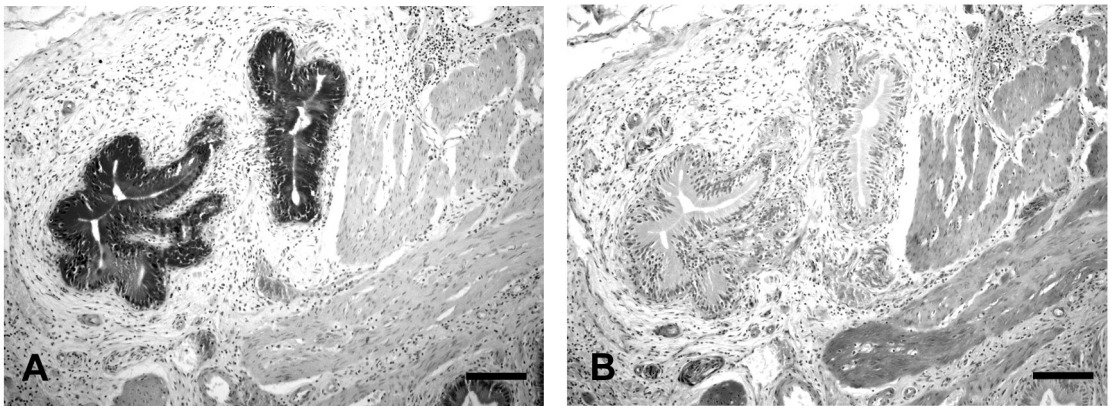

Fig. 4. Illustration of immunohistochemical staining patterns in sections of a human gallbladder with anti-galectin-2 antibody (**A**; please see also Fig. 5B) and anti-galectin-1 antibody (**B**) with its typical reactivity in stroma and muscle fibers. Bars: 80 µm.

brain given in Table 2 was confirmed by immunohistochemical detection (Fig. 5D). Tumoral astrocytes showed cytoplasmic staining in the four studied cases given in Table 1. Having herewith revealed galectin-2 presence in tissue sections and described the immunohistochemical staining profile, the question on suitability of application of the labeled tissue lectin remained to be answered.

Lectin histochemistry using galectin-2

To introduce galectin-2 as a tool to histochemical monitoring we biotinylated purified lectin under conditions not impairing the carbohydrate-binding activity. Prior to the histochemical processing we ascertained assumed maintenance of binding capacity by solid-phase and cell assays. Binding to glycans presented

Table 4. Immunohistochemical analysis with anti-galectin-2 antibody in normal tissues of epithelial origin and inflammatory diseases.[a]

HISTOLOGICAL TYPE	EPITHELIAL COMPARTMENT			STROMAL COMPARTMENT	
	staining intensity	pattern	sub-cellular pattern	staining intensity	Pattern
Normal tissues of epithelial origin					
Pulmonary system					
Lung	1	2	C	0	0
Pleura	1	1	C	0	0
Digestive system					
Salivary gland (mixed)	2	1	C	1	2
Gut					
Fundus	2	1	N+C	1	2
Antrum	1	1	C	1	2
Duodenum	2	1	C	2	2
Ileum	2	1	C	1	2
Colon	2	2	C	1	2
Liver	2	2	N+C	-	-
Biliary tract	2	2	N+C	0-1	0-2
Pancreas	2	2	C	1	2
Urogenital system					
Kidney	2	2	C+cm	1	2
Bladder	2	2	C+cm	0	0
Female					
Breast	2	2	N+C	2	2
Ovaries	1	1	C	1	2
Male					
Testis	2	2	N+C	1	2
Skin	2	2	N+C	1	2
Inflammatory diseases in epithelial tissues					
Thyroiditis	2	2	C	0-1	0-2
Interstitial pneumopathy	2	2	C	1	2
Sialadenitis	2	1	C	1	2
Barrett's esophagus without dysplasia	2	1	N+C	1	2
Gastritis (fundus)	2	1	N+C	1	2
Non-specific duodenitis	1	1	C	1	2
Coeliac sprue	2	1	C	1	2
Crohn's ileitis	2	1	C	2	2
Crohn's colitis	2	2	C	1	2
Hepatic rejection	2	2	N+C	-	-
Primary biliary cirrhosis	1	2	N+C	0	0
Chronic pancreatitis	2	2	C	1	2
Prostatitis	2	2	N+C	2	1
Lichen planus	2	2	N+C	1	2

[a]: for explanation of the grading system, see final section in Materials and methods.

Table 5. Immunohistochemical analysis with anti-galectin-2 antibody in benign and malignant tumors.[a]

HISTOLOGICAL TYPE	EPITHELIAL COMPARTMENT			STROMAL COMPARTMENT	
	staining intensity	pattern	sub-cellular pattern	staining intensity	Pattern
Benign tumors of epithelial origin					
Multinodular goiter of the thyroid	1	1	C	1	2
Microfollicular adenoma of the thyroid	2	2	C	1	2
Parathyroid adenoma	1	2	C	0	0
Pituitary gland adenoma	1	2	C	-	-
Pleomorphic adenoma of salivary gland	1	2	C	0	0
Mammary fibroadenoma	2	2	C	2	2
Pre-malignant conditions of epithelial origin					
Barrett's esophagus with dysplasia	2	2	N+C	2	2
Colon dysplasia	2	2	C	1	2
Prostatic intraepithelial neoplasia	2	2	C	2	2
Malignant tumors of epithelial origin					
Squamous: Skin- or epidermis-related tumors					
Spinocellular carcinoma	2	2	N+C	1	2
Basocellular carcinoma	2	2	N+C	1	2
Glandular: Urogenital or digestive system tumors					
Prostatic adenocarcinoma	1	2	C	0	0
Mammary adenocarcinoma	1	2	C	1	2
Ovary serous adenocarcinoma	2	2	C	1	2
Esophageal adenocarcinoma	2	1	C	1	2
Gastric adenocarcinoma	1	2	C	1	2
Carcinoma of ampulla of Vater	1	2	C	1	2
Colon adenocarcinoma	2	2	C	1	2
Cholangiocarcinoma	2	2	C	1	2
Pancreatic adenocarcinoma	2	2	C	1	2
Urogenital or digestive system tumors					
Urothelial bladder carcinoma	2	2	C	1	2
Grawitz tumor	2	2	C+cm	1	2
Seminoma	0-1	0-1	C	1	2
Hepatocarcinoma	1	2	C	1	2
Hepatoblastoma	1	1	C	1	2
Pulmonary or pleural tumors					
Adenocarcinoma	1	2	C	1	2
Epidermoid carcinoma	1	2	C	0	0
Large cell carcinoma	1	2	C	0	0
Mesothelioma	1	2	C	0	0
Thyroid tumors					
Papillary carcinoma	1-2	2	N+C	1	2
Follicular carcinoma	1-2	2	N+C	1	2
Anaplastic carcinoma	1-2	2	N+C	1	2

[a]: for explanation of the grading system, see final section in Materials and methods.

Table 6. Lectin histochemical analysis with biotinylated galectin-2 in normal human tissue types and malignant tumors.[a]

HISTOLOGICAL TYPE	EPITHELIAL COMPARTMENT			STROMAL COMPARTMENT	
	staining intensity	pattern	sub-cellular pattern	staining intensity	Pattern
Normal tissues of epithelial origin					
Biliary tract	2	2	C	1	2
Colon	1	2	C	1	2
Bladder	1	2	C	0	0
Malignant tumors of epithelial origin					
Cholangiocarcinoma	1-2	2	C	0-1	0-2
Colon adenocarcinoma	2	2	C	1	2
Urothelial bladder carcinoma	2	2	C	1	2

[a]: for explanation of the grading system, see final section in Materials and methods.

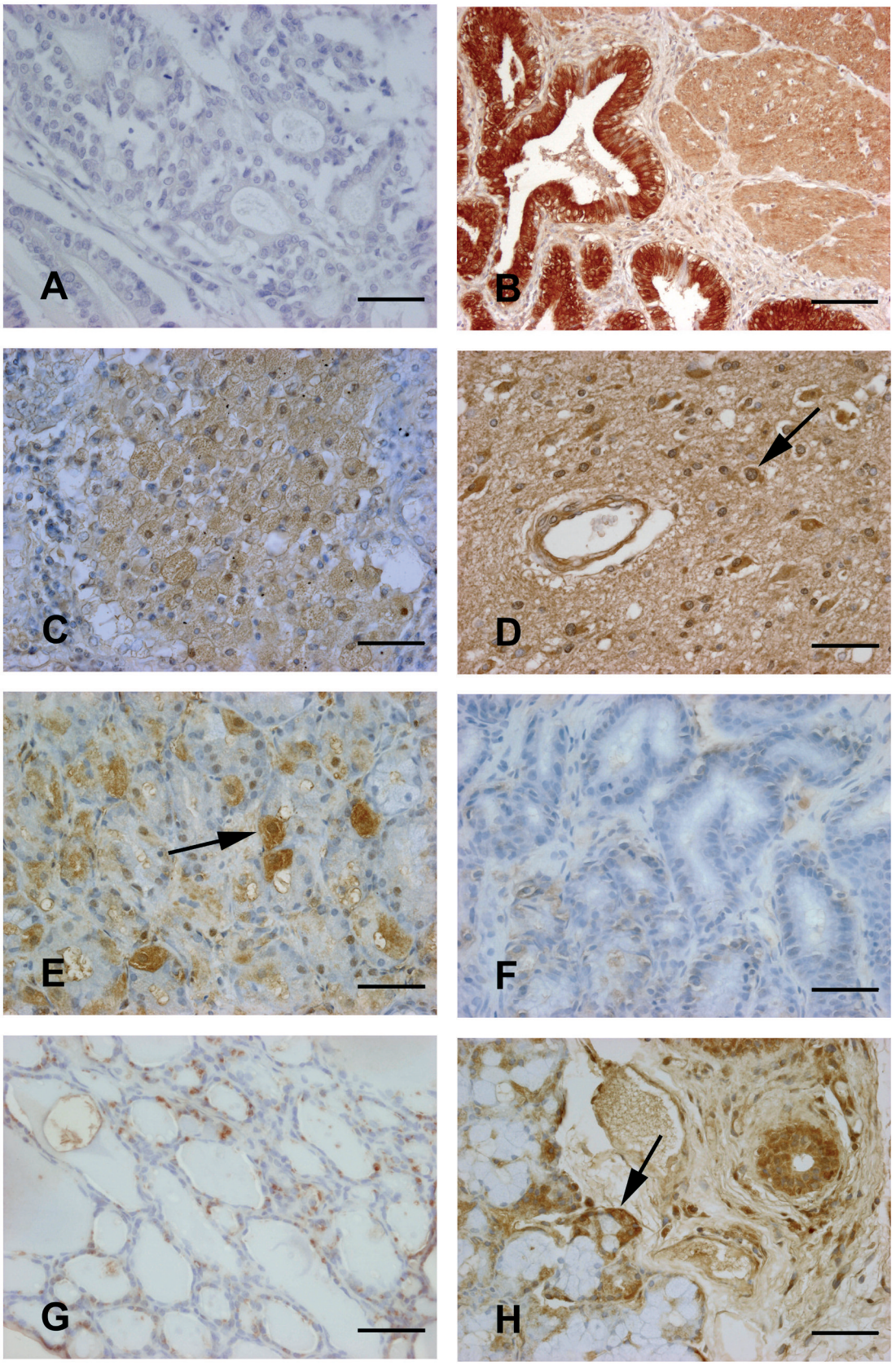

Fig. 5. Illustration of the immunohistochemical staining pattern with anti-galectin-2 antibody in selected human tissue types. **A**: negative control, section of a colon adenocarcinoma after routine processing lacking the incubation step with the primary antibody. Bar: 50 µm. **B**: mucosa of the normal biliary tract (gallbladder) showing the most intense staining of all epithelia analyzed in our study. Bar: 100 µm. **C:** macrophages, displaying a diffuse granular cytoplasmic and focal nuclear staining. Bar: 50 µm. **D:** gray matter, showing a diffuse staining of the neuropile and the blood vessel wall as well as focal cytoplasmic staining of the neuron bodies (arrowhead). Bar: 50 µm. **E**: normal fundic mucosa showing strong cytoplasmic staining of the parietal cells (arrowhead). Bar: 50 µm. **F:** normal antral mucosa displaying a spatially confined and less intense staining than the fundic mucosa. Bar: 50 µm. **G:** multinodular goiter of the thyroid, showing a focal cytoplasmic staining of the follicular cells. Bar: 50 µm. **H:** normal mixed salivary gland with cytoplasmic staining for the serous acini (arrowhead) but not of the mucous ones. Bar: 50 µm.

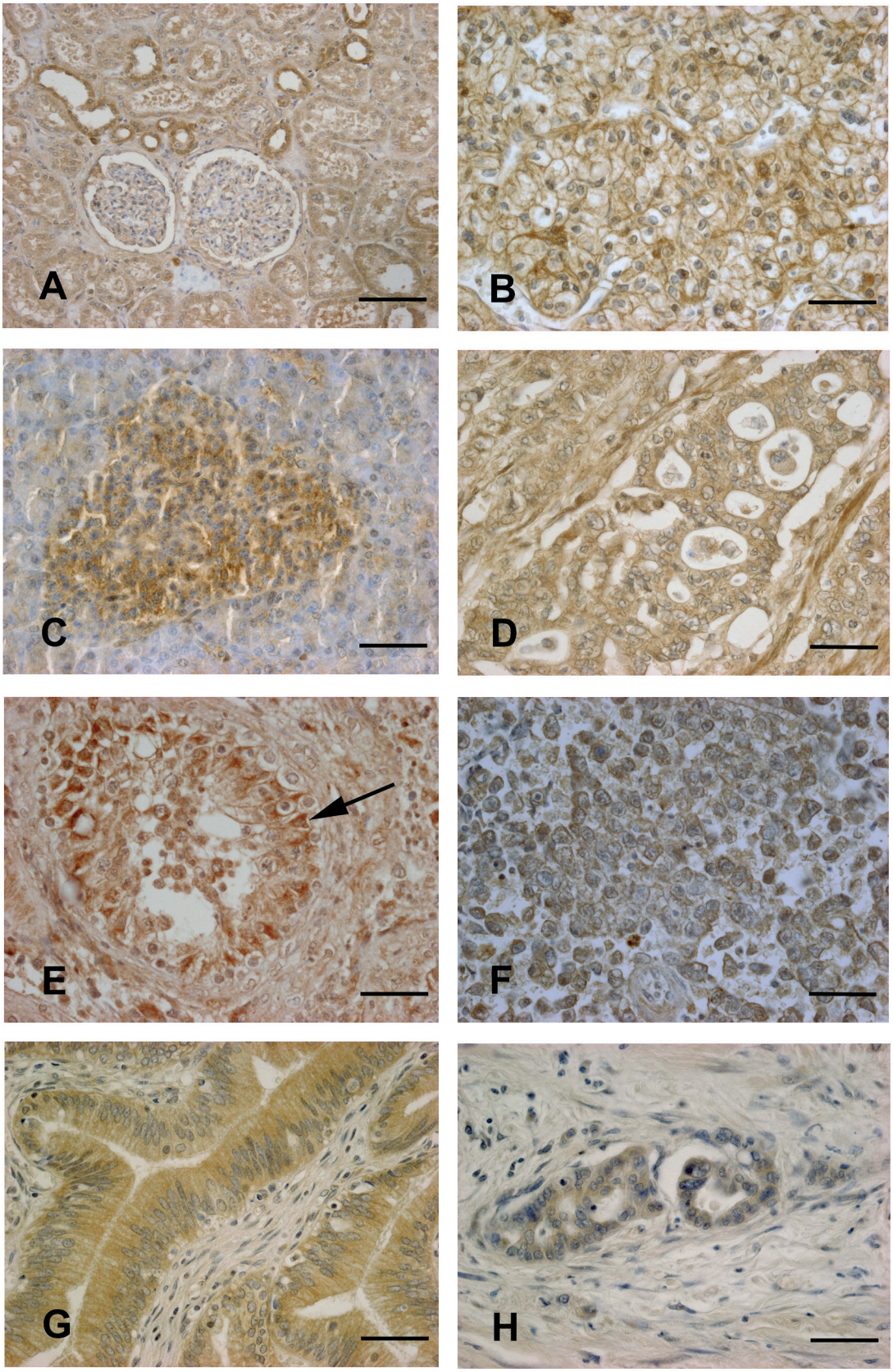

Fig. 6. Illustration of the immunohistochemical staining pattern with anti-galectin-2 antibody and the reactivity of labeled galectin-2 in a series of normal human tissue types and tumors. **A:** normal renal tissue showing diffuse and moderate to strong staining of the epithelial structures. Bar: 100 µm. **B:** Grawitz tumor cells displaying a distinct subcellular staining that underlines the cellular membrane. Bar: 50 µm. **C:** cells of the normal endocrine and exocrine pancreas. Bar: 50 µm. **D:** malignant glandular cells of pancreatic adenocarcinoma. Bar: 50 µm. **E:** normal seminiferous tubules showing cytoplasmic staining of the Sertoli cells (arrowhead). Bar: 50 µm. **F:** seminoma cells with a cytoplasmic staining of moderate intensity, which was unusual for the studied cases of this tumor entity. Bar: 50 µm. **G:** staining of galectin-2-binding sites in normal biliary mucosa (gallbladder) and **H**: staining of galectin-2-binding sites in malignant gland structures of a cholangiocarcinoma. Bars: 50 µm.

on a matrix or on tumor cell surfaces was invariably blocked by lactose, as also shown previously for activated T cells (Sturm et al., 2004). With respect to potential impact of ligand presentation by malignant transformation we analyzed three pairs of normal tissue and their corresponding malignant counterparts. As summarized in Table 6, processing with galectin-2 as a probe led to specific staining, for which examples are given in Fig. 6G,H. Reactivity was generally cytoplasmic, where blocking by lactose is at best partial, in contrast to cell surface staining. A tendency for increased staining intensity was seen in malignant tissue.

Discussion

The emerging complexity of the galectin network prompts a stepwise analytical approach, starting with initial sequence information and biochemical analysis. The next step toward elucidation of a) functions on the level of cells and tissues and b) extent of functional overlap/divergence among family members is expression profiling. Such a study on rat galectin-2 has described predominantly gastrointestinal presence with staining of gastric epithelial cells (Oka et al., 1999). Our experimental series using RT-PCR analysis clearly indicated the presence of galectin-2-specific mRNA in organs beyond the gastrointestinal tract. Considering the detrimental impact of tissue degradation it is noteworthy that we picked up such signals already in samples obtained from autopsy. Nonetheless, we added experiments by processing surgical specimens. They fully confirmed the positivity seen in the first part of our study. The concomitantly performed RT-PCR detection of other galectins extended our current knowledge of gene expression in human tissues. At this stage, it can not yet automatically be concluded that a positive signal in the agarose gel will account for presence of the protein which can then act as effector in the tissue. Thus, we proceeded to immunohistochemical processing. We first studied the inherent problem of cross-reactivity among members of this lectin family. Of note in ensuing expression profiling, the immunopositivity was preferentially epithelial, with the gallbladder's intense epithelial staining affording a model case of differential expression between galectins-1 and -2. Together with the initial indications from activated T cells lacking galectin-2 presence and from various tumor lines (Lahm et al., 2001, 2003; Sturm et al., 2004) these results solidify the notion that expression can well differ even between closely related family members. Consequently, the immunohistochemical mapping is a key step to gauge the range of cell-specific effects of related lectin proteins. This approach will be valuable also in other systems showing molecular diversification such as C-type lectins of dendritic cells, lymphocytes or macrophages or I- and P-type lectins (Kaltner and Stierstorfer, 1998; Angata and Brinkman-Van der Linden, 2002; Dahms and Hancock, 2002; Kanazawa et al., 2004; Marshall and Gordon, 2004; McGreal et al., 2005; Villalobo et al., 2005). A caveat concerns extrapolation of data on localization among species. It is not valid *a priori*. After all, occurrence of species-specific additions to the core family such as galectin-5 in rat and galectin-6 in mouse add unique aspects to the network in these cases (Cooper, 2002; Houzelstein et al., 2004).

With respect to medical aspects, our analysis revealed positivity in RT-PCR and immunohistochemical monitoring in various tumors. Due to the exploratory design at this stage of investigation it would certainly be premature to claim clinical relevance. However, our data underscore that the current status of detailed prognostic evaluation, as recently accomplished for colon tumors (Nagy et al., 2003), can now be refined by introducing galectin-2 to the panel established so far. Also, focusing on benign and malignant tumor types in distinct tumor entities will be helpful to spot cases where galectin-2 might be associated with the pathway of progression to the malignant phenotype, as previously illustrated for galectin-8 (Danguy et al., 2001). The histopathologic studies can be strategically flanked by transfection approaches to relate modulation of galectin-2 expression *in vitro* with functional aspects such as proliferation or resistance to apoptotic stimuli such as chemo-therapeuticals. In aggregate, these aspects of our study have answered three out of the four questions. The remaining question concerned the feasibility of introducing this galectin as a histochemical tool.

Toward this end, we combined bio- and histochemical work. Because we have answered the question positively as to whether galectin-2 itself, an effector for cell growth regulation in two systems (Sturm et al., 2004; André at al., 2005a), is suitable for ligand detection *in situ*, this approach should complement further immunohistochemical monitoring. In fact, the further use of galectin-2 as a histochemical tool is likely to reveal salient information relevant for figuring out cell reactivity and differences in ligand selection *in situ* among galectins. Our current knowledge in this area tells us that these differences can be assigned to a distinct preference for the carbohydrate sequence, conformation or mode of clustering (Wu et al., 2001, 2002, 2004; Ahmad et al., 2002; Hirabayashi et al., 2002; Siebert et al., 2003a,b). Equally important for cytoplasmic/nuclear presence of galectins, localization of protein ligands with functional impact could thus become possible. Mentioning oncogenic H-Ras as binding partner for galectin-1 and the thyroid-specific transcription factor TTF-1 or ß-catenin, a component of Wnt signaling for cyclin D_1/c-myc stimulation, as interaction sites for galectin-3 underscores the potential of this approach (Paz et al., 2001; Paron et al., 2003; Rotblat et al., 2004; Sharma et al., 2004; Shimura et al., 2004). Cytoplasmic staining with biotinylated galectin-2 is in principle in line with its reactivity to α- and ß-tubulins. These examples, too, highlight why tissue lectins should receive preference to plant lectins, which are often used in glycan detection (Spicer and Schulte, 1992;

Cummings, 1997; Rüdiger and Gabius, 2001), for monitoring binding sites *in situ*. The previous observation on differential reactivity of galectins-1 and -2 with the glycoproteins CD3 and CD7 of activated T cells (Sturm et al., 2004) is a strong incentive to pursue galectin histochemistry with different members of the family in addition to the localization of the galectin. In summary, these initial results encourage systematic diagnostic and prognostic study of galectin-2 by immuno- and lectin histochemistry in histopathology.

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