ONCOLOGY REPORTS 20: 1403-1408, 2008

SPANDIDOS PUBLICATIONS

Decreased expression of galectin-3 predicts tumour recurrence in pTa bladder cancer

MARIO WOLFGANG KRAMER[1], MARKUS ANTONIUS KUCZYK[2], JÖRG HENNENLOTTER[1], JÜRGEN SERTH[2], DAVID SCHILLING[1], ARNULF STENZL[1] and AXEL STUART MERSEBURGER[2]

[1]Department of Urology, Eberhard-Karls University, Hoppe-Seyler-Str. 3, D-72076 Tübingen;
[2]Department of Urology, Hannover University Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany

Received April 2, 2008; Accepted July 11, 2008

DOI: 10.3892/or_00000159

Abstract. Galectin-3 (gal-3) is a glycoprotein involved in various physiological cellular processes. Altered expression/loss of function of gal-3 is suggested to be involved in the pathogenesis and further progression of various human cancer entities. The aim of the present investigation was to elucidate the role of galectin-3 in the development and/or progression of non-muscle invasive (pTa, pT1) transitional cell carcinoma (TCC) of the urinary bladder. Gal-3 was analyzed by immunohistochemistry in 162 randomly selected non-muscle invasive bladder cancer specimens (pTa, 91; pT1, 71) using tissue microarray technique. It was compared with various patient and tumour characteristics (t-test). In addition, the role of gal-3 in association with tumour recurrence and progression was investigated (Log-rank test, Cox regression analysis). Gal-3 was found to be negatively correlated with tumour grade ($p<0.02$). Within the group of non-muscle invasive TCC, gal-3 could not differentiate between pTa and pT1 tumours ($p=0.50$), and within the subgroup of pTa tumours, loss of gal-3 determined the likelihood for the development of recurrent disease ($p<0.03$; Student's t-test). Furthermore, as demonstrated by Kaplan-Meier analysis, the expression level of gal-3 was identified to predict the duration of recurrence-free survival ($p=0.01$). In the multivariate analysis, gal-3 was found as an independent prognostic marker for predicting recurrence among the cohort of bladder tumours classified as pTa. In conclusion, loss of galectin-3 appears to be involved in the carcinogenesis of TCC and to serve as a valuable biological variable to identify a subgroup of Ta bladder cancer patients at high risk for the development of recurrent disease.

Correspondence to: Dr Mario Kramer, Department of Urology, Eberhard-Karls University, Hoppe Seyler Str. 3, D-72076 Tübingen, Germany
E-mail: mariokramer@gmail.com

Key words: transitional bladder cancer, transitional cell carcinoma, recurrence, prognosis, galectin-3, biomarker

Introduction

In the US, more than 67,000 new cases of bladder cancer, mostly transitional cell carcinoma (TCC), and 13,000 bladder cancer-related deaths were estimated to have occurred in 2007, with treatment costs amounting to approximately 2.9 billion dollars (1,2). Limiting tumour recurrence is one of the most cost-effective factors associated with the treatment of this disease, since 30-80% of bladder cancers tend to recur within the first 3 years following transurethral resection of the primary tumour (3). Thirty percent of low grade tumours recur at a higher grade (4,5), which is subsequently associated with an increased risk for the development of progressive/muscle-invasive disease. Besides recurrence, the unpredictable biological behaviour of superficial TCC, including the development of locally invasive tumour growth, the latter risk mainly affecting patients revealing pT1 tumours, represents one of the dilemmas faced during the treatment of the disease (6).

Galectin-3 (gal-3) is one of the most extensively investigated galectins. It has been demonstrated to be extensively involved in cellular processes such as cell-cell adhesion and cell-matrix interactions. Additionally, gal-3 was demonstrated to induce apoptosis, angiogenesis and mRNA splicing (7). However, the role of gal-3 in the development and/or progression of human malignant disease remains to be clarified (8).

Studies of gal-3 expression in urologic malignancies are sparse. For prostate cancer patients, decreased gal-3 expression appears to be negatively correlated with the biological aggressiveness of the primary tumours (9). In a study conducted by Francois *et al* (10), which determined gal-3 expression levels in primary renal cell carcinomas by an immunohistochemical approach, deceased gal-3 levels were directly correlated with tumour grade. In contrast, the involvement of gal-3 in the development and progression of transitional cell carcinoma of the urinary bladder has been rarely investigated. Although Cindolo *et al* (11) observed an increased expression of gal-3 in bladder cancer specimens when compared with non-malignant urothelium as detected at the mRNA level by Northern blot analysis, a correlation between mRNA levels and further tumour characteristics such as stage and grade was not evident.

Therefore, the aim of the present investigation was to further clarify the involvement of galectin-3 in superficial

bladder cancer (pTa, pT1), particularly to determine the capability of altered protein expression levels to predict the likelihood for tumour recurrence and progression following transurethral resection.

Materials and methods

Patients and specimens. A total of 162 patients with superficial bladder cancer treated by transurethral resection between 1993 and 2000 for whom paraffin blocks were available were included in the present investigation following approval by the Ethics Committee of Tübingen University. Follow-up information obtained from the patient charts included tumour stage, grade, the development of tumour recurrence and/or tumour progression, the presence of multifocal vs. unifocal tumour growth, the co-existence of carcinoma *in situ* (CIS) as well as patient gender and age. Only patients with a minimum follow-up of at least 3 years following initial transurethral treatment of the primary intravesical lesions were included in the present study. Recurrence was defined as reappearance of tumours of the same stage. Progression was defined as tumour development from non-muscle to muscle-invasive disease. All tumours were initially graded by pathologists according to the 1973 WHO grading system (12). As recommended by the EAU guidelines 2007 (13), this classification was used in the present study.

Tissue microarray and immunohistochemical analysis. Initially, tumour specimens were formalin-fixed, dehydrated and embedded in paraffin. The paraffin blocks were cut into 4-μm sections. H&E staining was performed for each tumour specimen to validate tumour stage and grade as well as for the onset of tissue microarrays (TMA). The TMAs were constructed as described previously (14,15).

For immunohistochemical analysis, the fresh cut 4-μm paraffin sections were deparaffinized, rehydrated and immersed in 3% hydrogen peroxide in purified water to block endogenous peroxidase activity. Antigen retrieval was accomplished by a steaming pretreatment for 23 min at 100˚C using a Target Retrieval Solution (S1699, Dako Cytomation Inc.). Galectin-3 was immunohistochemically detected by a commercially available anti-galectin-3 monoclonal mouse antibody (R&D Systems, clone 194804, MAB 1154). The optimal dilution of the anti-galectin-3 antibody was 1:300 in Dako background reducing dilutent (S3022, Dako Cytomation Inc.). Following overnight incubation (12 h), the sections were washed in PBS + 0.05% Tween-20 and incubated both with a universal secondary biotinylated antibody (K0690, Dako Cytomation Inc.) and streptavidin for 30 min, respectively. The DAB system (K3468, Dako Cytomation Inc.) was used for visualization according to the manufacturer's instructions. Sections were briefly rinsed in tap water, counter-stained with hematoxylin (Surgipath, Harris' formula) and mounted. For negative control, the primary anti-galectin-3 antibody was replaced by non-immune mouse serum. As a positive control, human lung cancer tissue sections were used.

The TMA slides were reviewed and classified by two independent investigators (M.W.K. and A.S.M) in a blinded manner. For statistical analysis, the immunohistochemical staining reaction was classified according to a semi-quantitative reference scale ranging from 0 to 3^+, depending on the intensity of galectin-3 protein expression. The relative amount of tumour cells that stained positively for galectin-3 (0-100%) in conjunction with the rating of the staining intensity, resulted in a staining score ranging from 0 to 300 as described previously (14). The concordance rate of the investigators was 89%.

Statistical analysis. The JMP program was used for statistical evaluations. A D'Augostino and Pearson omnibus normality test was performed to determine whether all the data sets to be compared were parametric or non-parametric. One-way ANOVA and the Student's t-test were applied to correlate gal-3 expression with various tumour characteristics. Time to event probabilities were estimated by the univariate Kaplan-Meier method. The Cox fit proportional hazard model was applied for the multivariate analysis. Statistical differences with p-values <0.05 were considered to be significant.

Results

Clinicopathological data. A total number of 162 superficial bladder cancer specimens obtained from transurethral resections were included in the present investigation [119 (73%) men and 43 (27%) female patients]. According to histopathologic evaluation, tumour stages were classified as pTa in 91 (56%) and pT1 in 71 (44%) cases, respectively. Tumour grade was as follows: G1, n=59 (36%); G2, n=90 (56%); and G3, n=13 (8%). The median follow-up interval was 58.5 months. The average time interval between the initial treatment and the observation of recurrent or progressive disease was 14 (3-72) and 23 (3-79) months, respectively (Table I).

Assessment of immunohistochemical staining of gal-3. Only the staining reaction within tumour cells was recognized for the classification of the immunohistochemical staining patterns. Gal-3 expression was preferably detected within the cytoplasm of tumour cells. In a few cases, nuclear staining was observed. However, it revealed no prognostic information for the present cohort of patients.

Correlation of gal-3 expression with clinicopathological parameters. Gal-3 protein expression patterns were correlated with various patient and tumour characteristics such as tumour stage, grade, unifocal vs. multifocal tumour growth, the detection of carcinoma *in situ* (CIS) in addition to the primary papillary lesion as well as patient age and gender. Gal-3 expression was negatively correlated with tumour grade ($p<0.02$; One-way ANOVA). Accordingly, the expression of tumours classified as G1 and G2 differed significantly from that observed in G3 lesions ($p<0.01$; Student's t-test). However, pTa and pT1 tumours could not be distinguished by gal-3 protein expression ($p=0.50$; Student's t-test) (Table II).

In contrast, gal-3 was neither correlated with multifocal tumour growth ($p=0.75$) nor the simultaneous detection of CIS for specimens with identical histology ($p=0.49$) or patient gender ($p=0.29$) and age ($p=0.90$) (Table II).

Frequency distribution of clinicopathological parameters and average galectin-3 expression levels.

Clinicopathological parameters	No. of patients (n=162)	%	Galectin-3 expression levels
Clinical data			
Male	119	73	205
Female	43	27	196
Age[a]	67.3 (mean)	69 (median)	200
Carcinoma *in situ* (+)	8	5	119
Multifocality (+)	60	37	189
Lymph node (+)[b]	x	x	x
Pathological distribution			
pTa	91	56	214
pT1	71	44	206
G1	59	36	211
G2	90	56	210
G3	13	8	170
Follow-up data			
Time to recurrence[a]	14.6 (mean)	10 (median)	x
Time to progression[a]	23.1 (mean)	12 (median)	x
pTa+pT1 recurrence (+)	84	52	194
pTa+pT1 recurrence (-)	78	48	209
pTa recurrence (+)	47	52	190
pTa recurrence (-)	44	48	226
pTa+pT1 progression (+)	28	17	182
pTa+pT1 progression (-)	134	83	204

[a]Assessed in months. [b]None of the patients with non-muscle invasive bladder cancer were assessed for positive lymph nodes.

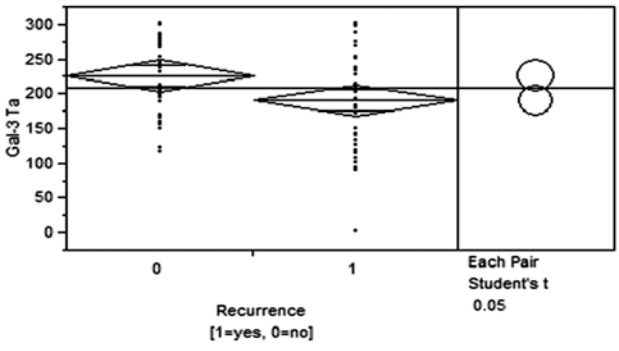

Figure 1. Galectin-3 (gal-3) expression is significantly decreased in recurrent pTa TCC (p<0.03; Student's t-test).

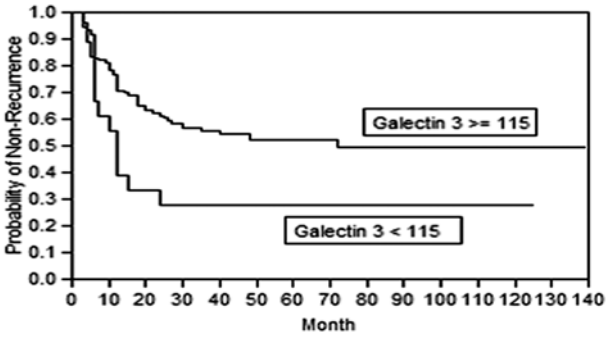

Figure 2. In the subgroup of pTa tumours, decreased galectin-3 protein expression was significantly correlated with the likelihood for the development of tumour recurrence as well as the duration of the recurrence-free survival following transurethral resection of the primary intravesical lesion (p=0.01; Log-rank test).

Correlation between gal-3 expression and the tendency towards tumour recurrence and/or progression. All patients included in this study had follow-up information of at least 36 months following transurethral resection of the primary lesion. Eighty-four (52%) revealed at least one recurrence as detected during subsequently performed examination by cystoscopy. Of these, 47 patients with pTa disease showed recurrent tumours. Twenty-eight (17%) patients revealed progression of the previously detected tumour (Table I).

For pTa patients, decreased expression of gal-3 in the primary tumour was significantly correlated with an increased risk for the development of recurrent disease (p<0.03; Student's t-test) as confirmed by Chi-square analysis (Figs. 1, 3 and 4; Table II). For statistical analysis and to determine the prognostic value of altered gal-3 expression to predict the duration of the patient recurrence-free survival, patients were

Table II. p-values of the univariate and multivariate analyses using the Student's t-test and the Cox proportional hazard fit.

Parameters	p-value	Chi-square	Odds ratio	Std Error	Lower 95%	Upper 95%
Univariate analyses: Student's t-test						
Fit Y by X (Y=Gal-3)						
Gender	0.29	1.26	1.00	0.00	0.00	0.01
Age	0.90	0.81	0.99	0.09	-0.45	0.08
Carcinoma *in situ*	0.49	0.97	4.51	0.01	0.00	0.02
Multifocality	0.75	0.10	0.79	0.00	0.00	0.00
Ta vs. T1	0.50	0.46	1.00	0.00	0.00	0.01
G1 vs. G2	0.79	0.07	0.99	0.00	0.00	0.00
G1 vs. G3	**<0.01**	1.69	6.20	0.00	0.00	0.02
G2 vs. G3	**<0.01**	1.89	4.00	0.00	0.00	0.01
Recurrence vs. non-recurrence (Ta+T1)	0.22	6.38	6.22	0.00	0.00	0.01
Recurrence vs. non-recurrence (Ta)	**<0.03**	7.84	20.27	0.00	0.00	0.02
Recurrence vs. non-recurrence (T1)	0.59	0.47	1.77	0.00	0.00	0.01
Progression vs. non-progression (Ta+T1)	0.25	4.07	7.37	0.00	0.00	0.01
Progression vs. non-progression (Ta)	0.93	0.26	2.11	0.00	0.00	0.01
Progression vs. non-progression (T1)	0.13	4.47	11.60	0.00	0.00	0.02
Multivariate analysis: Cox proportional hazard fit						
Censor: recurrence of Ta tumors						
Galectin-3 expression	**<0.01**	9.59	X	0.00	-0.01	0.00
Tumour stage	0.61	0.26	X	0.15	-0.22	0.38
Grade	0.62	0.95	X	0.39	-0.80	0.89
Multifocality	0.27	1.21	X	0.18	-0.55	0.16
Carcinoma *in situ*	0.32	1.01	X	0.59	-1.69	0.85
Gender	0.06	3.52	X	0.23	-0.01	0.89
Age	0.10	2.75	X	0.02	0.00	0.06

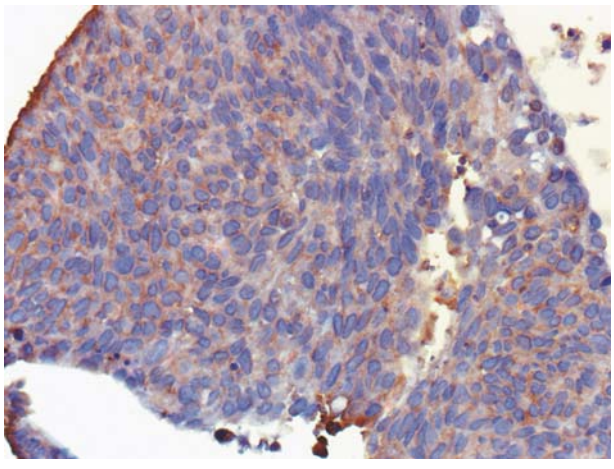

Figure 3. pTa G1 TCC specimen from patient with recurrence 5 months after initial TUR-BT; low galectin-3 expression.

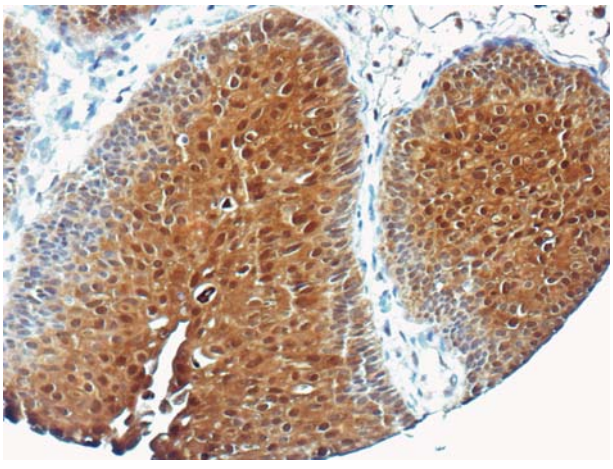

Figure 4. High galectin-3 expression in a pTa bladder cancer specimen from a patient who remained disease-free following resection of the primary tumour.

classified into two groups according to the characteristics of the immunohistochemical staining reaction (group A, staining score ≥115; group B, staining score <115). The duration of the recurrence-free survival was significantly decreased for patients from group B (45 and 13 months for patients from group A and B, respectively) (p=0.01; Log-rank test) (Fig. 2).

As detected using multivariate statistical analysis (Cox logistic regression), gal-3 was identified as an independent prognostic parameter to predict the likelihood for the development of recurrent tumour growth for the TCC tumour subgroup pTa. Further parameters included in the analysis were tumour stage, tumour grade, multifocality, the co-

of carcinoma *in situ*, gender and age. None of these ·s were statistically significant (Table II).

Twenty-eight (17%) patients showed tumour progression (Table I). However, no significant correlation was noted with gal-3 protein expression levels in both univariate and multivariate analyses (Table II).

Discussion

Increased galectin-3 expression was first observed in thyroid and breast cancer as well as in squamous head and neck carcinomas (16-18). Notably, these first observations were not consistent in certain cases. Subsequently performed studies on breast cancer described decreased gal-3 expression when the expression levels in cancer and non-malignant tissue specimens were compared (19). Conflicting data also became evident for thyroid cancer. The most recently reported studies, further questioning the initial suggestion of galectin-3 to be specifically overexpressed in human malignant disease, described an elevated gal-3 expression also for benign thyroid tumours and cases of Hashimoto thyroiditis (20,21).

More recent investigations on urologic malignancies have helped to determine gal-3 expression patterns more precisely. Whereas gal-3 was observed to be retained or even elevated in normal prostate glands, down-regulated or absent protein expression was described for the vast majority of prostate cancer specimens. In addition, a shift from a nuclear to an intracytoplasmic localization of the protein was correlated, as described for renal cell carcinomas, with an increased tendency towards disease progression (10,22). For renal cell cancer, as described by Francois *et al* (10), gal-3 expression was observed to be decreased in correlation with increasing tumour stage (stage I/II vs. II/III).

Limited data are available for gal-3 expression in transitional cell carcinomas of the urinary bladder. In contrast to the above mentioned findings in prostate and renal cell cancer, Cindolo *et al* (11) observed increased gal-3 mRNA levels in TCC detected by Northern blot analysis. The findings were grade and stage independent. However, there was no attempt to confirm these results at the protein level. Therefore, the present investigation was the first to determine gal-3 expression in non-muscle invasive TCC (pTa, pT1) and to correlate protein expression patterns with their tendency towards the development of recurrent and/or progressive disease following transurethral resection of the primary vesical lesion. Hereby, decreased gal-3 protein expression was demonstrated to be negatively correlated with tumour grade ($p<0.02$, One-way ANOVA). Additionally, for pTa tumours the observation of decreased gal-3 expression predicted both the likelihood for the development of recurrent disease ($p=0.03$) and the duration of the recurrence-free survival following transurethral resection of the primary tumour. In light of a recurrence-free survival of 45 vs. 13 months for patients with vs. without retained protein expression, the duration of the recurrence-free survival was significantly decreased for patients revealing loss of gal-3 ($p=0.01$; Log-rank test). Furthermore, the multivariate model (Cox) revealed that the expression of gal-3 indicates whether pTa transitional carcinomas of the bladder might have an increased risk of recurrence.

Discrepancies regarding gal-3 expression levels in various tumour entities including breast, thyroid, colon and now transitional cell cancer of the urinary bladder, might be explained by a non-specific involvement of the protein in tumour development and/or progression or, much more likely, by the different investigational approaches applied so far. Whereas Cindolo *et al* (11) determined gal-3 mRNA levels in TCC by Northern blot analysis, gal-3 expression was investigated at the protein level by immunohistochemistry in the current study. Therefore, according to the promising results obtained herein and to further determine the value of galectin-3 to predict the aggressiveness of at least a subgroup of non-muscle invasive bladder cancer, further investigations must be performed prospectively including comparative analytical approaches both at the protein and RNA levels.

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J and Thun MJ: Cancer statistics, 2007. CA Cancer J Clin 57: 43-66, 2007.
2. Brown ML, Riley GF, Schussler N and Etzioni R: Estimating health care costs related to cancer treatment from SEER-Medicare data. Med Care 40 (Suppl 8): IV-104-117, 2002.
3. Heney NM: Natural history of superficial bladder cancer. Prognostic features and long-term disease course. Urol Clin North Am 19: 429-433, 1992.
4. Droller MJ: Bladder cancer: state-of-the-art care. CA Cancer J Clin 48: 269-284, 1998.
5. Raghavan D and Huben R: Management of bladder cancer. Curr Probl Cancer 19: 1-64, 1995.
6. Van Tilborg AA, de Vries A, de Bont M, Groenfeld LE, van der Kwast TH and Zwarthoff EC: Molecular evolution of multiple recurrent cancers of the bladder. Hum Mol Genet 9: 2973-2980, 2000.
7. Califice S, Castronovo V and Van Den Brule F: Galectin-3 and cancer (Review). Int J Oncol 25: 983-992, 2004.
8. van den Brule F, Califice S and Castronovo V: Expression of galectins in cancer: a critical review. Glycoconj J 19: 537-542, 2004.
9. Califice S, Castronovo V, Bracke M and van den Brule F: Dual activities of galectin-3 in human prostate cancer: tumor suppression of nuclear galectin-3 vs tumor promotion of cytoplasmic galectin-3. Oncogene 23: 7527-7536, 2004.
10. Francois C, van Velthoven R, De Lathouwer O, Moreno C, Peltier A, Kaltner H, Salmon I, Gabius HJ, Danguy A, Decaestecker C and Kiss R: Galectin-1 and galectin-3 binding pattern expression in renal cell carcinomas. Am J Clin Pathol 112: 194-203, 1999.
11. Cindolo L, Benvenuto G, Salvatore P, Pero R, Salvatore G, Mirone V, Prezioso D, Altieri V, Bruni CB and Chiariotti L: Galectin-1 and galectin-3 expression in human bladder transitional-cell carcinomas. Int J Cancer 84: 39-43, 1999.
12. Mostofi F, Sorbin L and Torloni H: Histological typing of urinary bladder tumours. In: International Classification of Tumours. 19th edition. World Health Organisation, Geneva, 1973.
13. Van der Meijden A, Böhle A, Lobel B, Oosterlinck W, Rintala E, Solsona E and Sylvester R: European Association of Urology Pocket Guidelines. EAU, Arnhem, pp5-13, 2007.
14. Kramer MW, Merseburger AS, Hennenlotter J and Kuczyk M: Tissue microarrays in clinical urology - technical considerations. Review. Scand J Urol Nephrol 41: 478-484, 2007.
15. Merseburger AS, Hennenlotter J, Schilling D, Sievert KD, Kufer R and Kuczyk MA: Application of tissue microarrays for the diagnosis, prognosis and therapeutic decision making in renal cell carcinoma. Urologe A 45: 323-327, 2006.
16. Gillenwater A, Xu XC, el-Naggar AK, Clayman GL and Lotan R: Expression of galectins in head and neck squamous cell carcinoma. Head Neck 18: 422-432, 1996.
17. Xu XC, el-Naggar AK and Lotan R: Differential expression of galectin-1 and galectin-3 in thyroid tumors. Potential diagnostic implications. Am J Pathol 147: 815-822, 1995.
18. Gabius HJ, Brehler R, Schauer A and Cramer F: Localization of endogenous lectins in normal human breast, benign breast lesions and mammary carcinomas. Virchows Arch B Cell Pathol Incl Mol Pathol 52: 107-115, 1986.

19. Castronovo V, Van Den Brule FA, Jackers P, Clausse N, Liu FT, Gillet C and Sobel ME: Decreased expression of galectin-3 is associated with progression of human breast cancer. J Pathol 179: 43-48, 1996.
20. Martins L, Matsuo SE, Ebina KN, Kulcsar MA, Friguglietti CU and Kimura ET: Galectin-3 messenger ribonucleic acid and protein are expressed in benign thyroid tumors. J Clin Endocrinol Metab 87: 4806-4810, 2002.
21. Niedziela M, Maceluch J and Korman E: Galectin-3 is not an universal marker of malignancy in thyroid nodular disease in children and adolescents. J Clin Endocrinol Metab 87: 4411-4415, 2002.
22. Van den Brule FA, Waltregny D, Liu FT and Castronovo V: Alteration of the cytoplasmic/nuclear expression pattern of galectin-3 correlates with prostate carcinoma progression. Int J Cancer 89: 361-367, 2000.