

ORIGINAL**Clinical significance of Galectin-3 in clear cell renal cell carcinoma**

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Abstract : Overexpression of galectin-3 in a variety of cancer cell lines has been shown to correlate with tumor progression and metastasis. In this study, we investigated the expression of galectin-3 in clear cell renal cell carcinoma (CC-RCC) and evaluated the relationship between galectin-3 expression levels and the clinicopathological features of CC-RCC. Expression of galectin-3 in the kidney cancer cell lines Caki-1, Caki-2, A704, ACHN and KPK-1 were evaluated using western blot analysis, while galectin-3 expression in CC-RCC tissues and normal parenchyma were measured by real-time PCR and immunohistochemistry. We found that galectin-3 was overexpressed in the Caki-1, Caki-2, A704, ACHN and KPK-1 cell lines and that the expression level in CC-RCC was also significantly higher than that in renal parenchyma obtained from the same patient samples ($p=0.039$). Galectin-3 expression in CC-RCC with distant metastasis was also significantly higher than that in CC-RCC without distant metastasis ($p=0.045$). In conclusion, we revealed that galectin-3 is highly expressed in CC-RCC, especially in CC-RCC with distant metastasis, suggesting that galectin-3 may serve as a novel target molecule for predicting CC-RCC metastasis. *J. Med. Invest.* 57 : 152-157, February, 2010

Keywords : clear cell renal cell carcinoma, galectin-3, prognosis

INTRODUCTION

Galectins are members of the animal lectins, a family of carbohydrate-binding proteins characterized by their affinity for β -galactoside and by a conserved sequence of the carbohydrate recognition domain. Fourteen members of the galectin family have been identified to date (1-3). Galectin-3 is a 31-kDa gene product that serves as an intracellular and extracellular lectin and that is thought to interact with glycoproteins located on the cell surface

matrix (1-2). Galectin-3 is broadly expressed in numerous tumors, including renal cell carcinoma (RCC), and has been shown to be involved in numerous cellular processes including growth, proliferation, differentiation, adhesion, cell-cycle progression, angiogenesis and apoptosis, mainly through its binding to glycoproteins (4-11). Clinical evidence has shown that the expression of galectin-3 is also associated with carcinogenesis and malignant potential in the head and neck, thyroid, breast, gastrointestinal tract, pancreas, uterus and bladder (12-18).

Recent array studies have revealed that galectin-3 is up-regulated in conventional RCC (19-20). In the present study, we investigated the expression of galectin-3 in clear cell RCC (CC-RCC) and revealed significantly higher galectin-3 expression in CC-RCC than in renal parenchyma obtained from the

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same patients. We also found that the expression level of galectin-3 in CC-RCC with distant metastasis was significantly higher than that in CC-RCC without distant metastasis.

MATERIALS AND METHODS

Cell Culture

The human kidney cancer cell lines Caki-1, Caki-2, A704, ACHN and KPK-1 were obtained from ATCC (Manassas, VA, USA). The cells were maintained in RPMI 1640 containing 2 mM glutamine, penicillin-streptomycin (Life Technologies, Inc., Grand Island, NY, USA) and 10% fetal bovine serum (FBS) in 5% CO₂ at 37°C.

Patient Characteristics

We studied CC-RCC samples obtained from 66 patients with CC-RCC who underwent nephrectomy between 1996 and 2006 at the Department of Urology, the University of Tokushima, Japan. Informed consent was obtained from all patients prior to inclusion in this study, and the Research Ethics Committee of the University of Tokushima Faculty of Medicine approved the study. Of the 66 patients, 41 were male and 25 were female. The patients had a mean age of 61.9 years (range : 40 to 81 years). Any patients with a renal tumor that was histologically different from CC-RCC were excluded from the study. None of the patients had received neoadjuvant chemotherapy or interferon and/or interleukin-2 (IL-2) immunotherapy. Tumors were staged according to the TNM staging classification of the International Union Against Cancer (21). Staging procedures included physical examination, chest radiography, ultrasonography and computerized tomography. If caval invasion was suspected, magnetic resonance scanning was performed. Patients with elevated serum alkaline phosphate levels or skeletal symptoms were assessed using bone scintigraphy and radiography. As presented in Table 1, the patients were staged as follows : 27 in stage I, 21 in stage II, 4 in stage III and 14 in stage IV. Fourteen patients had tumors with distant metastasis including 13 with lung metastasis, 3 with bone metastasis, 1 with liver metastasis, 1 with pancreatic metastasis, 1 with adrenal metastasis and 1 with lymph node metastasis. Five patients demonstrated multiple organ metastasis. Tumor nuclear grading was performed according to the 1997 TNM grading system (22). The grade distribution was as follows :

Table 1 Clear cell renal cell carcinoma patient characteristics

Characteristics	No. of patients
Age	
40-81 years (mean, 61.9 years)	
Sex	
Male	41
Female	25
Clinical stage	
I	27
II	21
III	4
IV	14
Histologic grade (G)	
G1	18
G2	32
G3	16
Metastasis (M)	
M0	52
M1	14
Follow-up period	
6.9-129.0 months (median, 38.6 months)	

18 in grade 1, 32 in grade 2 and 16 in grade 3. Follow-up examination ranged from 6.9 to 129.0 months (median : 38.6 months).

Western Blot Analysis

Western blot analyses were performed using the enhanced chemiluminescence (ECL) detection system (GE Healthcare, Piscataway, NJ, USA) as described previously (9-10). Aliquots of protein (20 µg) were separated on a 12.5% SDS-PAGE gel using the method of Laemmli and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 5% non-fat dry milk in phosphate buffered saline (PBS)-0.1% Tween 20 at 4°C overnight. The membrane was then incubated with primary antibody for 1 hour. Immunoreactivity was detected by sequential incubation with horseradish peroxidase (HRP)-conjugated secondary antibody and ECL reagents. Antibodies were obtained from the following sources : anti-galectin-3 monoclonal antibody (TIB166 ; American Type Culture Collection) ; anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Sigma-Aldrich, St. Louis, MO, USA), horseradish peroxidase (HRP)-conjugated anti-mouse antibody and anti-rat antibody (Invitrogen, Carlsbad, CA, USA).

Relative Semi-Quantitative RT-PCR

Expression of galectin-3 in the 66 CC-RCC tissues and normal parenchyma obtained from patients with CC-RCC were measured using real-time PCR as described previously (22). Tissue samples for RNA extraction were immediately submerged in RNA Later, incubated at 4°C overnight and stored at -80°C until required. Total RNA was then extracted using the RNeasy kit (Qiagen, Inc., Valencia, CA) following the manufacturer's instructions. To avoid DNA contamination, RNA was digested with RNase-free DNase (Qiagen) as recommended by the supplier. Total RNA was quantified using a UV spectrophotometer, and the quality and integrity was assessed on a 1.5% agarose gel. Total RNA (1 µg) was then reverse transcribed using the First-Standard cDNA Synthesis Kit (GE Healthcare, Piscataway, NJ, USA) according to the protocol of the manufacturer. To examine the expression levels of galectin-3 and the housekeeping gene GAPDH, real-time quantitative PCR was performed using the Roche LightCycler® System (Roche, Diagnostic GmbH, Mannheim, Germany) and SYBR Green I dye. The sense and antisense primer sequences for galectin-3 have been described previously (22). For GAPDH, the PCR reaction was carried out using the LightCycler™-Primer Set ready-to-use amplification primer mix for RT-PCR. For each sample, the amount of target and endogenous control (GAPDH) were determined using a calibration curve. The amount of the target was calculated as a ratio of the number of target (T) gene copies to the housekeeping (H) gene copies.

Immunohistochemical Staining

Tissue samples obtained from patients with CC-RCC were frozen in OCT compound (Tissue-Tek, Sakura Fine Chemical, Tokyo, Japan) and stored at -80°C as described previously (22). Serial 10-µm sections were cut using a cryotome (CM-501, Tissue-Tek) and mounted on glass slides. Sections were then dried and fixed with 4% paraformaldehyde in 0.1 M PBS for 15-30 min at 20°C. The sections were incubated with 1% bovine serum albumin (BSA) in PBS for 1 h at 20°C and then with rat monoclonal antibody against galectin-3 (1 : 500 ; TIB166 ; American Type Culture Collection) for 2-3 hours at 20°C. After rinsing in PBS, the sections were further incubated with fluorescein isothiocyanate (FITC)-labeled anti-rat IgG (1 : 200 ; Jackson ImmunoResearch Laboratories Inc., West Grove,

PA, USA) in 1% BSA in PBS for 2 h at 20°C. 4', 6-diamidino-2-phenylindole (DAPI, Kierkegaard and Perry Laboratories, Gaithersburg, MD, USA) was then applied for 15 min to stain the nuclei. After this, sections were mounted with Vectashield (Vector Laboratories, INC, Burlingame, CA, USA) and examined using a confocal laser-scanning light microscope (Bio-Rad Radiance 2000, Bio-Rad Laboratories, Hercules, CA, USA).

Statistical Analysis

Statistical analyses were performed using StatView 5.0 software (Abacus Concepts, Berkeley, CA, USA). Real-time PCR data is presented as the mean ± SE for each of the clinicopathological variables such as stage and grade, and the statistical significance was determined using Student's *t* test and differences were considered statistically significant at $p < 0.05$. The Kaplan-Meier method and log-rank test were used to compare group differences in cause-specific survival. Values are presented as the mean (SEM) and differences were considered significant at $p < 0.05$.

RESULTS

The expression of galectin-3 in kidney cancer cell lines and CC-RCC tissue

Overexpression of galectin-3 was observed in all of the human RCC cell lines investigated, including Caki-1, Caki-2, A704, ACHN and KPK-1 (Fig. 1).

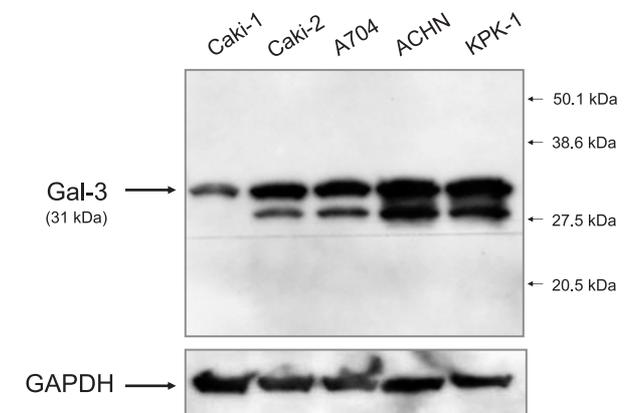


Fig. 1 Expression of galectin-3 in the renal cell carcinoma (RCC) cell lines Caki-1 Caki-2, A704, ACHN and KPK-1 by western blot analysis with 20 µg of protein loaded into each lane. The expression of anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was measured as an internal control. Overexpression of galectin-3 (31 kDa) was detected in all human RCC cell lines investigated.

The expression level of galectin-3 in CC-RCC obtained following nephrectomy was significantly higher than that in the normal renal parenchyma obtained from the same kidney (n=66, p=0.039, Fig. 2A). The expression level of galectin-3 in CC-RCC with distant metastasis (M1) was significantly

higher than that in CC-RCC without distant metastasis (M0, p=0.045, Fig. 2B). There was no significant correlation between galectin-3 expression and T category, N category, presence of tumor thrombus, number of metastases and metastatic organs (data not shown). There was also no difference in galectin-3 expression between CC-RCC with lung metastasis and CC-RCC with multiple organ metastasis (data not shown). Though the expression level of galectin-3 in grade 3 CC-RCC tended to be higher than that in grade 1 CC-RCC (p=0.098), there was no significant difference in expression levels of galectin-3 in each nuclear grade (G1 vs G2, p=0.43 and G2 vs G3, p=0.12, Fig. 2C).

To validate the RT-PCR data, we stained CC-RCC tissue samples with a galectin-3 antibody using immunofluorescence. We detected weak expression in the organ-confined low-grade (T1N0M0/G1) CC-RCC samples (Fig. 3A, 3C) and intense expression in advanced disease and high-grade (T3N0M1/G2>G3) CC-RCC samples (Fig. 3B, 3D). To determine whether galectin-3 expression was correlated with patient outcome, we divided the CC-RCC samples into subgroups of high and low expression using a variety of cutoff values. There was no significant difference between galectin-3 expression and clinical outcome (data not shown).

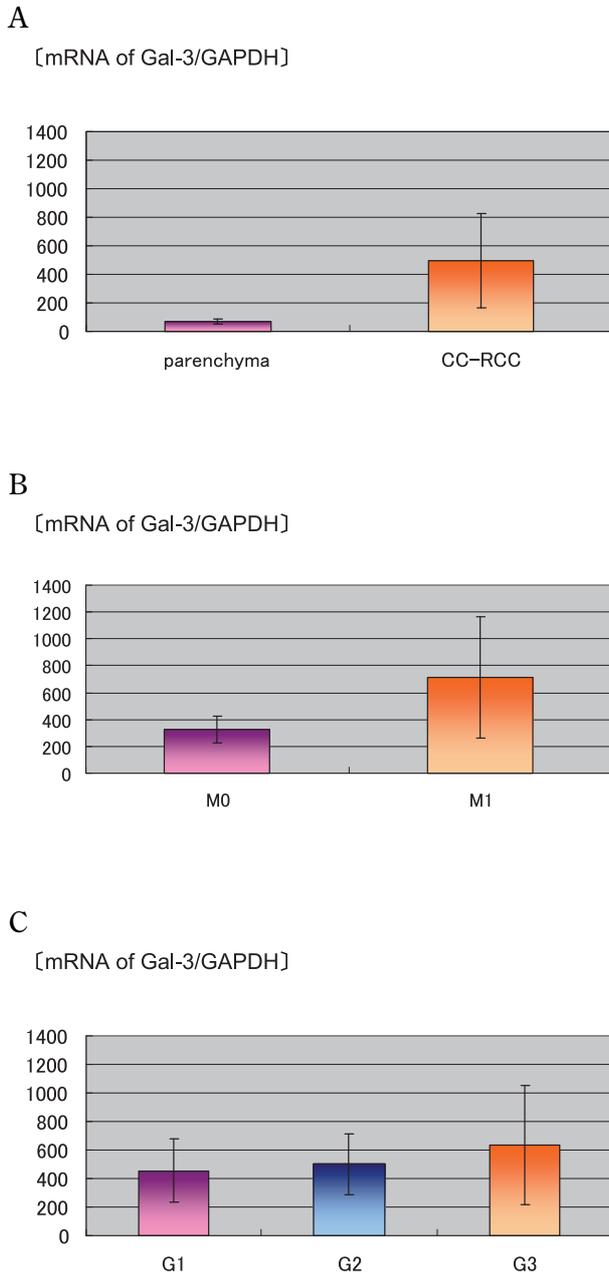


Fig. 2 Galectin-3 expression in normal parenchyma and clear cell renal cell carcinoma (CC-RCC) tissue samples obtained following nephrectomy. A, CC-RCC vs normal parenchyma. A significantly higher mean ratio of galectin-3 to GAPDH expression in the CC-RCC tumors was observed than in the normal kidney parenchyma tissues (p=0.039). B, M0 vs M1. Patients with M1 demonstrated a significantly higher galectin-3 expression level than patients with M0 (p=0.045). C, The various histological grades of galectin-3 expression. Patients with grade 3 (G3) CC-RCC tended to be higher than those in grade 1 (G1) CC-RCC (p=0.098).

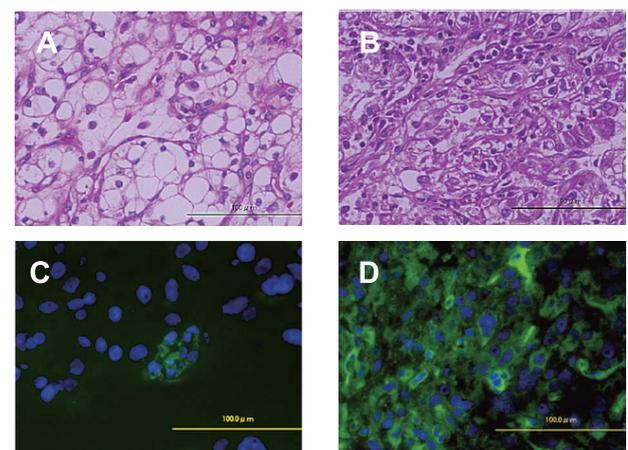


Fig. 3 Microscopic analysis of the CC-RCC samples. A and C, sections obtained from a T1N0M0/G1 CC-RCC sample and B and D, sections obtained from a T3aN0M1/G2>G3 CC-RCC sample. Hematoxylin and eosin were used to stain the sections presented in A and B. Representative images of the immunofluorescence staining of CC-RCC tissues using monoclonal anti-galectin-3 antibody are presented in C and D. We observed weak expression of galectin-3 (green) in the T1N0M0/G1 CC-RCC sample (C) and intense expression in the T3aN0M1/G2>G3 CC-RCC sample (D). 4', 6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei (blue). Immunohistochemical analysis of galectin-3 expression revealed both cell surface and cytoplasmic staining in the RCC specimens. Scale bar=100 μm.

DISCUSSION

Galectin-3 is a member of the galectin gene family and is expressed at elevated levels in a variety of cancers (23). In renal cell carcinoma, it has been shown that galectin-3 expression was significantly higher in conventional CC-RCCs and chromophobe RCCs (19, 20). Galectin-3 expression has also been associated with tumor progression and metastasis through its role in the regulation of cellular growth, adhesion, proliferation, cell-cycle progression, angiogenesis and apoptosis (4-11). Galectin-3 is localized not only in intracellular spaces such as the cytoplasm and the nucleus, but also in extracellular spaces such as the cell surface and the extracellular matrix (24). We have reported previously that secreted extracellular galectin-3 induced apoptosis of T-cells, suggesting that galectin-3 may be important for the immune escape mechanisms operating during tumor progression (10). Therefore, galectin-3 may play an important role in CC-RCC progression as CC-RCC is sensitive to immune therapy treatments including interferon and IL-2 administration.

In the present study, we evaluated galectin-3 expression in both RCC cell lines and surgical specimens of CC-RCC using real-time quantitative RT-PCR and analyzed its relationships with the clinicopathological factors of CC-RCC. We revealed that galectin-3 was overexpressed in adenocarcinoma (A704, ACHN and KPK-1) and CC- (Caki 1, Caki 2) RCC cell lines. In addition, galectin-3 expression was up-regulated in CC-RCC compared to that in normal kidney tissues, suggesting that the up-regulation of galectin-3 is an early event in CC-RCC development. The expression level of galectin-3 was also higher in advanced disease (M1) than in organ-confined (M0) tumors, suggesting that galectin-3 might be associated with RCC metastasis. Results from the confocal laser microscopy examination confirmed intense expression of galectin-3 in advanced tumors compared to organ-confined tumors. These results were also supported by our RT-PCR results. We found that there was no significant correlation in the survival rate between high galectin-3-expressing CC-RCC and low galectin-3-expressing CC-RCC. Taken together, these findings demonstrate that the expression of galectin-3 correlates with the presence of metastasis. If galectin-3 secreted from RCC induces apoptosis in T cells as we have previously shown (10), this induction of T-cell apoptosis by secreted galectin-3 potentially provides novel

insight into the immune escape mechanisms underlying CC-RCC. Further examination is required to fully investigate these hypotheses.

CONCLUSIONS

Galectin-3 is highly expressed in kidney cancer cell lines and CC-RCC and especially in CC-RCC with distant metastasis. We found that there was no positive correlation between galectin-3 expression and prognosis in CC-RCC. These results suggest that galectin-3 serves as a target molecule for predicting metastasis of RCC. Further, more detailed study may lead to the elucidation of galectin-3-regulated microenvironments in CC-RCC.

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REFERENCES

1. Barondes SH, Cooper DN, Gitt MA, Leffler H : Galectins : structure and function of a large family of animal lectins. *J Biol Chem* : 269 : 20807-20810, 1994
2. Barondes SH, Castronovo V, Cooper DN, Cummings RD, Drickamer K, Feizi T, Gitt MA, Hirabayashi J, Hughes C, Kasai K, Leffler H, Liu F, Lotan R, Mercurio AM, Monsigny M, Pillai S, Poirer F, Raz A, Rigby PWJ, Rini JM, Wang JL : Galectins : a family of animal β -galactoside-binding lectins (Letter). *Cell* 76 : 597-598, 1994
3. Hughes RC : Galectins as modulators of cell adhesion. *Biochimie* 83 : 667-676, 2001
4. Perillo NL, Marcus ME, Baum LG : Galectins : versatile modulators of cell adhesion, cell proliferation, and cell death. *J Mol Med* 76 : 402-412, 1998
5. Yang RY, Liu FT : Galectins in cell growth and apoptosis. *Cell Mol Life Sci* 60 : 267-276, 2003
6. Inohara H, Raz A : Functional evidence that cell surface galectin-3 mediate homotypic cell adhesion. *Cancer Res* 55 : 3267-3271, 1995
7. Inohara H, Akahani S, Raz A : Galectin-3 stimulates cell proliferation. *Exp Cell Res* 245 :

- 294-302, 1998
8. Nangia-Makker P, Honjo Y, Sarvis R, Akahani S, Hogan V, Pienta KJ, Raz A : Galectin-3 induces endothelial cell morphogenesis and angiogenesis. *Am J Pathol* 156 : 899-909, 2000
 9. Yoshii T, Fukumori T, Honjo Y, Inohara H, Kim HRC, Raz A : Galectin-3 phosphorylation is required for its anti-apoptotic function and cell cycle arrest. *J Biol Chem* 277 : 6852-6857, 2002
 10. Fukumori T, Takenaka Y, Yoshii T, Kim HR, Hogan V, Inohara H, Kagawa S, Raz A : CD29 and CD7 mediate galectin-3-induced type II T-cell apoptosis. *Cancer Res* 63 : 8302-8311, 2003
 11. Fukumori T, Oka N, Takenaka Y, Nangia-Makker P, Elsamman E, Kasai T, Shono M, Kanayama HO, Ellerhorst J, Lotan R, Raz A : Galectin-3 regulates mitochondrial stability and antiapoptotic function in response to anticancer drug in prostate cancer. *Cancer Res* 66 : 3114-3119, 2006
 12. Lotan R, Ito H, Yasui W, Yokozaki H, Lotan D, Tahara E : Expression of a 31-kDa lactoside-binding lectin in normal human gastric mucosa and in primary and metastatic gastric carcinomas. *Int J Cancer* 56 : 474-480, 1994
 13. Xu XC, el-Naggar AK, Lotan R : Differential expression of galectin-1 and galectin-3 in thyroid tumors. *Am J Pathol* 147 : 815-822, 1995
 14. Choufani G, Nagy N, Saussez S, Marchant H, Bisschop P, Burchert M, Danguy A, Louryan S, Salmon I, Gabius HJ, Kiss R, Hassid S : The levels of expression of galectin-1, galectin-3, and the Thomsen-Friedenreich antigen and their binding sites decrease as clinical aggressiveness increases in head and neck cancers. *Cancer* 86 : 2353-2363, 1999
 15. Schwarz G Jr, Remmelink M, Decaestecker C, Gielen I, Budel V, Burchert M, Darro F, Danguy A, Gabius HJ, Salmon I, Kiss R : Galectin fingerprinting in tumor diagnosis. Differential expression of galectin-3 and galectin-3 binding sites, but not galectin-1, in benign vs. malignant uterine smooth muscle tumors. *Am J Clin Pathol* 111 : 623-631, 1999
 16. Castronovo V, Van Den Brùle FA, Jackers P, Clausse N, Liu FT, Gillet C, Sobel ME : Decreased expression of galectin-3 is associated with progression of human breast cancer. *J Pathol* 179 : 43-48, 1996
 17. Shimamura T, Sakamoto M, Ino Y, Shimada K, Kosuge T, Sato Y, Tanaka K, Sekihara H, Hirohashi S : Clinicopathological significance of galectin-3 expression in ductal adenocarcinoma of the pancreas. *Clin Cancer Res* 8 : 2570-2575, 2002
 18. Cindolo L, Benvenuto G, Salvatore P, Pero R, Salvatore G, Mirone V, Prezioso D, Altieri V, Bruni CB, Chiariotti L : Galectin-1 and galectin-3 expression in human bladder transitional cell carcinomas. *Int J Cancer* 84 : 39-43, 1999
 19. François C, van Velthoven R, De Lathouwer O, Moreno C, Peltier A, Kaltner H, Salmon I, Gabius HJ, Danguy A, Decaestecker C, Kiss R : Galectin-1 and galectin-3 binding pattern expression in renal cell carcinomas. *Am J Clin Pathol* 112 : 194-203, 1999
 20. Young AN, Amin MB, Moreno CS, Lim SD, Cohen C, Petros JA, Marshall FF, Neish AS : Expression profiling of renal epithelial neoplasms : a method for tumor classification and discovery of diagnostic molecular markers. *Am J Pathol* 158 : 1639-1651, 2001
 21. Fleming ID : *AJCC Cancer Staging Manual*, Ed. 5, Lippincott-Raven, Philadelphia 1997, pp. 231-232
 22. Elsamman EM, Fukumori T, Tanimoto S, Nakanishi R, Takahashi M, Toida K, Kanayama HO : The expression of prostate stem cell antigen in human clear cell renal cell carcinoma : a quantitative reverse transcriptase-polymerase chain reaction analysis. *BJU Int* 98 : 668-673, 2006
 23. Takenaka Y, Fukumori T, Raz A : Galectin-3 and metastasis. *Glycoconj J* 19 : 543-549, 2004
 24. Wang JL, Werner EA, Laing JG, Patterson RJ : Nuclear and cytoplasmic localization of a lectin-ribonucleoprotein complex. *Biochem Soc Trans* 20 : 269-274, 1992