Clinicopathological significance of galectin-1 expression and percentage of galectin-1-expressing T cells in clear-cell renal cell carcinoma

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Abstract

Introduction: This study investigates the clinical significance of galectin-1 expression in carcinoma tissues, plasma, and lymphocytes of patients with clear-cell renal cell carcinoma (RCC).

Methods: Galectin-1 expression was investigated, using immuno-histochemistry, in 91 clear-cell RCC tissue sections, five angioleiolipomas tissue sections, and three oncocytomas tissue sections. As controls, normal tissue sections adjacent to each tumour and six benign renal tumour sections were examined. Plasma galectin-1 levels as measured by ELISA were compared in 39 patients. Proportions of galectin-1 expressing CD4$^+$ and galectin-1 expressing CD8$^+$ T lymphocytes in peripheral blood of these patients were detected by flow cytometry.

Results: The positive expression rate of galetin-1 in 91 clear-cell RCC tissues sections by immunohistochemistry was 87 (95.6%), with weak expression rate of 35.2 (32/91), moderate expression rate of 51.6% (47/91), and strong expression rate of 13.2% (12/91); whereas 25% (2/8) of renal benign tumour sections showed weak galectin-1 expression, 91.2% (83/91) of non-tumor tissues adjacent to carcinomas had negative expression of galectin-1, and another six (75%) renal benign tumour sections had negative galectin-1 expression. Plasma galectin-1 levels between patients with clear-cell RCC and with benign tumours were not significantly difference (p>0.05). In patients with clear-cell RCC, we found a significantly higher proportion of galectin-1-expressing CD4$^+$ lymphocytes (p<0.05) and galectin-1-expressing CD8$^+$ lymphocytes (p<0.05) than in patients with benign tumours. Moreover, the level of galectin-1 expression was positively associated with stage and Fuhrman grade of clear-cell RCC.

Conclusions: Our results suggest that high level of galectin-1 expression in clear-cell RCC tissues may be a useful marker for clear-cell RCC. Our findings also reveal a new clinical significance of galectin-1 — that high proportions of galectin-1-expressing CD4$^+$ and CD8$^+$ lymphocytes were positively associated with poor clinicopathological features.

Introduction

Renal cell carcinoma (RCC) accounts for approximately 3% of adult malignancies.[1] The incidence of RCC increased over the last two decades in all regions of the world and across ethnic groups. Although RCC develops insidiously, over 200 000 new cases of kidney cancer are diagnosed worldwide each year.[2] This improved detection is attributed to medical evaluation for unrelated conditions, routine health checkups, and improved methods of detection, such as computed tomography (CT) or magnetic resonance imaging (MRI). Despite increased detection of RCC, its prognosis varies widely; about 30% of patients have metastatic disease when diagnosed and approximately 30% of patients present with local or distant recurrence after radical or partial nephrectomy.[3,4] Apart from RCC being highly resistant to chemotherapy, radiation, and immunotherapy, there are also few predictive or prognostic markers identified. The identification of a prognostic marker for RCC would hasten the development of new therapeutic strategies and improve survival of patients with RCC.

The galectins are a family of 15 mammalian galactoside-binding proteins that share a consensus amino acid sequence in their carbohydrate recognition domains (CRDs).[5] Galectin-1 was the first described member of the galectins family; it is a 14 KDa protein consisting 135 amino acids.[6] Galectin-1 is expressed in some malignant tumours and is involved several biological activities, including cell adhesion, cell migration, cell apoptosis, cell proliferation, regulation of immunity, and tumour angiogenensis.[7]

RCC is classified as an immunogenic cancer because it shows spontaneous regression and occasionally responds to immunotherapy. However, for unknown reasons, the overall response rate of RCC to therapeutics remains low. And thus, it is common for RCC to evade immune surveillance. A previous study indicated that galectin-1 modulates T cell immune responses by virtue of a pro-apoptotic effect on Th subsets, induction of IL-10 synthesis, and Th2 cyto-

kines skewing. Moreover, galectin-1 promotes the formation of immune-privileged sites.[8] Little is known about role of galectin-1 in RCC. Therefore, in this study, we investigated galectin-1 expression in RCC and non-RCC tissue sections. Concurrently, we assessed galectin-1 expression on peripheral blood T lymphocytes.

Methods

Patient characteristics

Ninety-nine patients with a renal tumour were enrolled in our study at the Department of Urology, Harbin Medical University Cancer Hospital from 2013–2015. Of these patients, 99 underwent nephrectomy or partial nephrectomy. Informed consent was obtained from all patients before inclusion in this study. The study was conducted in accordance with the Declaration of Helsinki. Kidney cancer was staged according to the TNM staging classification of the International Union against Cancer. All of the patients were confirmed to be free of any malignancies other than renal tumour. None of the patients had received any type of therapy before being admitted to the hospital. The mean age of the patients was 53 years (range 36–67). Of the 99 patients, 91 patients were confirmed as clear-cell RCC. Postoperative pathological examinations were done on 63 male and 28 female patients, from which another eight patients were diagnosed with renal benign tumour that consisted of five angioleiolipomas and three oncocytomas. The patients were staged as follows: 48 in stage T1, 29 in stage T2, 11 in stage T3, and three in stage T4. The Fuhrman grade distribution was as follows: 43 were grade 1, 29 were grade 2, 16 were grade 3, and three were grade 4. There were no statistically significant differences in age and body mass index (BMI) among participants.

Blood samples preparing

Blood samples (10 ml) were obtained from the antecubital vein into heparinized tube of 93 patients on the first day after hospital admission. For control samples, we obtained 10 blood samples from health donors. Thereafter, plasma was prepared by centrifugation of peripheral blood at 3000 g for 25 minutes, followed by storage at -80 °C until use. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood samples by the standard Ficoll-Hypaque (Shanghai Reagent Secondary Factory, Shanghai, China) density-gradient centrifugation method and were used immediately for flow cytometry analysis.

Flow cytometry analysis

The isolated mononuclear cells were washed twice using phosphate-buffered saline (PBS). Non-specific binding sites were blocked by incubation with mouse serum for 10 minutes at room temperature. Cells were stained with mononuclear antibody PE-labeled anti-human CD4 and mononuclear antibody (APC)-labeled anti-human CD8 (BD Pharmingen, San Diego, CA, U.S.) for 15 minutes in the dark at room temperature and then washed twice with PBS. Cells were centrifuged and the supernatant was removed. Cells were incubated in 1000 μl 1 × permeabilizing solution (eBioscence, San Diego, C, U.S.) for 10 minutes at room temperature in the dark. Thereafter, cells were washed twice with PBS and were treated with rabbit anti- human galectin-1 (LGALS1) antibody (Epitomics, Burlingame, CA, U.S.) for 30 minutes at room temperature in the dark. After washing off unbound antibodies, the cells were stained with FITC-labeled anti-rabbit IgG (H+L) antibody (KPL, Gaithersberg, MD, U.S.) for 15 miutes at room temperature in the dark. After washing twice with PBS, the cells were resuspended in 4% paraformaldehyde in PBS. Flow cytometry analysis was performed on BD FACSCanto II (BD Accuri Cytometers, MI, U.S.) and data was processed using CellQuest Pro software (BD Biosciences).

Immunohistochemistry

All tissue samples were collected immediately after surgery. All sample tissues included the primary tumour and the adjacent normal-appearing tissue up to 0.5–2 cm from the margin of tumour site.[9] Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded, 4 μm thick tissue sections. Tissue sections were deparaffinized three times in xylene for a total of 15 minutes and subsequently rehydrated. To block endogenous peroxidase activity, the sections were incubated with 3% hydrogen peroxidase for 10 minutes. The rabbit anti- human galectin-1 (LGALS1) antibody incubation was carried out for two hours at 37 °C at an antibody dilution of 1:300. The sections were washed triple with PBS. Next, the sections were incubated for 30 minutes at room temperature with a biotinylated goat anti-rabbit IgG antibody (1:200; Vector Laboratories Inc., Burlingame, CA, U.S.). And finally, samples were incubated with peroxidase-conjugated streptavidin (Zhongshan gold bridge, Beijing, China). Colour was developed by incubating the slides for 5 min with diaminobenzidine and nuclei were counterstained with hematoxylin. For negative controls, the primary antibodies were replaced with PBS. Positive controls were provided by the kit supplier. The results were assessed by two independent pathologists who had no knowledge of the patient clinical status.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from RCC tissues and adjacent kidney tissues using Trizol reagent (Invitrogen, Beijing China). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using a SYBR Premix Ex Taq™ Reverse Transcription-PCR kit (TaKaRa, Shiga, Japan). The primers for human galectin-1 were 5'-GAGGTGGCTCCTGACGCTAA-3' (forward) and 5'-CCTTGCTGTTGCACACGATG-3' (reverse). The primers for β-actin were 5'-AGAAAATCTGGCACCACACC-3' (forward) and 5'-TAGCACAGCCTGGATAGCAA-3' (reverse). To synthesize first-strand cDNA from total RNA, each mixture reaction was incubated at 41°C for 30 minutes. Thereafter, qPCR was performed at 95°C for 30 seconds pre-denaturation, followed by 40 cycles of 95 °C for five seconds, 60 °C for 34 seconds. The ratio of galectin-1 to β-actin expression was calculated. All samples were run in triplicate.

Western blotting analysis

Western blotting was performed according to the previously described. Briefly, the tissues were lysed in SDS buffer and 100 μg proteins was separated on 10% gradient SDS-polyacrylamide gels, transferred to PVDF, and incubated with rabbit anti-galectin-1 antibody (1:500) overnight at 4 °C. After incubation with peroxidase-conjugated rabbit anti-mouse IgG antibody (Cell Signaling Technologies, Beverly, MA, U.S.), β-actin was used as the loading control. After incubation with enhanced chemoluminescence reagent (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.), protein expression was visualised after exposure to X-ray film.

ELISA

Patient plasma galectin-1 levels in were determined with a specific enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, U.S.), as previously described ELISA protocol.[10] A 96-well plate was coated overnight at 4 °C with mouse monoclonal anti-galectin-1 antibody and locked with 1% bovine serum albumin/PBS for one hour at room temperature before applying plasma for one hour at room temperature. Subsequently, wells were incubated with rabbit polyclonal anti-galectin-1 antibody for one hour followed by 30-minute incubation with biotin-conjugated goat anti-rabbit IgG antibody (1:400). Finally, horseradish peroxidase (HRP)-conjugated streptavidin was applied for 30 minutes and staining reaction was initiated by adding TMB staining solution. Reaction was stopped by adding one volume of 1 mol/L H_2SO4, and absorbance was measured at 450 nm. During all incubation steps, the wells were washed.

Statistical analysis

Plasma galectin-1 concentrations and percent of galectin-1 positive CD4+T cells or galectin-1 positive CD8+ T cells were compared between the RCC group and the renal benign tumour group. Statistical analysis was performed using SSPS software (version 19.0; Inc, IBM). The significance of differences was tested by various non-parametric methods, such as the Mann-Whitney test. Spearman correlation test was used to evaluate the correlation between tumour nuclear grade and T cells expressed galectin-1. $P<0.05$ was considered significant.

Results

Expression of galectin-1 in RCC tissues and adjacent non-carcinoma tissues

Using immunohistochemistry, we examined galectin-1 expression in 91 clear-cell RCC tissue sections. As controls, normal tissue sections adjacent to each tumour and eight benign renal tumour sections were examined. The benign renal tumours included three oncocytomas and five angioleiolipoma. We analyzed the amount of galectin-1 expression in different tissues and found that galectin-1 expression was positive in 87/91 (95.6%) of clear-cell RCC samples. Of all of RCC samples, weak, moderate, and strong intensity of galectin-1 expression was seen in 32 (35.2%), 47 (51.6 %), and 12 sections (13.2%), respectively. In contrast, there was negative or weak expression of galectin-1 in both peritumoural normal tissue sections or in renal benign tumour sections; only 2/8 (25%) renal benign tumours showed a weak expression of galectin-1, and 91.2% (83/91) non-tumor tissues adjacent to carcinomas had negative expression of galectin-1. The results are shown in Fig. 1 A. Furthermore, we evaluated galectin-1 expression at protein and molecule levels in RCC, non-RCC, and oncocytoma, respectively. Expression levels of galectin-1 protein and mRNA in RCC were significantly higher when compared to the surrounding normal kidney and oncocytoma tissues (Figs. 1B, C, and D).

Intracellular galectin-1 expression of peripheral blood lymphocytes in patients with RCC and with non-RCC

Using flow cytometry, intracellular galectin-1 expression was measured in freshly isolated peripheral blood lymphocytes from 91 patients with RCC and eight patients with non-RCC. As shown in Figs. 2A and 2B, our results revealed that a higher proportion of galectin-1-expressing CD4+ helper and CD8+ cytotoxic T cells were present in patients with RCC compared to patients with non-RCC, such as oncocytoma and angioleiolipoma ($p<0.05$), however, there was no differ-
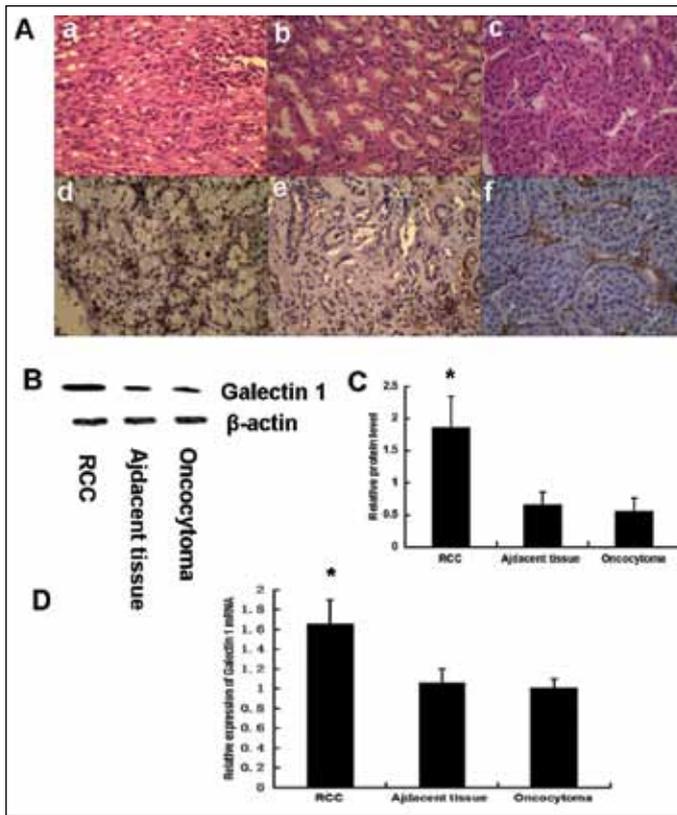
Fig. 1. Immunohistochemical evaluation of galectin-1 expression in renal cell carcinoma (RCC) tissues and adjacent normal tissues. **(A)(a–c)** Representative H&E staining of samples of RCC tissue, the renal benign tumour (oncocytoma) tissue and the adjacent normal renal tissues (original magnification 400×); **(d)** immunohistochemical evaluation showing increased galectin-1 expression in RCC compared with **(e)** adjacent normal tissue and **(f)** renal oncocytoma tissue (original magnification 400×); **(B and C)** Western blot analysis showing increased expression of galectin-1 protein in RCC; **(D)** real-time PCR showing increased galectin-1 mRNA expression in RCC when compared to the adjacent normal tissue samples and benign renal tumour samples (*p<0.05).

ence in the number of galectin-1-expressing CD4$^+$ or CD8$^+$ T cells between patients with RCC (p>0.05). Representative examples are displayed in Fig. 3A and B, and an isotype control is shown in Fig. 3C.

Relationship between Fuhrman grade and peripheral blood T cells expressed galectin-1

According to the Spearman correlation test, a significant positive correlation was seen between the percentage of galectin-1$^+$ CD4$^+$ T cells (p<0.01) or the percentage of galectin-1$^+$ CD8$^+$ T cells (p<0.01) and the Fuhrman grade. Similarly, no matter what percentage of galectin-1$^+$CD4$^+$ T cells or galectin-1$^+$CD8$^+$ T cell was present, there was a positive correlated with tumour stage (all p<0.01); however, there was no correlation with tumour side, age, or BMI (all p>0.05). Our results indicate that percentage of galectin-1$^+$ T cells may be a valuable predictive factor in clear-cell RCC. The correlations between galectin-1 expression and clinico-pathological features are shown in Table 1.

Circulating galectin-1 levels in patients with RCC or with non-RCC

Plasma galectin-1 levels ranged from 13.61–36.01 ng/ml (mean 23.62) and 22.62–29.09 ng/ml (mean 25.69) in RCC and non-RCC patients, respectively, prior to surgery for removal of tumours. As shown in Fig. 4, the results showed no statistically significant difference among the patient groups (p=0.436). In addition, clinical features of patients with RCC (i.e., tumour size and TNM stage) were not related to the circulating levels of galectin-1.
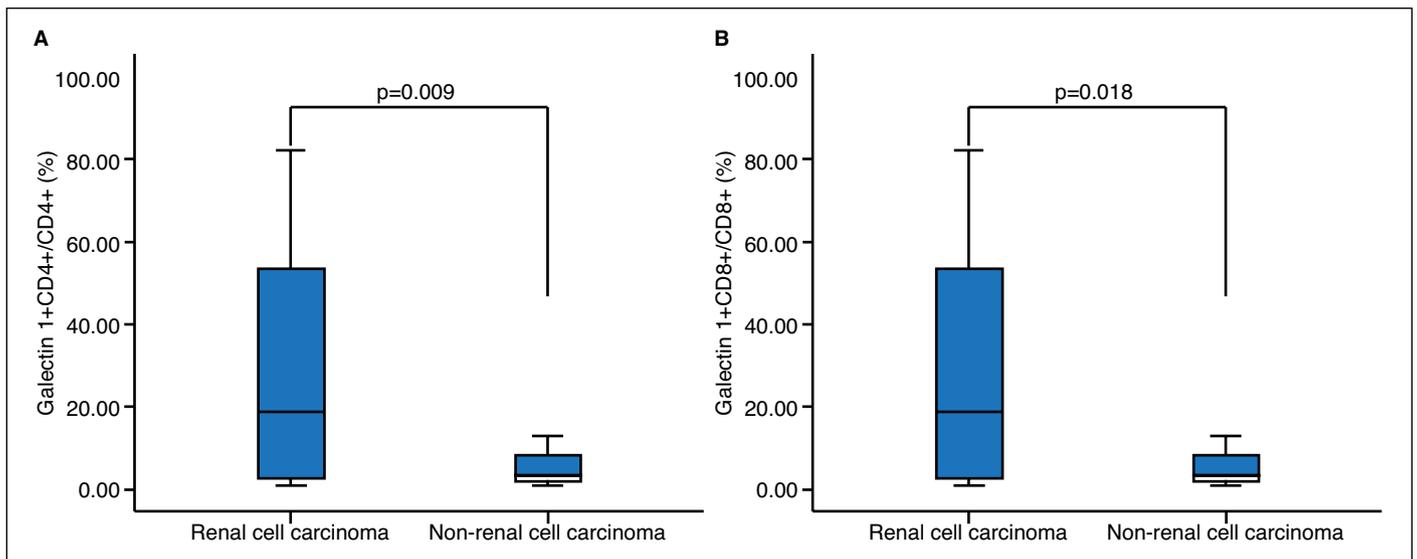

Fig. 2. Proportions of galectin-1-expressing CD4+ lymphocytes and galectin-1-expressing CD8+ lymphocytes in peripheral blood of patients with renal cell carcinoma and patients with non-renal cell carcinoma. **(A)** Proportions of galectin-1-expressing CD4+ lymphocytes; **(B)** proportions of galectin-1-expressing CD8+ lymphocytes.
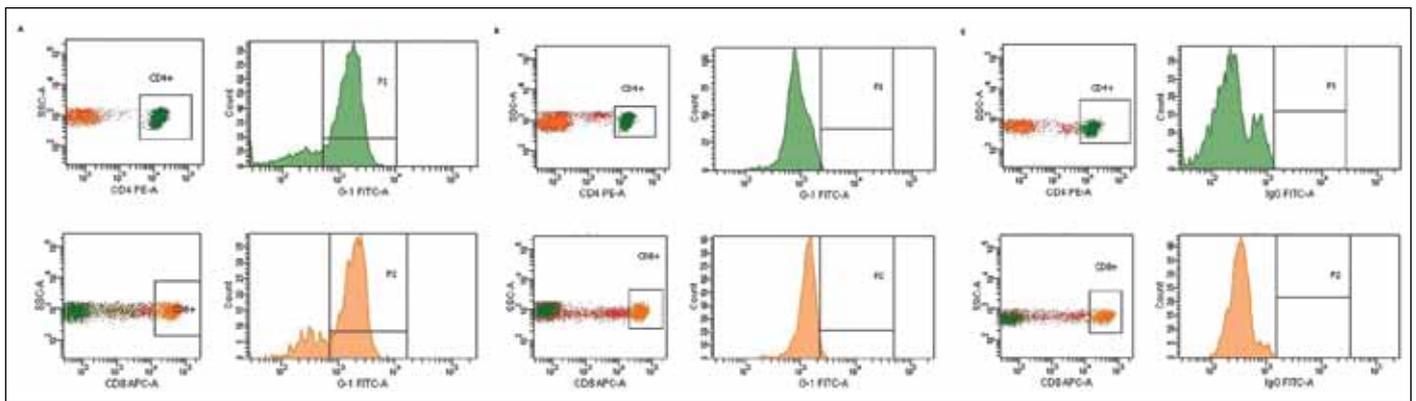
Fig. 3. Representative flow cytometric pattern showing intracellular galectin-1 expression of peripheral blood CD4+ and CD8+ lymphocytes in patients with renal cell carcinoma (RCC) and oncocytoma. *(A)* RCC patient; *(B)* oncocytoma patient; *(C)* isotype control.

Discussion

In this study, we investigated the relationship between galectin-1 and RCC. We demonstrated for the first time that high galectin-1 is expressed in tumour sections of patients with RCC, whereas galectin-1 expression appeared negative or weak in the peritumoural normal tissues and benign kidney

Table 1. Patients' characteristics

	Number	%
Gender		
Male	20	51.28
Female	19	48.72
Histology		
Clear-cell carcinoma	30	76.92
Chromophobic carcinoma	1	2.56
Mucinous tubular and spindle carcinoma	2	5.13
Renal benign tumour	6	15.38
Stage (T)		
T1a	22	66.67
T1b	9	27.27
T2a	1	3.03
T2b	1	3.03
T3	0	0.00
T4	0	0.00
Stage (N)		
N-	11	33.33
N+	0	0.00
Nx	22	66.67
Stage (M)		
M-	32	96.97
M+	1	3.03
Furhman grade		
1	18	54.55
2	9	27.27
3	6	18.18
4	0	0.00

tumour sections assessed. A recent report also indicated that expression of galectin-1 mRNA was significantly higher in RCC compared to the adjacent normal kidney tissue,[10] which is in line with our results. Moreover, a large proportion of peripheral blood CD4+ and CD8+ T cells had high galectin-1 expression. In addition, galectin-1 expression intensity is positively related to TNM stage and the nuclear Fuhrman grade of RCC. However, our study also revealed that circulating galectin-1 levels were not significantly altered in patients with RCC as compared to patients with benign tumours.

Our results support the idea that galectin-1 expression can reflect RCC aggressiveness because moderate to strong galectin-1 expression was observed in the RCC tissue sections in this study. According to the findings of several previous studies, galectin-1 expression was increased in several types of cancer, such as lung, colon, breast, prostate, ovarian cancer, and head and neck cancers.[11-16] It suggests that galectin-1 correlates with the malignant behaviour of many types of carcinoma. However, clinical significance concerning galectin-1 in RCC remains unclear. Our data provide the evidence that galectin-1 may be an important biomarker for RCC. Huang et al suggest that galectin-1 promotes kidney cancer progression through upregulation of CXCR4 via NF-κB; they concluded that both factors may be novel prognostic factors for RCC.[17] However, another study showed that galectin-1 mediates aggressiveness of RCC through the HIF-1α-mTOR signaling axis.[18]

We believe galectin-1 could affect progression of RCC through multiple signaling pathways; further studies are warranted to explore the relationship between galectin-1 expression and long-term survival of patients with RCC, and the possible regulation of progression by galectin-1 in RCC.

One of the most important findings of this study is that a significantly higher proportion of circulating CD4+ and CD8+ T cells expressed galectin-1 in patients with RCC than in patients with benign tumours. It is well-known that RCC is an immunogenic cancer resistant to current chemotherapy and radiation therapy. Moreover, low response to immunothera-
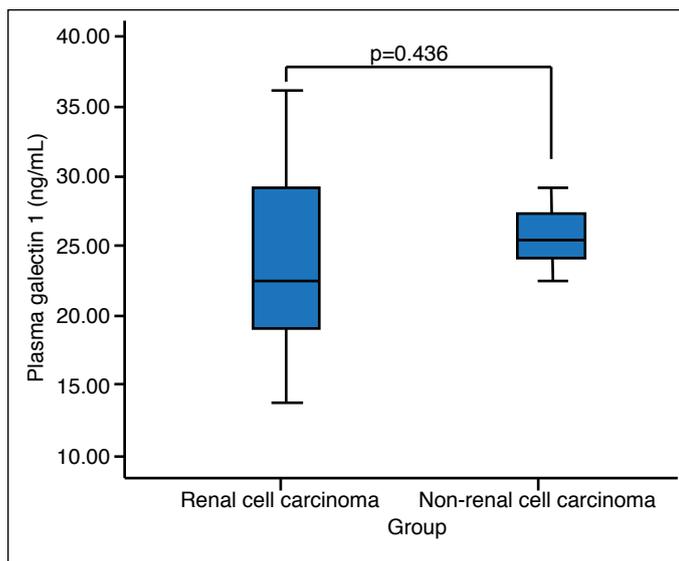
Fig. 4. Plasma concentrations of galectin-1 between patients with renal cell carcinoma and patients with non-renal cell carcinoma.

py also critically affects prognosis of patients with advanced and metastatic RCC. It is possible that many factors contribute to weaken the anti-RCC immune response. For example, our previous study indicated RCC can inhibit maturation of dendritic cells and increase regulatory T cells.[19] Both CD4+ and CD8+ T cells are important for effective anti-tumour immunity. However, high galectin-1 expression may result in impairment or attenuation of immune function in CD4+ and CD8+ T cells. According to previous studies, galectin-1 can block T cell activation, suppress T cell proliferation, and induce T cell death.[20-23] Furthermore, galectin-1 binding to activated Th cells also might be able to promote Tr1 cell differentiation.[24-26] Those combined effects, taken together with our new findings, suggest that galectin-1 may play an important role in escaping immune surveillance and promoting the establishment of immune-privileged sites in RCC. Additionally, low rates of response to immunotherapy in RCC may correlate with galectin-1 expression. It may help to evaluate or predict efficacy of immunotherapy by measuring galectin-1 expression in RCC. The fact that we did not find a significant difference in circulating plasma galectin-1 levels between patients with RCC and patients with benign kidney tumours could be explained by the findings that galectin-1 expression was mainly located in cytoplasm or that only a small amount of galectin-1 was secreted from either RCC cells or T cells. However, this contradicts recent research that showed plasma levels of galectin-1 were significantly higher in RCC patients than in healthy controls.[27] Differences in results between our study and the previous study could be due to patients enrolled in the study A major limitation of this study is the small sample size. The lack of long-term followup data on these patients is another limitation. It remains unclear how these galectin-1-expressing T cells

are promoting to immune-evasion in clear-cell RCC, which should be addressed in the future.

Conclusion

Our study suggested that expression levels of galectin-1 are significantly higher in RCC tissue sections than in adjacent normal tissue sections or in benign kidney tumours tissue sections. Moreover, galectin-1 expression is positively correlated with TNM stage and Fuhrman grade in RCC. More interestingly, we found that the majority of circulating CD4+ and CD8+ T cells showed intracellular galectin-1 expression in patients with RCC. In conclusion, galectin-1 may be an important marker in evaluating aggressive behaviour in RCC and may be involved in mediating tumour-immune privilege, thus making it a potential target for RCC immunotherapy in the future.

This paper has been peer-reviewed.

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