Ecto-5'-nucleotidase expression is associated with the progression of renal cell carcinoma

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Abstract. Renal cell carcinoma (RCC) is a common tissue tumor that occurs across all age groups and has become one of the types of cancer with the fastest increasing incidence. Due to the resistance of RCC chemo- and radiotherapy, surgery is the only currently effective treatment. Therefore, specific markers for the diagnosis and prognosis of RCC are expected to result in novel methods of treatment. Ecto-5'-nucleotidase, also termed cluster of differentiation (CD)73, is a protein that is activated in several types of aggressive cancer and may promote cancer progression. CD73 was examined in the present study to determine the association between the protein and RCC. The expression levels of CD73 in 159 RCC tissue sections and 30 paratumorous normal renal tissue samples obtained from 235 patients that underwent nephrectomy were examined by immunohistochemical staining. By contrast, the expression level of P-glycoprotein (P-gp), a potential prognostic factor in RCC, was also examined in 85 RCC and 13 normal tissue samples. Intense CD73 expression was identified in 75 out of 159 RCC cell membranes compared with normal renal tissues. In contrast, there was high P-gp expression in the blood vessels of 42 out of 85 RCC tissues and there was no significant difference between the P-gp expression identified in RCC cells (34 out of 85) and the cell membrane of normal renal cells (2 out of 13). The expression level of CD73 in RCC cells was significantly associated with tumor type, tumor node metastasis (TNM) stage, and tumor grade. However, the expression of P-gp in RCC cells was only associated with the TNM stage and tumor grade. Using a multivariable Cox regression analysis, it was found that the median survival rate of RCC patients with intense CD73 expression in RCC cells was 62.06±5.35 months, which was drastically shorter compared with rare CD73 expression (103.72±3.67 months). In conclusion, the expression level of CD73 is significantly associated with RCC tumor progression and may serve as a favorable marker for the diagnosis and prognosis of RCC, in addition to being a therapeutic target for the treatment of RCC.

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Introduction

Renal cell carcinoma (RCC) is the most common malignant kidney neoplasm that occurs across all age groups, and the disease accounts for ~3% of all cancers worldwide (1). RCC is the seventh leading cause of cancer in the USA, resulting in ~54,390 novel cancer cases and ~14,000 mortalities each year in the USA (2). In China, RCC has become one of the cancer types with the fastest increasing incidence, increasing at an annual rate of 6% (3). Improvements in diagnostic imaging technology have enabled more RCC cases to be diagnosed at an early stage and the five-year survival rate of patients with RCC has reached 67% (4). However, as RCC is insensitive to chemo- and radiotherapy, advanced RCC with systemic metastasis demonstrates a poorer prognosis and the five-year survival rate is <10% (5). RCC comprises several histological subtypes and ~75% of RCC lesions are clear cell carcinoma, which is associated with the worst prognosis (6). Therefore, specific markers for clear cell RCC are required to improve the early diagnosis and targeted therapy of RCC, and for patients to eventually demonstrate a good prognosis and outcome.

P-glycoprotein (P-gp) is a drug efflux pump that is widely associated with the chemoresistance of a variety of tumors (7,8). The expression of P-gp has been reported in untreated RCC (9,10) and is strongly associated with a differentiated RCC tumor phenotype (11-13). However, the use of P-gp as a specific biomarker for RCC remains controversial. Thus, additional specific biomarkers for RCC are required.

Ecto-5'-nucleotidase, also termed cluster of differentiation (CD)73, is a glycosyl phosphatidylinositol-linked membrane

protein found on the surface of a variety of cell types (14). CD73 was initially identified as a cell surface antigen that was specific to hematopoietic stem cells and functioned in a number of physiological processes in various normal human tissues, including hypoxic adaptation, ischemic preconditioning and inflammation (15-17). CD73 is reportedly activated in aggressive breast cancer, gastric and pancreatic cancers and lymphoma, and may be used as a prognostic marker for these tumors (18,19). The protective role of CD73 in renal ischemia (20) and the absence of tubuloglomerular feedback responses in CD73-deficient mice (14) have indicated the important role of CD73 in the function of renal tissue. Furthermore, abnormal expression of CD73 may be associated with the progression of RCC. However, the association between CD73 expression and RCC is unclear. The present study aimed to investigate the expression of CD73 and P-gp in clear cell RCC and to assess their potential significance for the clinical diagnosis and prognosis of RCC.

Patients and methods

Patients. Pathological tissues were collected from patients with RCC who had undergone surgery between January 2004 and July 2012 at the Second Hospital of Lanzhou University (Lanzhou, China). A total of 235 patients aged between 35 and 87 years of age (median, 58 years) that were diagnosed with RCC and 30 transitional-normal tissues collected from certain patients were selected for the present retrospective study. Pathological confirmation was conducted on standard sections stained with hematoxylin and eosin according to the World Health Organization guidelines (21). Clinical follow-up data were available for the majority of patients. The medical records of these patients were reviewed and the demographic characteristics, date of diagnosis, date of surgery, resection margin status, tumor stage, tumor size, tumor differentiation and five-year survival rates were retrieved. The tumor stage was assessed according to the pathological tumor-node-metastasis (TNM) staging system based on the tumor size and progression status, involvement of the lymph nodes, presence of distant metastasis and tumor differentiation, according to the medical records. In addition to these characteristics, the mortality reports were obtained by maintaining regular contact with close relatives of the enrolled patients. All clinical information was independently confirmed. This study was approved by the ethics committee of the Second Hospital of Lanzhou University and written informed consent was obtained from the families of the patients.

Immunohistochemical assay. The expression levels of CD73 and P-gp were determined using a two-step immunohistochemical assay procedure. Briefly, P-gp was detected using a PV-6000 Polymer Detection System (Zhongshan Golden Bridge Biotechnology, Co., Ltd., Beijing, China). Surgically resected specimens were fixed in 10% formalin and cut into 4-μm thick slices. The sections were subsequently mounted on Superfrost Plus slides (Zhongshan Golden Bridge Biotechnology, Co., Ltd.), de-waxed with xylene and gradually hydrated. Antigen retrieval was then achieved by pressure-cooking the samples in 0.01 M citrate buffer (pH 6.0) for 2 min, cooling to room temperature, washing with PBS and incubating in 3% hydrogen peroxide for 10 min. Monoclonal rabbit anti-human CD73 (cat. no. ab115289; Abcam, Cambridge, MA, USA) and monoclonal mouse anti-human P-gp (cat. no. zm0189; Zhongshan Golden Bridge Biotechnology, Co., Ltd.) antibodies were used as primary antibodies at a 1:200 dilution, with 50 μl of solution being used for each section. Mouse IgG (Abcam) was used as a control. The sections were incubated with the primary antibodies for 2 h at 37˚C, washed with PBS, incubated with horseradish peroxidase-conjugated polyclonal sheep anti-rabbit (cat. no. pv6000) or anti-mouse (cat. no. pv6000) IgG secondary antibodies (Zhongshan Golden Bridge Biotechnology, Co., Ltd.) for 30 min at 37˚C and then washed three times in 0.1% Tween-20 (Zhongshan Golden Bridge Biotechnology, Co., Ltd.). Subsequently, the sections were developed with diaminobenzidine tetrahydrochloride (DAB) and selected samples were counterstained with hematoxylin. The immunohistochemical assays were performed within seven days of section preparation. To prevent antigen degradation, the sections were stored at 4˚C prior to analysis.

Table I. Percentage and intensity grade of staining.

Level	Percentage of intensively stained cells, %	Intensity of staining
0	0	Low
1	<25	Weak
2	25≤ x <50	Moderate
3	50≤ x <100	Strong

Immunostaining evaluation. The results from the immunohistochemical staining of tissue slides were independently evaluated by two pathologists in a double-blind process. Immunohistochemical staining of the tissue slides indicated whether the stained CD73 and P-gp proteins were located in the cytoplasm, cell membrane or vascular wall. A semi-quantitative scoring system was developed (Table I) based on the staining intensity, with low, weak, moderate and strong staining being classified as levels 0, 1, 2 and 3, respectively, and the percentage of cells with intense staining, with levels 0, 1, 2 and 3 being defined as no cells positively stained, <25% cells stained, 26-50% cells stained and >50% cells stained, respectively. For each slide, three fields were evaluated. In addition, the slides were re-evaluated, classified according to the resulting scores, and the highest score was used for subsequent analyses.

Statistical analysis. The data were analyzed using SPSS software, version 19.0 (IBM, Armonk, NY, USA). Categorical data were reported as counts and percentages. Fisher's exact test and the χ^2 test for trends were used to assess the significance of associations between the expression of CD73 or P-gp. Associations between clinicopathological parameters were tested using the Mann-Whitney U test and the Kruskal-Wallis H test. Univariate survival analysis was performed according to the Kaplan-Meier method and differences in survival curves were assessed. Multivariate survival analysis was performed on all important parameters (14) using Cox's regression model. In

Table II. Immunohistochemical staining properties of CD73 and P-gp for two kinds of kidney cancer and transitional-normal tissues.

A, CD73 expression and location in different renal cell carcinoma

Location	Expression level	Normal renal tissue, n (%)	Clear cell RCC, n (%)	Uroepithelial carcinoma, n (%)	χ^2	P-value
Cell membrane	Rare	30 (100.00)	64 (51.61)	20 (57.14)	22.568	0.000
	Intensive	0 (0.00)	60 (48.39)[a]	15 (42.86)[a]		
Vascular wall	Rare	26 (86.67)	110 (88.71)	35 (100.00)	4.989	0.074
	Intensive	4 (13.33)	14 (11.29)	0 (0.00)		

B, P-gp expression and location

Location	Expression level	Normal renal tissue, n (%)	Clear cell RCC, n (%)	Uroepithelial carcinoma, n (%)	χ^2	P-value
Cell membrane	Absent	11 (84.6)	39 (65.0)	12 (48.0)	4.72	0.095
	Intensive	2 (15.4)	21 (35.0)	13 (52.0)		
Vascular wall	Absent	13 (100.0)	27 (45.0)	16 (64.0)	16.30	0.000[b]
	Intensive	0 (0.0)	33 (55.0)	9 (36.0)		

[a]P<0.05 between normal renal cells and different RCC, [b]P<0.05 between normal renal cells and different RCC. CD73, cluster of differentiation 73; P-gp, P-glycoprotein; RCC, renal cell carcinoma.

a visual evaluation of survival plots, no violation of proportional hazards was observed. Results are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significantly difference.

Results

Patients. A total of 235 patients were included in the present study, which used the intact data from 205 RCC tissues, comprising 157 clear cell cancer and 48 urothelial carcinoma tissues, and 30 normal renal tissues. The patients ranged in age between 35 and 87 years (58.1±5.2 years) and the male to female ratio was 137:98. The mean tumor size was 7.6±3.2 cm. A total of 89 RCC patients possessed lesions classified as stage I, 78 were classified as stage II, 33 were classified as stage III and 5 were classified as stage IV. The median follow-up time was 78 months (range, 1-118 months) and a total of 14 patients (6.83%) succumbed to the disease.

Expression of CD73 and P-gp in RCC and normal renal tissue samples. The analysis of CD73 expression was performed in 159 tumor samples, consisting of 124 clear cell RCC and 35 uroepithelium cell carcinoma tissues, and 30 paratumorous normal renal tissue samples (Table II). CD73 expression was low in normal renal cells (Fig. 1A). In RCC tissues, CD73 expression was most frequently observed in the cell membrane and cytoplasm (Fig. 2), whereas little signal was observed in the vascular smooth muscle and leukomonocytes (Fig. 3). The majority of samples from RCC patients exhibited intense CD73 staining (75 out of 159; Table IIA), and 48.39% (60 out of 124) of clear cell RCC samples and 42.86% (15 out of 35) of uroepithelial cell carcinoma samples exhibited intense CD73 staining. In addition, CD73 expression in clear cell RCC and uroepithelial cell carcinoma was markedly increased compared with the expression in normal renal cells (P<0.001 and P=0.001, respectively; Table IIA). In addition, there was a high expression of CD73 in the vascular wall in 4 out of 30 normal tissues and 14 out of 159 tumor tissues (Table IIA) but the difference was not significant (P=0.074). P-gp protein expression, determined in 85 tumors and 13 surrounding normal renal tissues (Table IIB), was mainly observed in the blood vessels of tumor tissues (42 out of 85) in cytomembrane and cytoplasmic compartments (Fig. 4). P-gp expression was also observed in the glomerulus, proximal convoluted tubule and lymphocytes in tumors (Fig. 5). The P-gp expression in the RCC blood vessels was significantly increased compared with the expression in the blood vessels in normal tissue (P<0.001; Table V).

Cells were classified into three levels according to the percentage and intensity of CD73 staining (Table III). In the group with clear cell RCC, 43.3% (68 out of 157) scored as Level 1, 4.5% (7 out of 157) as Level 2 and 3.8% (6 out of 157) as Level 3. By contrast, in uroepithelial carcinoma cases, 35.4% (17 out of 48) scored as Level 1, 8.3% (4 out of 48) as Level 2 and 6.3% (3 out of 48) as Level 3. The classification of patients based on the percentage and intensity of P-gp staining was examined (Table IV). In the group of patients with clear cell RCC, 25.3% (19 out of 157) scored as Level 1, 10.7% (8 out of 157) as Level 2 and 4.0% (3 out of 157) as Level 3. In addition, 37.9% (11 out of 48) of the uroepithelial carcinoma cases scored as Level 1, 10.3% (3 out of 48) as Level 2 and 0% as Level 3. Therefore, the expression level of CD73 is more strongly associated with the classification of RCC than the expression of P-gp.

Table III. CD73 expression in RCC and its association with clinicopathological factors.

Variable	Number of samples, n	Low expression, n (%)	Intensive expression, n (%)			χ^2	P-value
			Level 1	Level 2	Level 3		
Gender							
Male	119	73 (61.3)	42 (35.3)	3 (2.5)	1 (0.8)	0.677	0.498
Female	70	41 (58.6)	21 (30.0)	6 (8.6)	2 (2.9)		
Tumor type							
Normal	30	30 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	30.184	0.000
Clear cell RCC	157	76 (48.4)	68 (43.3)	7 (4.5)	6 (3.8)		
Urothelium carcinoma	48	24 (50.0)	17 (35.4)	4 (8.3)	3 (6.3)		
TNM stage							
T1N0M0	73	35 (47.9)	35 (47.9)	2 (2.7)	1 (1.4)	12.186	0.002
T2N0M0	70	45 (64.3)	21 (30.0)	1 (1.4)	3 (4.3)		
T3N0M0	28	8 (28.6)	14 (50.0)	5 (17.9)	1 (3.6)		
T4N0M0 N^+M^+	2	0 (0.0)	1 (50.0)	1 (50.0)	0 (0.0)		
Tumor grade							
1	49	33 (67.3)	12 (24.5)	4 (8.2)	0 (0.0)	12.821	0.002
2	95	48 (50.5)	43 (45.3)	1 (1.1)	3 (3.2)		
3	53	17 (32.1)	28 (52.8)	5 (9.4)	3 (5.7)		

RCC, renal cell carcinoma; TNM, tumor-node-metastasis; N^+, lymph node involvement; M^+, presence of metastasis.

Table IV. P-glycoprotein expression in RCC and its association with clinicopathological factors.

Variable	Number of samples, n	Low expression, n (%)	Intensive expression, n (%)			χ^2	P-value
			Level 1	Level 2	Level 3		
Gender							
Male	63	35 (55.6)	20 (31.7)	7 (11.1)	1 (1.6)	0.232	0.816
Female	42	25 (59.5)	11 (26.2)	4 (9.5)	2 (4.8)		
Tumor type							
Normal	13	11 (84.6)	0 (0.0)	2 (15.4)	0 (0.0)	8.616	0.196
Clear cell RCC	75	45 (60.0)	19 (25.3)	8 (10.7)	3 (4.0)		
Urothelium carcinoma	29	15 (51.7)	11 (37.9)	3 (10.3)	0 (0.0)		
TNM stage							
T1N0M0	42	29 (69.0)	10 (23.8)	3 (7.1)	0 (0.0)	8.463	0.015
T2N0M0	33	19 (57.6)	11 (33.3)	1 (3.0)	2 (6.1)		
T3N0M0	18	6 (33.3)	6 (33.3)	5 (27.8)	1 (5.6)		
T4N0M0 N^+M^+	2	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)		
Tumor grade							
1	25	20 (80.0)	4 (16.0)	1 (4.0)	0 (0.0)	7.282	0.026
2	38	20 (52.6)	12 (31.6)	5 (13.2)	1 (2.6)		
3	42	20 (47.6)	15 (35.7)	5 (11.9)	2 (4.8)		

RCC, renal cell carcinoma; TNM, tumor-node-metastasis; N^+, lymph node involvement; M^+, presence of metastasis.

Association between the expression of CD73 and P-gp and clinicopathological features. The present analyses revealed that clinicopathological parameters, including the tumor type and an advanced tumor stage, were significantly associated with CD73 expression in RCC tissue cells. CD73 expression was also associated with an increased tumor grade (Table III). CD73 was intensely expressed in 32.65% (16 out of 49) grade 1, 49.47% (47 out of 95)

Table V. Association of tumor specific characteristics with CD73 and P-gp expression in tumor blood vessel.

	CD73, n (%)				P-gp, n (%)			
Variable	Low	Intensive	χ^2	P-value	Low	Intensive	χ^2	P-value
Gender								
Male	113 (90.4)	12 (9.6)	1.323	0.250	34 (53.13)	30 (46.88)	0.000	0.994
Female	74 (94.9)	4 (5.1)			23 (54.76)	19 (45.24)		
Tumor type								
Normal	26 (86.7)	4 (13.3)	4.365	0.113	13 (100.00)	0 (0.00)	14.590	0.001
Clear cell RCC	141 (89.81)	16 (10.19)			36 (48.00)	39 (52.00)		
Urothelium carcinoma	45 (97.8)	1 (2.2)			20 (66.67)	10 (33.33)		
Stage								
T1N0M0	61 (85.9)	10 (14.1)	4.261	0.235	19 (45.24)	23 (54.76)	3.226	0.358
T2N0M0	67 (95.7)	3 (4.3)			18 (52.94)	16 (47.06)		
T3N0M0	25 (89.3)	3 (10.7)			11 (61.11)	7 (38.90)		
T4N0M0-N+M+	2 (100.0)	0 (0.0)			2 (100.00)	0 (0.00)		
Grade								
1	43 (87.8)	6 (12.2)	2.310	0.315	7 (28.00)	18 (72.00)	11.948	0.003
2	87 (91.6)	8 (8.4)			21 (53.85)	18 (46.15)		
3	49 (96.1)	2 (3.9)			30 (71.43)	12 (28.57)		

CD73, cluster of differentiation 73; P-gp, P-glycoprotein; RCC, renal cell carcinoma; TNM, tumor-node-metastasis; N^+, lymph node involvement; M^+, presence of metastasis.

Table VI. Multivariate Cox proportional hazards model of RCC.

Variable	HR	95% CI	P-value
Gender	3.061	0.888-10.552	0.076
Age	0.974	0.909-1.043	0.453
Type of tumor	1.524	0.265-8.776	0.637
Pathological stage	3.537	0.952-13.139	0.059
Histological grade	3.929	1.098-14.062	0.035
Tumor size	0.870	0.696-1.089	0.224
CD73 intensive	3.989	1.089-14.611	0.037
P-gp intensive	0.703	0.184-2.679	0.605

CD73, cluster of differentiation 73; P-gp, P-glycoprotein; HR, hazard ratio; CI, cluster of differentiation.

grade 2 and 67.92% (36 out of 53) grade 3 RCC tissue samples. Based on the TNM analysis, CD73 expression in RCC tissue cells was also found to be associated with tumor differentiation. The incidence of CD73 expression in RCC tissue cells was 52.05% (38 out of 73) and 35.71% (25 out of 70) in tumor stages T1N0M0 and T2N0M0, respectively. However, the incidence of CD73 expression was 71.43% (20 out of 28) and 100% (2 out of 2) in tumor stages T3N0M0 and T4N0M0 N^+M^+, respectively. P-gp was found to be intensely expressed in 20% (5 out of 25) of grade 1, 47.37% (18 out of 38) of grade 2 and 52.38% (22 out of 42) of grade 3 RCC tissues (Table IV).

Based on analysis of the TNM stage, P-gp expression in RCC tissue cells was also found to be associated with tumor differentiation (Table IV). The incidence of P-gp expression in RCC tissue cells was 30.95% (13 out of 42) and 42.42% (14 out of 33) in stages T1N0M0 and T2N0M0, respectively. However, the incidence of P-gp expression was 66.67% (12 out of 18) and 100% (2 out of 2) in stages T3N0M0 and T4N0M0 N^+M^+, respectively.

Association between the expression of CD73 and P-gp and the survival of RCC patients. At the end of the five-year follow-up, 14 out of 189 patients had succumbed to RCC. Therefore, the overall five-year survival rate of RCC patients was 80.60%. The five-year survival rate of patients with intense CD73 expression in RCC tissue was 67.6%, whereas the rate was 91.7% in patients with low CD73 expression ($P<0.001$). The median survival of RCC patients with intense CD73 expression was 78.00 months (range, 37.63-118.37 months; Table VII). The survival time of RCC patients with intense CD73 expression was 62.06±5.35 months, which is markedly decreased compared with RCC patients with low CD73 expression (103.72±3.67 months; Table VII). A significant difference was observed between the survival rate of patients with intense CD73 expression compared with patients with low CD73 expression (Log-rank and Breslow tests, $P<0.001$), indicating that the lack of CD73 expression is associated with an increased five-year survival rate in RCC patients.

The five-year survival rate of patients with intense P-gp expression was 44.1%, which was not significantly increased compared with the five-year survival rate of 27.1% in patients with low P-gp expression (Log-rank analysis, $P=0.957$;

Table VII. Means and medians of survival time of renal cell carcinoma patients with rare and intensive CD73 expression.

	Mean[a]				Median			
			95% confidence interval				95% confidence interval	
CD73	Estimated	SE	Lower bound	Upper bound	Estimated	SE	Lower bound	Upper bound
Rare	103.719	3.667	96.532	110.907				
Intensive	62.058	5.354	51.564	72.553	78.000	20.597	37.630	118.370
Overall	89.460	4.021	81.579	97.342				

[a]Estimation is limited to the largest survival time if patients succumbed prior to reaching the mean survival time. CD73, cluster of differentiation 73; SE, standard error.

Table VIII. Means and medians of survival time of RCC patients with rare and intensive P-gp expression.

	Mean[a]				Median			
			95% confidence interval				95% confidence interval	
P-gp	Estimated	SE	Lower bound	Upper bound	Estimated	SE	Lower bound	Upper bound
Rare	46.608	4.352	38.079	55.137	45.000	4.734	35.722	54.278
Intensive	47.408	5.582	36.468	58.348	47.000	3.656	39.834	54.166
Overall	46.918	3.414	40.227	53.610	47.000	2.381	42.333	51.667

[a]Estimation is limited to the largest survival time if patients succumbed prior to reaching the mean survival time. P-gp, P-glycoprotein; SE, standard error.

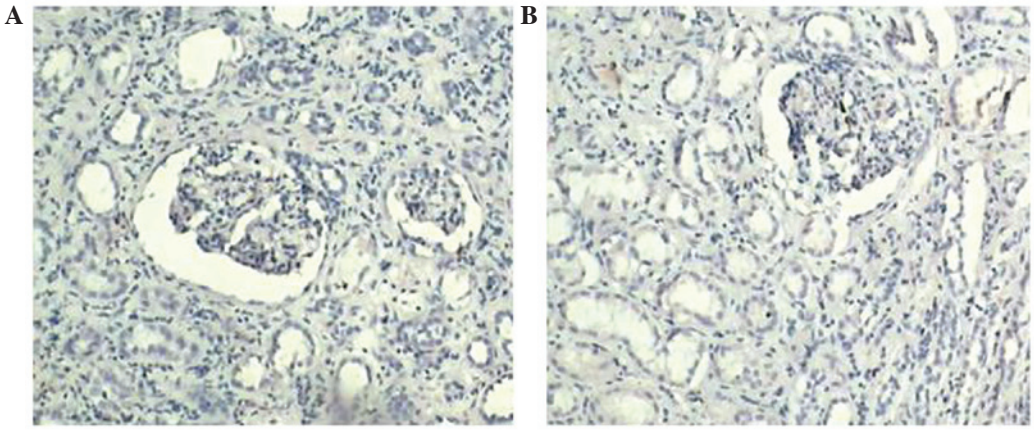

Figure 1. The low expression of CD73 and P-gp processed by immunohistochemistry in normal renal tissue. (A) CD73 (10x20); (B) P-gp (10x20).

Breslow test, P=0.795). Patients with RCC that exhibited intense P-gp expression demonstrated a median survival of 47.00 months (range, 39.83-54.17 months; Table VIII). The survival time of RCC patients with intense P-gp expression was 47.41±5.58 months, which was similar to the survival time of RCC patients with low P-gp expression (46.61±4.35 months; Table VIII). No significant difference was observed in the survival of RCC patients based on P-gp expression (Log-rank and Breslow tests, P>0.05).

In addition, multivariable Cox regression analysis, including gender, age, tumor type, TNM, tumor stage and grade, indicated that CD73 expression and histological grade, but not P-gp expression, were strongly associated with RCC prognosis (Table VI).

Discussion

In the present study, the expression levels of CD73 and P-gp were examined in RCC tumor and surrounding normal kidney tissues. An association was identified between intense CD73 expression and clear cell RCC, high TNM stage, high tumor grade and a low five-year survival rate, which indicates

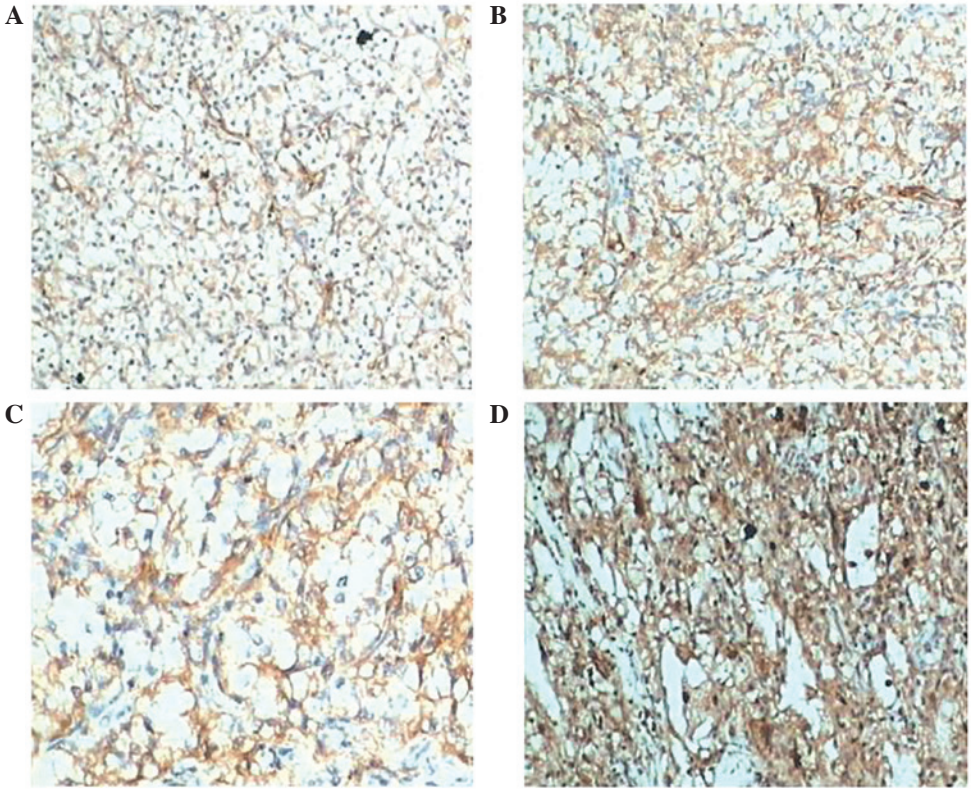

Figure 2. Expression of cluster of differentiation 73 in clear cell renal cell carcinoma. (A) Level 1 (magnification, x100); (B) Level 2 (magnification, x100); (C) Level 2 (magnification, x200); and (D) Level 3 (magnification, x100).

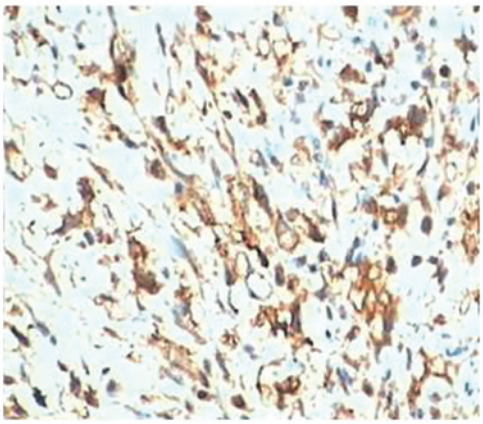

Figure 3. Cluster of differentiation 73 expression in vascular smooth muscle and leukomonocytes in renal cell carcinoma tissue (magnification, x200).

that CD73 is involved in the progression of clear cell RCC and may be used as a biomarker for the diagnosis and prognosis of RCC.

Previous studies have revealed that CD73 is applicable as a cell-surface marker of malignant tumors, including those in bladder cancer (22), leukemia (23), glioma (24), melanoma (25), and ovarian (26), colon, breast (7,24,27-29), thyroid (30), esophageal (31) and prostate cancers (32). However, to the best of our knowledge, the present study is the first to report that CD73 may also be a biomarker for clear cell RCC. All normal kidney samples did not express CD73, as determined by immunohistochemical staining. In addition, 48.4% of clear cell RCC and 42.9% of uroepithelial cell carcinoma tissues exhibited intense CD73 staining. Thus, the expression of CD73 is increased in RCC and may act as an important marker for RCC patients.

An increasing quantity of novel evidence has revealed that cancer stem cells (CSCs) may exist in RCC (33). However, few studies have focused on the identification of renal CSCs, and studies have been performed to investigate and verify initial markers on the cell surface of CSCs in RCC. The present study found that *in vitro* cultured and *in vivo* RCC $CD73^+$ cells possess a stronger tumorigenic capacity compared with $CD73^-$ cells (data not shown). Therefore, a patent has been obtained for the use of CD73 as a renal clear cell carcinoma stem cell surface marker (34). CSCs, known for their resistance to chemotherapeutic agents and their tumor initiating ability (35), may exhibit distinct pro-angiogenic and micro vesicular properties and play relevant roles in the pathogenesis and prognosis of RCC tumors. Previous studies have hypothesized that well-differentiated cancer cells tend to produce a higher level of CD73 (36,37). Since clear cell carcinoma and uroepithelium carcinoma mainly originate from the renal tubular epithelial and urinary tract epithelial cells, RCC CSCs possibly originate from mutated normal stem cells in the kidney and conserve the expression of CD73 during differentiation into endothelial cells.

A high level of CD73 expression is associated with a poor prognosis in colorectal cancer (38). However, the role of CD73 expression in cancer cells in determining the prognosis of patients remains controversial in breast cancer (29,38-40), which may be due to the conversion of adenosine that is promoted by CD73 in tumors. In the present cohort of RCC patients, intense CD73 protein expression in cancer cells was associated with a

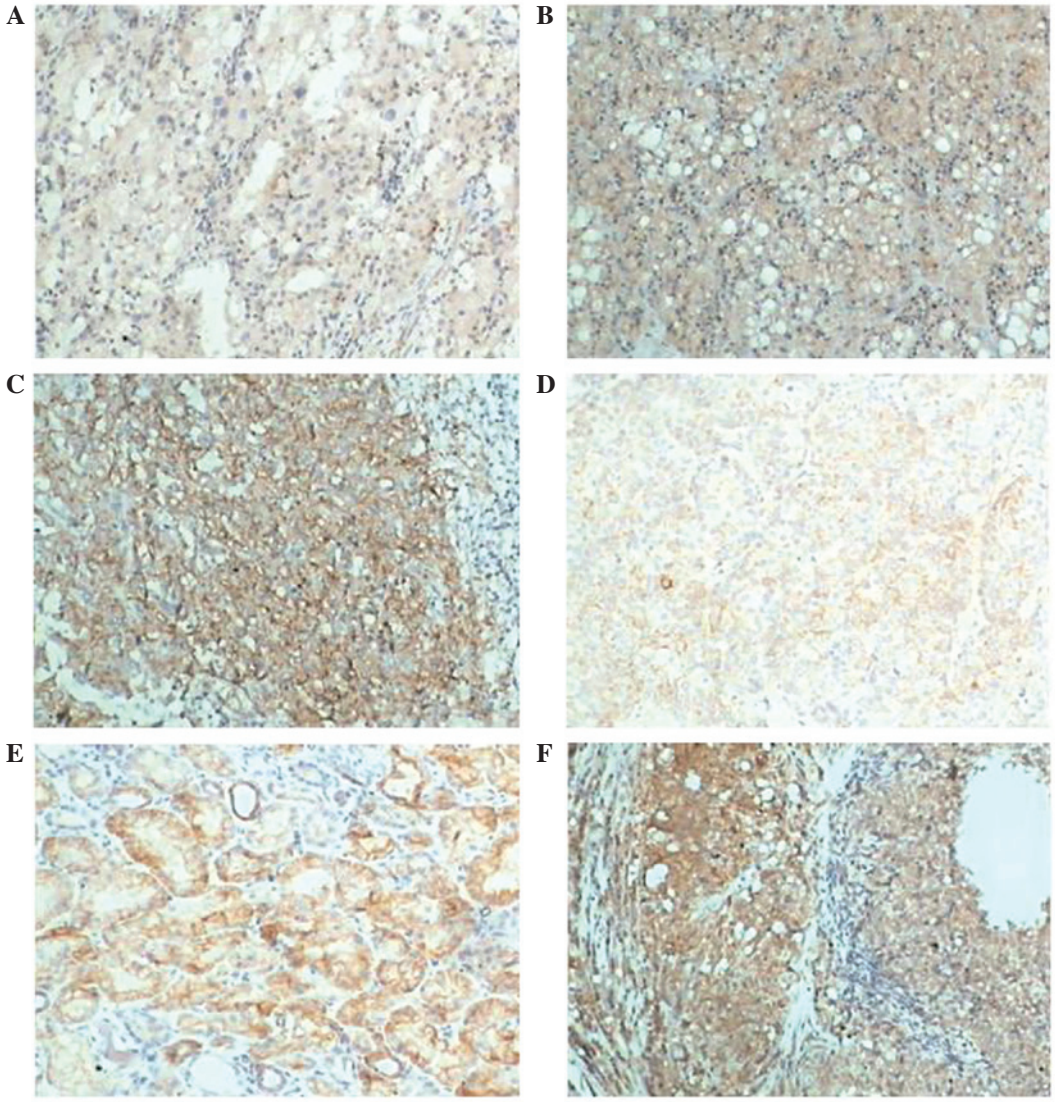

Figure 4. P-glycoprotein staining in RCC tissues using immunohistochemical staining. (A) Level 1 clear cell RCC tissue (magnification, x100). (B) Level 2 clear cell RCC tissue (magnification, x100). (C) Level 3 clear cell RCC tissues (magnification, x100). (D) Level 1 uroepithelial carcinoma tissue (magnification, x200). (E) Level 2 uroepithelial carcinoma tissues (magnification, x200). (F) Level 3 urothelial carcinoma tissue (magnification, x100).

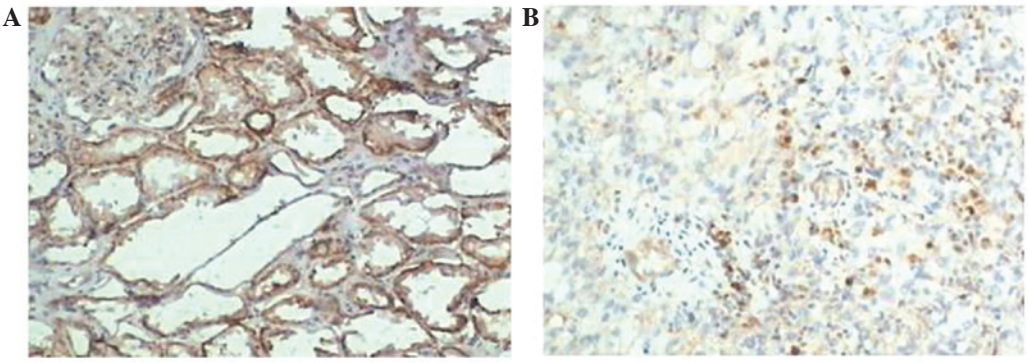

Figure 5. Immunohistochemical staining of P-glycoprotein in (A) glomeruli, tubules and (B) lymphocytes in renal cell carcinoma tissues (magnification, x200).

worse prognosis, increased tumor grade and increased TNM stage compared with cells that did not express CD73. Currently, the present results support that CD73-expressing RCC lesions are more aggressive compared with lesions that do not express CD73.

Inflammatory hypoxia (14), hypoxia inducible factor-1 (HIF-1) (41) and Wnt signaling (42,43) are potent transcriptional stimuli for CD73 expression, which implies that the microenvironment of malignant tumors elevates CD73 expression. At present, it is challenging to estimate the

relative contribution of cancer cells compared with other cells or exosomes that contribute to adenosine production in the tumor microenvironment *in vivo*. However, the majority of findings strongly indicate that CD73 functions at multiple levels in limiting antitumor effects (44).

Although the vascular expression of CD73 in RCC samples was not significantly different from that of normal renal cells, CD73 may play other roles in RCC progression. CD73 is known to participate in leukocyte extravasation from blood in endothelial cells and lymphatics (45) and to regulate endothelial hemostasis (46). In addition, CD73 overexpression may play a role in the immune system response (47) or also result from a selective pressure exerted by the immune system. Cells positive for CD73 through the production of immunosuppressive adenosine are more able to evade anti-tumor immune responses (48). These findings indicate that CD73 is associated with tumor proliferation, metastasis and invasion (7,8,44,49). By contrast, the suppression of CD73 induces apoptosis and cell-cycle arrest in human breast cancer cells (50), and the inhibition of adenosine production by CD73 could be a therapeutic target for the prevention of tumor angiogenesis and metastasis (14). Therefore, although the association between CD73 and tumor progression requires further investigation, the present results indicate that CD73 is involved in tumor progression and is a potential target for tumor therapy.

RCC is resistant to antitumor drugs, implying high expression levels of membrane transport proteins that inhibit the cellular influx and increase the efflux of chemotherapeutic drugs. P-gp is a well-known plasma membrane drug efflux pump involved in the chemo resistance of numerous types of tumors (7,8). However, the association between RCC and P-gp is disputed (9-13). In the present study, it was found that P-gp was mainly expressed on the blood vessels in RCC tissues, and was particularly associated with the tumor type and tumor grade. Normal human tissues from the majority of secretory organs have been analyzed for P-gp expression (51) and it has been found that in the normal kidney, P-gp is immunoreactive on the epithelial cells of the proximal tubules, but not within the capillaries of the glomerular tuft and endothelial cells of arterioles (52). The most notable observations have been found in the endothelial cells of capillary blood vessels at blood-tissue barrier sites, including in the central nervous system, papillary dermis and choroid plexus (53). Recently, the *MDR1* C3435T-associated variable P-gp expression and function were also identified in peripheral blood mononuclear cells (54,55). This indicates that the shifting expression of P-gp in blood vessels may have important implications in cancer chemotherapy and reinforced self-protection in RCC. The present findings revealed that P-gp expression in tumor vessels of clear cell RCC is associated with a worse tumor grade and poorer clinical treatment effect. The role of P-gp-mediated chemoresistance in RCC may therefore not change with the expression quantity, but the migration of P-gp to the blood vessels for recycling may strengthen the barrier function, exerting its cytotoxic action rapidly, prior to intrinsic resistance mechanisms being activated. In the present study, however, P-gp expression levels were not significantly associated with the prognosis of patients with RCC. Therefore, P-gp remains controversial as a prognostic biomarker for RCC, but may be an important factor in the failure of treatment for RCC. Solidifying the role of P-gp in metastatic RCC in all patients may therefore continue to aid in optimizing treatment for RCC.

In the present study, it was found that the expression level of CD73 in RCC tumors was associated with the tumor stage, tumor grade and patient survival. The expression of CD73 in clear cell RCC tumor tissues was associated with a high tumor stage and tumor grade, but was associated with low patient survival, indicating the potential application of CD73 as a novel diagnostic and prognostic marker of clear cell RCC.

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