RESEARCH ARTICLE

Overexpression of Cyclooxygenase-1 Correlates with Poor Prognosis in Renal Cell Carcinoma

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Abstract

The aim of this study was to evaluate expression of COX-1 in renal cell carcinoma (RCC) and its prognostic value. mRNA of COX-1 was detected in 42 paired RCC and adjacent normal tissues with quantitative real-time polymerase chain reaction (qRT-PCR). Expression of COX-1 was also evaluated in 196 RCC sections and 91 adjacent normal tissues with immunohistochemistry. Statistical analysis was performed to assess COX-1 expression in RCC and its prognostic significance. The results of qRT-PCR showed mRNA levels of COX-1 in RCC tissues to be significantly higher than that in adjacent normal tissues ($p < 0.001$). Immunohistochemical assays also revealed COX-1 to be overexpressed in RCC tissues ($p < 0.001$). Statistical analysis demonstrated high expression of COX-1 was correlated with tumour size ($p = 0.002$), pathological stage ($p = 0.003$), TNM stage ($p = 0.003, 0.007, 0.027$, respectively), and tumour recurrence ($p < 0.001$). Survival analysis indicated patients with high expression of COX-1 had shorter survival time ($p < 0.001$), and COX-1 was an independent predictor. This is the first study to reveal overexpression of COX-1 in RRC and point to use as a prognostic marker in affected patients.

Keywords: Cyclooxygenase-1 - renal cell carcinoma - prognostic marker

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Introduction

Renal cell carcinoma (RCC) is the second most common tumor in urinary system, accounting for 3% of adult malignant tumor (Jemal et al., 2010; Siegel et al., 2012). About 30% of patients have metastatic disease when diagnosed with RCC and radical nephrectomy remains the main treatment for RCC patients because of the tumor's resistance to radiation and chemotherapy. Even so, approximately 30% of RCC patients experience local or distant recurrence after radical nephrectomy (Skinner et al., 1971; Patel et al., 2012). Histological grade combined with clinical stage, which is considered to be the golden standard of prediction of patient's prognosis, cannot predict patient's prognosis accurately when used alone (Amin et al., 2002; Choi et al., 2007). In recent years, identification of molecular markers that predict prognosis of patients with cancer becomes a much-talked-about topic, and many markers were found to be associated with patient's prognosis (Shvarts et al., 2005; Skolarikos et al., 2005; Sejima et al., 2006; Mittal et al., 2012; Wei et al., 2013).

Cyclooxygenase-1 (COX-1), also named prostaglandin-endoperoxide synthase 1, is one of two isoenzymes of cyclooxygenase, which is the key rate-limiting enzyme in the synthesis of prostaglandin from arachidonic acid. The other is Cyclooxygenase-2 (COX-2), it was found to be related to various forms of human cancer such as lung cancer, mammary cancer, gastrointestinal and bladder cancers, also including RCC (Liu et al., 1996; Ristimaki et al., 1997; Hida et al., 1998; Hashimoto et al., 2004; Diamantopoulou et al., 2005). Generally, COX-1 is believed to participate in the normal physiological process and protection function. However, some studies have reported that COX-1 was over expressed in epithelial ovarian cancer and it was the major prostanoid generating pathway operative in ovarian cancers of epithelial origin (Gupta et al., 2003; Daikoku et al., 2005). The researchers suggested COX-1 derived prostaglandins promote tumor growth via being involved in the downstream signaling pathways and influencing cell proliferation and apoptosis. There have been quite a few studies on the role of COX-2 in RCC, but the action of COX-1 in RCC is still unclear up to present. The aim of present study was, therefore, to investigate the expression of COX-1 in normal human kidney and in different types of RCC, and to test its

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ability to predict long-term prognosis. From the point of methodology, qRT-PCR was used to detect the mRNA of COX-1 in 42 pairs of RCC tumors and adjacent normal tissues. Furthermore, immunohistochemical assay was applied to determine the COX-1 protein in 196 RCC tumors and 91 paired normal tissues.

Materials and Methods

Patients and tissue specimen collection

Forty-two fresh RCCs and matched normal tissues, including 36 clear cell renal cell carcinoma (ccRCC) tissues and 6 papillary carcinoma tissues, surgically resected between 2009 and 2011 at the Department of Urology, Peking University Shenzhen Hospital, were used for quantitative real-time polymerase chain reaction (qRT-PCR). Among these patients, there were 23 men and 19 women, with an average age of 53.3 years old, ranging from 30-76 years old. All of these specimens were immediately immersed in RNAlater (Qiagen, Germany) after resection and subsequently stored at -80°C. Additionally, 196 paraffin-embedded RCC samples and 91 adjacent normal tissues from patients of the Department of Urology, Peking University Shenzhen Hospital between 2000 and 2009 were used for immunohistochemical assay to detect the expression of COX-1. All of these tumors were diagnosed and classified or reclassified according to the 2002 American Joint Committee on Cancer (AJCC) staging system (Greene et al., 2002). Clinical and pathological characteristics of these 196 patients are listed in Table 1. The study was approved by the institutional review board and ethical committee of Peking University Shenzhen hospital. Informed consent was written and obtained from all the patients in this study.

Quantitative real-time polymerase chain reaction (qRT-PCR) assay for mRNA of COX-1

After extracted from the fresh tissue with Trizol solution (Invitrogen, USA) according to the manufacturer's protocol, 1μg total mRNA of each sample was used for reverse transcription with Omniscript RT kit (Qiagen, Germany). The reactions of q-PCR were performed and analyzed with the ABI PRISM 7000 Fluorescent Quantitative PCR System. Reaction mixture was set up in a total volume of 20μl, consisting of 1μl of cDNA template synthesized previously, 10μl SYBR Green master mix (Invitrogen; USA), 1μl of each primer (sense and antisense primer) and RNase-free water. The COX-1 sense primer and anti-sense primer were 5'-GGAGTTTGTCAATGCCACCT-3', 5'-GCAACTGCTTCTTCCCTTTG-3', respectively. GAPDH was used as internal control and the corresponding primers were as follows: sense primer: 5'-GGAGTCCACTGGCGTCTTCACC-3', antisense primer: 5'-GAGGAGTGGGTGTCGCTGTTG-3'. Cycling parameters were set as 95°C for 2 min, followed by 40 cycles of 95°C (15sec), 55°C (30 sec), and 72°C (40 sec). Relative expression of COX-1 was normalized and the data was analyzed with the comparative threshold cycle ($2^{-\Delta CT}$, $\Delta CT = CT_{COX-1} - CT_{GAPDH}$) method (Schmittgen et al., 2001).

Immunohistochemical assay for COX-1

The immunohistochemical assay for COX-1 was performed according to standard procedures. Paraffin-embedded samples were cut into 5μm sections and baked at 65°C for 1h, then deparaffinized in xylene and rehydrated in descending ethanol series, followed by antigen retrieval. This step was done by heating the sections in a microwave oven for 2 × 15 min in 0.01M citrate buffer (pH 6.0) antigen retrieval buffer. Then the slides were immersed in 3% hydrogen peroxide solution for 20 minutes, washed in phosphate buffered saline (PBS) triple for 5 minutes, treated in 10% bovine serum albumin for 30min in 37°Cto block non-specific protein binding. For the immunostaining of COX-1, the specimens were treated with rabbit monoclonal antibody anti-COX1 (Epitomics, California, USA) at 1:200 dilution and overnight at 4°C. Rinsed with PBS for 3 times, the samples were incubated with anti-Rabbit IHC Kit (Maixin Bio; Fujian, China) at 37°C for 30 min. Finally, the slides were stained with 3'3-diaminobenzidine tetrahydrochloride (DAB) for 3 min, counterstained with hematoxylin, dehydrated, and mounted. Negative controls were performed with omission of the primary antibodies.

Staining evaluation of each sample was carried out by two independent observers blinded to clinicopathologic variables. Intensity of staining was graded: 0, no staining; 1, weakly stained; 2, moderately stained; 3, highly stained (Bao et al., 2004; Saussez et al., 2006). Percentage of cells showing positive staining was graded: 1, 0-5%; 2, 6-25%; 3, 26-50%; 4, 51-75% and 5, > 75% (Tsuchiya et al., 2003). All of these paraffin-embedded sections were given final scores based on the multiplications of intensity scores and percentage scores. In case of any discrepancy, specimens were reviewed by the two observers together and a final score was agreed upon. The optimal cut-off value was calculated with log-rank test on the basis of a measure of heterogeneity in overall survival rates and final score of more than 5 was considered as high expression of COX-1 and < 5 as low expression.

Statistical analysis

Paired -sample t test was used to analyze the significance of differences in mRNA and protein expression of COX-1 between tumors and adjacent normal tissues. Relationships between expression of COX-1 and clinicopathologic variables were calculated using χ^2 test. Kaplan-Meier method and log-rank test were used to plot survival curves and to test statistical significance between stratified survival groups. To assess variables affecting overall survival, univariate and multivariate Cox proportional hazards models were used. In all tests, $p < 0.05$ was considered statistically significant and all statistical analyses were performed with the IBM SPSS Statistics (version 17.0) software package.

Results

Assay of COX-1 mRNA level by qRT-PCR

The mRNA level of COX-1 in 42 tumor tissues and paired adjacent normal tissue samples were detected by qRT-PCR. Paired -sample t test showed that the relative

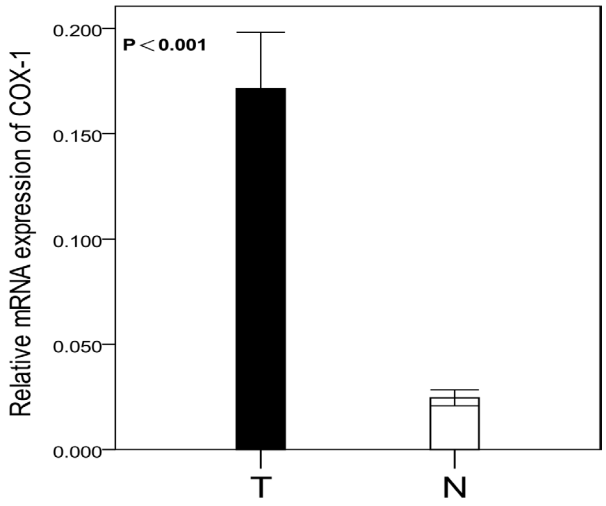

Figure 1. Relative mRNA Expression of COX-1 by qRT-PCR Analysis. In RCC tumor (T) tissues, the relative expression of COX-1 mRNA was higher than that in paired adjacent normal (N) tissues (n = 42, $P < 0.001$)

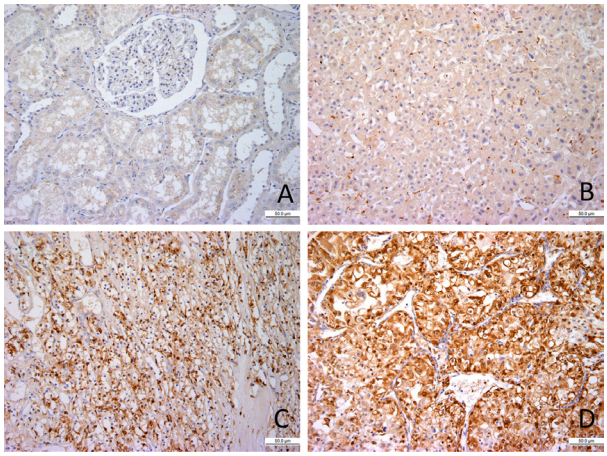

Figure 2. Immunohistochemical Staining of COX-1 in RCC Tissues and Normal Tissues. Staining of COX-1 was mainly located in the cytomembrane and kytoplasm in renal cancer cells. (A): Staining of COX-1 in adjacent normal tissue was negative or at a low level (200×). (B): Weak staining of COX-1 in RCC cells (200×). (C): Moderate COX-1 staining in tumor tissues (200×). (D): Strong staining in most of tumor cells (200×)

In tumors smaller than 7cm, only 58.1% showed high expression of COX-1 whereas approximately 80% of tumors that > 7cm showed high expression ($p = 0.002$, χ^2 test). Respectively, high expression of COX-1 was found in 58.1 percent, 73.2 percent, 86.8 percent of tumors in T1, T2, T3/T4 stage groups ($p = 0.003$, χ^2 test). It's noteworthy that N+ (N1, N2) group of RCCs showed comparative high expression of COX-1 (87.5%) compared with N0 (62.8%) group ($p = 0.007$, χ^2 test). Similarly, approximately 90% of RCCs with metastasis showed high expression (89.5%) compared to RCCs without metastasis ($p = 0.027$, χ^2 test). Of 57 patients with recurrence, 50 (87.7%) showed high expression of COX-1 and the difference was statistically significant ($p < 0.001$, χ^2 test).

Survival analysis

Of 196 patients with RCC included in survival analysis, 122 were alive at the end of follow-up. The

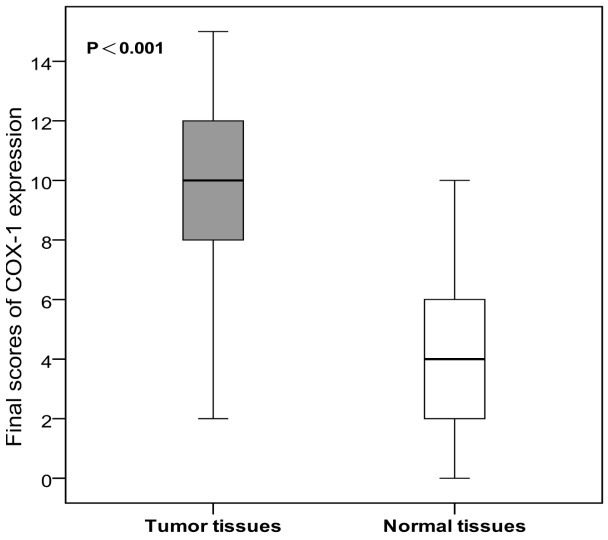

Figure 3. Relative Protein Expression of COX-1 by Immunohistochemical Assay. COX-1 expression in tumors was higher than that in adjacent normal tissues (n = 91, $P < 0.001$). The bottom and the top of the box represent the lower and upper quartiles, respectively, and the band near the middle of the box is the median. The ends of the whiskers represent the 2.5th percentile and the 97.5th percentile

mRNA level of COX-1 in tumors was significant higher than that in the adjacent normal tissue ($p < 0.001$, paired -sample t test, Figure 1).

Immunostaning of COX-1 in 91 RCC tissues and adjacent normal tissues

COX-1 immunostaining was presented in all tumor specimens and paired normal tissues. As showed in Figure 2, staining of COX-1 was mainly located in cytomembrane and kytoplasm in renal cancer cells, and there was an obvious contrast between stained tumor tissues and normal tissues. Compared with tumor tissues, expression of COX-1 in adjacent normal tissues were negative or at low level (score < 5). Uniformly, paired-sample t test showed COX-1 expression in tumor tissues was higher than that in adjacent normal tissues ($p < 0.001$, Figure 3).

COX-1 expression in 196 RCC tissues by immunohistochemistry

To further investigate the correlations between expression of COX-1 and clinicopathologic variables, all of these RCC slides were divided into two groups according to the level of COX-1 immunostaining and χ^2 test was used. Of 196 RCC samples stained in this study, 131 showed high expression of COX-1 and 65 tumor tissues showed low expression, as showed in Table 1. The results revealed high expression of COX-1 was correlated with tumor size ($p = 0.002$), pathological stage ($p = 0.003$), TNM stage ($p = 0.003, 0.007, 0.027$, respectively), recurrence of tumor ($p < 0.001$). Even though there was a trend suggesting that COX-1 expressed more commonly in clear cell renal cell carcinoma (68.2% versus 60.9%), the difference did not reach statistical significance ($p = 0.741$, χ^2 test). Compared with pathologicalI/IIgroups (57.7%, 64,5%), III/IV (89.9%, 85.2%) groups showed obvious high expression of COX-1 ($p = 0.003$, χ^2 test).

Table 1. Clinical and Pathological Characteristics of 196 RCC Patients and Correlation with Expression Level of COX-1

Characteristics	No. of patients(n=196)	COX-1 expression Low(n=65)	High(n=131)	chi-square value	*P* value
Gender					
Male	130	42 (32.3%)	88 (67.7%)	0.127	0.721
Female	66	23 (34.8%)	43 (65.2%)		
Age(years)					
≤50	104	35 (33.7%)	69 (66.3%)	0.024	0.877
>50	92	30 (32.6%)	62 (67.4)		
Histology					
Clear cell	151	48 (31.8%)	103 (68.2%)	0.6	0.741
Papillary	22	8 (36.4%)	14 (63.6%)		
Others	23	9 (39.1%)	14 (60.9%)		
Size(cm)					
≤7	117	49 (41.9%)	68 (58.1%)	9.951	0.002
>7	79	16 (20.3%)	63 (79.7%)		
Pathologic stage					
I	111	47 (42.3%)	64 (57.7%)	14.319	0.003
II	31	11 (35.5%)	20 (64.5%)		
III	27	3 (11.1%)	24 (89.9%)		
IV	27	4 (14.8%)	23 (85.2%)		
T stage					
T1	117	49 (41.9%)	68 (58.1%)	11.614	0.003
T2	41	11 (26.8%)	30 (73.2%)		
T3,T4	38	5 (13.2%)	33 (86.8%)		
N stage					
N0	164	61 (37.2%)	103 (62.8%)	7.367	0.007
N+	32	4 (12.5%)	28 (87.5%)		
Metastasis					
No	177	63 (35.6%)	114 (64.4%)	4.864	0.027
Yes	19	2 (10.5%)	17 (89.5%)		
Recurrence					
No	139	58 (41.7%)	81 (58.3%)	15.813	<0.001
Yes	57	7 (12.3%)	50 (87.7%)		

mean survival time overall was 73.56 months (95% CI, 66.96-80.17 months). The longest follow-up time was 115 months. Relationship between expression of COX-1 and survival state was evaluated using Kaplan-Meier analysis and log-rank test. The mean survival time of patients with low expression of COX-1 was 105.5 months (95%CI, 97.70-113.42 months), obviously longer than patients with high expression (mean survival time 61.2 months, 95%CI 54.15-68.30 months). Log-rank test showed the survival rates were significant different between patients with high expression of COX-1 and patients without ($p < 0.001$, log-rank test, Figure 4). Additionally, univariate Cox regression analysis showed pathologic stage, tumor size, TNM stage, metastasis and expression of COX-1 were related to the patient's survival (Table 2). Multivariate Cox regression analysis revealed that only pathologic stage and expression of COX-1 were independent clinical predictors of overall survival of RCC patients ($p < 0.001$, Table 2).

Discussion

Prognosis of patient with renal cell carcinoma is undesirable because of its high rates of metastasis at initial diagnose and recurrence (Motzer et al., 1996; Shindo et al., 2013). In recent years, number of molecular markers emerged with their potential values in predicting patients' prognoses and molecular-targeted therapy. For instance, RNA-binding protein IMP3 expression was associated with metastasis in RCC and it was an attractive prognostic marker for this tumor (Jiang et al., 2006). A study showed that carbonic anhydrase 9 (CA9) had a 100% diagnostic specificity in solid renal tumors and would be a promising molecular marker for diagnosis, prognosis and therapy of clear cell renal cell carcinoma (Tostain et al., 2010). However, few utility of these molecular markers for RCC exist till now, probably because of lack of knowledge at the molecular level regarding the biology of renal cell carcinogenesis and progression (Wood 2006; Nogueira et al., 2008).

Studies have showed COX-1 was over expressed in ovarian cancer (Gupta et al., 2003). The researchers discovered dramatic elevations of COX-1, not COX-2, protein and mRNA in a majority of the ovarian cancer samples and positive regulating action in VEGF production in ovarian epithelial cells. Subsequently, some researchers found ovarian surface epithelial cells and tumors comprised of these cells expressed high level of COX-1 but not COX-2 in mouse model of epithelial ovarian cancer (Daikoku et al., 2005). Moreover, they found SC-560 (a COX-1-selective inhibitor) dramatically inhibited PGI2 production and reduced the growth of tumors, indicating that COX-1 could become a potential target for prevention and treatment of ovarian cancer (Daikoku et al., 2005). Up to now, there is no research to compare the expression of COX-1 in RCC tissue and

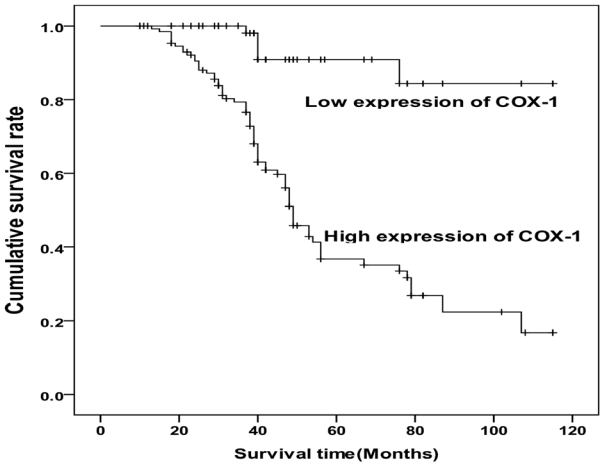

Figure 4. Overall Survival of Patients with RCC (n = 196), Subdivided According to Expression Level of COX-1. The longest follow-up time was 115 months. Log-rank test showed the survival rates were significant different between patients with high expression of COX-1 and patients without ($P < 0.001$)

Table 2. Overall Survival Analyses of Prognostic Factors in Patients with RCC by Cox Regression Analysis

Variables	Univariate analysis		Multivariate analysis	
	P value	Hazard ratios(95%CI)	P value	Hazard ratios(95%CI)
Pathologic stage	<0.001	2.173 (1.645, 2.776)	<0.001	1.814 (1.399, 2.352)
Tumor size	<0.001	2.871 (1.794, 4.594)	0.798	1.177 (0.356, 3.891)
T stage	<0.001	2.037 (1.550, 2.676)	0.975	0.987 (0.437, 2.229)
N stage	<0.001	3.891 (2.316, 6.535)	0.413	1.387 (0.634, 3.034)
Metastasis	<0.001	4.469 (2.362, 8.457)	0.163	1.743 (0.789, 3.810)
COX-1 expression	<0.001	10.411 (3.796; 28.553)	<0.001	8.302 (3.002, 22.964)

normal tissue and to reveal the relationship between COX-1 and RCC.

In our study, qRT-PCR was used to detect the mRNA of COX-1 in RCC tissues and paired adjacent normal tissue samples. The result showed mRNA of COX-1 was up-regulated in tumors compared to normal tissues. Furthermore, immunostaining of RCC tissue sections and adjacent normal tissues revealed COX-1 was over expressed in tumors, in accord with the mRNA level. Additionally, correlations between COX-1 expression and clinicopathologic variables were tested with χ^2 test and it was found to be related with pathological stage, tumor size, TNM stage, recurrence of tumor. To further assess the prognostic value of COX-1 in RCC patients, survival curve was plotted and Cox regression analysis was used. The result indicated patients with high expression of COX-1 had shorter survival time. To investigate which factors affect the patient's survival actually, multivariate Cox regression analysis was used and showed expression level of COX-1 and pathologic stages were independent clinical predictors, implying that COX-1 may be a potential prognostic marker for RCC patients.

As to the value of being a target in the treatment of RCC, studies have discovered that SC-560 could reduce the growth of tumors and may be a potential targeting drug for tumor treatment, prompting that COX-1 may be a new site for molecular target therapy of RCC (Daikoku et al., 2005; Tatokoro et al., 2011). But like other studies of molecular markers, more researches are needed to explore the molecular mechanism of COX-1 regarding the biology of renal cell carcinogenesis and progression. To our knowledge, this is the first study to discover the overexpression of COX-1 in RCC and evaluate its ability to predict prognoses of RCC patients.

It must be pointed out that our study was a retrospective study and the number of patients included in this study was limited. Moreover, as a semi-quantitative method, immunohistochemical assay for protein expression of COX-1 is not as favorable as western blot, which may because of the specificity of the antibody used or the rough estimation by bare eyes. Besides, some inscrutable factors may exist and influence the outcomes of this study. Although we found the protein levels of COX-1 in tumors were correlated with the prognostic outcomes of RCC patients, further explorations are needed before the clinical utility of COX-1 protein as a biomarker for prognosis in RCC patients. More investigations are demanded to explore the impact of expression of COX-1 on RCC and its molecular mechanisms in tumorigenesis, proliferation and progression.

In conclusion, our study firstly revealed the overexpression of COX-1 in renal cell carcinoma and it was correlated with several clinicopathologic characteristics. Patient with high expression of COX-1 has shorter survival time and worse prognosis. These results indicated that COX-1 may be a prognostic marker for renal cell carcinoma.

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