RESEARCH COMMUNICATION

RALY RNA Binding Protein-like Reduced Expression is Associated with Poor Prognosis in Clear Cell Renal Cell Carcinoma

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Abstract

The molecular mechanisms involved in the progression of clear cell renal cell carcinomas (ccRCCs) are still unclear. The aim of this study was to analyse the relationships between expression of RALYL and clinical characteristics. In 41 paired samples of ccRCCs and adjacent normal tissues, we used real-time qPCR to evaluate the expression of RALYL mRNA. RALYL protein levels were determined in 146 samples of ccRCC and 37 adjacent normal tissues by immunohistochemistry. Statistical analysis was used to explore the relationships between expression of RALYL and the clinical characteristics (gender, age, tumor size, T stage, N stage, M stage, survival times and survival outcome) in ccRCC. In addition, these patients were follow-up period 64 months (range: 4~116months) to investigate the influence on prognosis. We found significantly differences between ccRCC tissues and normal tissues ($p<0.001$, paired-sample t test) in mRNA levels of RALYL. Immunohistochemistry analyses in 146 ccRCC samples and 37 adjacent normal tissues showed significantly lower RALYL protein levels in ccRCC samples ($\chi 2$-test, $p<0.001$), inversely correlating with tumour size ($p=0.024$), T stage (0.005), N stage ($p<0.001$) as well as M stage ($p=0.019$), but not age ($p=0.357$) and gender ($p=0.348$) . Kaplan-Meier survival analysis demonstrated that people with lower level of RALYL expression had a poorer survival rate than those with a higher level of RALYL expression, significantly different by the log-rank test ($p=0.011$). Cox regression analysis indicated that RALYL expression ($p=0.039$), N stage ($p=0.008$) and distant metastasis ($p<0.001$) were independent prognosis factors for the overall survival of ccRCC patients. We demonstrated that the expression of RALYL was significantly low in ccRCC and correlated with a poor prognosis in a large number of clinical samples. Our findings showed that RALYL may be a potential therapeutic target as well as a poor prognostic factor.

Keywords: Clear cell renal cell carcinomas - RALYL - clinical characteristics - prognosis

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Introduction

Renal cell carcinoma (RCC) is the most common malignant tumour of adult kidney (Eble et al., 2004), and RCC's histopathology is subdivided into various categories, which clear cell renal cell carcinoma (ccRCC) is the most common pathological category, accounting for 70-80% of all RCCs (Young et al., 2001; Matsuura et al., 2011). In the last decades, there have been immense improvements in the treatment of ccRCC, but approximately 30% of the patients would develop to metastatic after surgical therapy (Janzen et al., 2003; Cohen et al., 2005; Chen et al., 2011). Although some environmental and genetic factors have been found to be associated with ccRCC, the molecular mechanisms of ccRCC occurrence and development are still unclear (Janzen et al., 2003).

RALY RNA binding protein-like (RALYL) belongs to the RALY subfamily. RALYL locates in 8q21.2, the function of which is RNA-binding and nucleotide binding. The RALYL high expression in the normal adrenal, kidney and brain (Shyamsundar et al., 2005). Furthermore, RALYL expression down-regulated correlates with mental disorder (Lee et al., 2007), Parkinson's disease (Moran et al., 2006), brain cancer (Maris et al., 2008), adrenal cancer (Giordano et al., 2009) and kidney cancer (Higgins et al., 2003). However, there was not published report on the relationships between the expression of RALYL and

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clinical manifestation and prognosis. In this study, we aimed to explore the expression of RALYL and its clinical significance in ccRCC.

Materials and Methods

Patients and tissue specimens

Written informed consent was obtained from all patients, and the study was approved by the institutional review board of Peking University Shenzhen hospital. For real-time RT-PCR, we collected 37 paired samples of ccRCCs and adjacent normal tissues from patients who underwent radical nephrectomy between November 2010 and June 2011, and the normal kidney sample were defined as tissues located 2.0 cm outside of visible ccRCC lesions. The 41 patients included 36 men and 5 women. The fresh tissues were immediately immersed in RNAlater (Qiagen; Germany) after surgical resection, which were stored at 4°C overnight to allow thorough penetration of the tissue and then frozen at -80 °C.

In addition, we performed an immunohistochemistry assay of 146 paraffinembedded samples of ccRCC and 37 adjacent normal renal tissue samples collected from patients between 2001 and 2008. The characteristics of these 146 patients are listed in Table 1. None of the patients underwent radiotherapy or chemotherapy before surgery. The histological and clinical diagnoses of the tumours in all these patients were performed by department of urology of the Peking University Shenzhen hospital. All the 146 patients' survival information was collected on telephone. Patients' clinical characteristics (gender, age, tumour size and TNM stage) were obtained from the medical records. The disease stage of each patient was classified or reclassified according to the 2002 American Joint Committee on Cancer (AJCC) staging system (Greene 2002) .

Real-time qPCR

Total RNA was extracted from ccRCC samples and the normal tissue using TRIZOL (Invitrogen, US) according the manufacture's protocol. Then we used Omniscript Reverse Transcriptase kit (Qiagen, Hilden, Germany) to synthesize the first-strand cDNA. The total reaction volume was 20μl including 1ug RNA, and the reaction mixture was incubated at 42 °C for 60 min, heated at 95°C for 10 min and then cooled on ice. Both the RNA and cDNA were evaluated according Agilent 2100 Bioanalyzer (Agilent Technologies, US).

Both RALYL and U6 (as an internal control) olionucleotide primers were designed by Primer 5, based on their mRNA sequences. The corresponding primers sequence as follows showed: RALYL sense strand: 5'-GAGTCTAGTGCTGATCCAAG-3', RALYL antisense strand: 5'-CCTCCTCTATCCCATCTGT-3', U6 senses strand: 5'-CTCGCTTCGGCAGCACA-3', U6 antisense strand: 5'-ACGCTTCACGAATTTGCGT-3'. Real-time PCR was carried out with SYBR Green dye in 7000 Sequence Detection System (Applied Biosystems). The 20 μl real-time PCR reaction mixture contained 1 μl of cDNA (synthesized as described above), 10 μl SYBR Green master mix (Invitrogen; Carlabad, CA), and upstream and downstream primer each add 1μl. The amplification conditions were 95 °C (2 min) for 1 cycle and 95 °C (5 sec), 57 °C (30 sec), and 68 °C (30 sec) for 40 cycles. Relative expression levels of the target genes were normalized to the geometric mean of the internal control gene, U6. The data were analysed using the comparative threshold cycle ($2^{-\Delta CT}$) method.

Immunohistochemistry (IHC)

An immunohistochemistry assay was performed to examine RALYL expression in the 146 ccRCC samples and 37 paired samples of adjacent normal renal tissue. All procedures were performed using classical protocols. Briefly speaking, paraffin-embedded specimens were cut into 5μm sections and baked at 65 °C for 30 min. The sections were deparaffinized in 100% xylene and re-hydrated in descending ethanol series (100%, 90%, 80%, 70% ethanol) and water according to standard protocols. Then antigen retrieval submerged into 0.01 M citrate buffer (pH 6.0) for 2 min at 100 °C. They were then treated with 3% hydrogen peroxide in methanol to quench the incubation with 10% bovine serum albumin to block nonspecific binding.

The RALYL protein was detected by using a mouse monoclonal antibody against RALYL (Abcam; Cambridge, MA, USA). The specimens were incubated overnight at 4 °C with anti-RALYL antibody (1:250). The negative control for immunohistochemistry analysis was obtained by replacing the primary antibodies with an antibody diluent. After washed in phosphate buffered saline (PBS), the sections were treated with MaxvisionTM HRP-plymer anti-Mouse IHC Kit (Maixin Bio; Fujian, China) at 37 °C for 20 min. The tissue sections were immersed in 3-amino-9-ethyl carbazole, counterstained with Mayer's hematoxylin, dehydrated, and finally mounted in Crystal Mount.

The formalin-fixed, paraffin-embedded sections were reviewed for the degree of immune-staining and scored by 2 independent observers. The proportion of cells expressing RALYL varied from 0% to 100%, and the intensity of staining varied from weak to strong. The proportion of RALYL expression tumour cells was scored as follows: 0, no positive cells; 1, 1%-10%; 2, 11%-50%; 3, 51%-75%; and 4, >75% according to Tsuchiya et al. The staining intensity was graded according to the mean optical density (Tsuchiya et al., 2003; Bao et al., 2004; Saussez et al., 2006): 0, no staining; 1, weak staining (light yellow); 2, moderate staining (yellow brown); and 3, strong staining (brown). Staining index was calculated as the multiplication of staining intensity score and the proportion of RALYL-positive tumour cells. We evaluated RALYL expression in benign kidney tissue and malignant lesions on the basis of the staining index values, with scores of 0, 1, 2, 3, 4, 6, 8, 9 and 12. The cut-off values for RALYL expression were chosen on the basis of a measure of heterogeneity in overall survival rates, which were calculated using the log-rank test. An optimal cut-off value was identified: a staining index score 6, 8, 9 and 12 was considered as high RALYL expression, whereas a staining index score of <=4 was considered as low RALYL expression.

Statistical analysis

All statistical analysis was carried out with the SPSS 17.0 statistical software package. In the real-time RT-PCR paired-sample t test were used to analyse the significance of the differences in mRNA between ccRCC and the adjacent normal tissues. The χ^2-test for proportion was used to analyse the relationship between RALYL expression and clinical significance. Survival curves were plotted by the Kaplan-Meier method and compared with the log-rank test. Multivariate analyses were performed according to Cox proportional hazards regression mode. P<0.05 was considered to be statistically significant.

Results

Real time RT-PCR

Real-time PCR was performed to measure the expression of RALYL mRNA in 41 ccRCC tumour tissues and the paired adjacent normal tissue samples. Compared with normal tissues, 36 ccRCC tumour tissues were significantly lower expression at mRNA levels (p<0.001, paired-sample t test, Figure 1).

Immunohistochemistry analysis of RALYL protein expression in ccRCC samples and the paired normal renal tissue

In normal renal tissue, RALYL protein was localized mainly in epithelial cells' cytoplasm of renal tubule. We performed immunohistochemistry analysis to assess the expression of RALYL protein in 146 ccRCCs tissue blocks and 37 normal renal tissues. RALYL protein were high expression in 33 normal tissues or 89.2% (33/37), significantly higher than 36% (52/146) in ccRCC tissues (χ^2-test, p<0.001) (Figure 2).

The relationships between RALYL protein expression and clinical features

The correlation between RALYL expression and various clinic-pathological parameters were shown in Table 1. In the 146 ccRCC samples, RALYL decreased expression in 94 specimens (scores<=4) and increased expression in 52 cases (scores<=5). The RALYL protein expression in ccRCC tissues were correlated with tumour size (χ^2=5.084, p=0.024), T stage (χ^2=10.491, p=0.005),

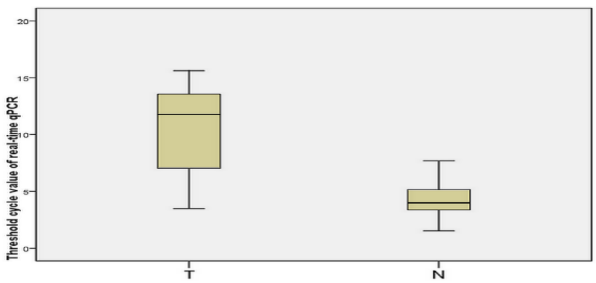

Figure 1. Real-time RT-PCR Analysis of RALYL Expression. The threshold cycle value of real-time qPCR in 36 RCC tumour tissue samples was higher than that in the paired adjacent normal tissue samples (n=41, p<0.001). The bottom and the top of the box represent the box represent the 25th and the 75th percentile, respectively, and the band near the middle of the box is the 50th percentile (the median). The ends of the whiskers represents the minimal and the maximal value

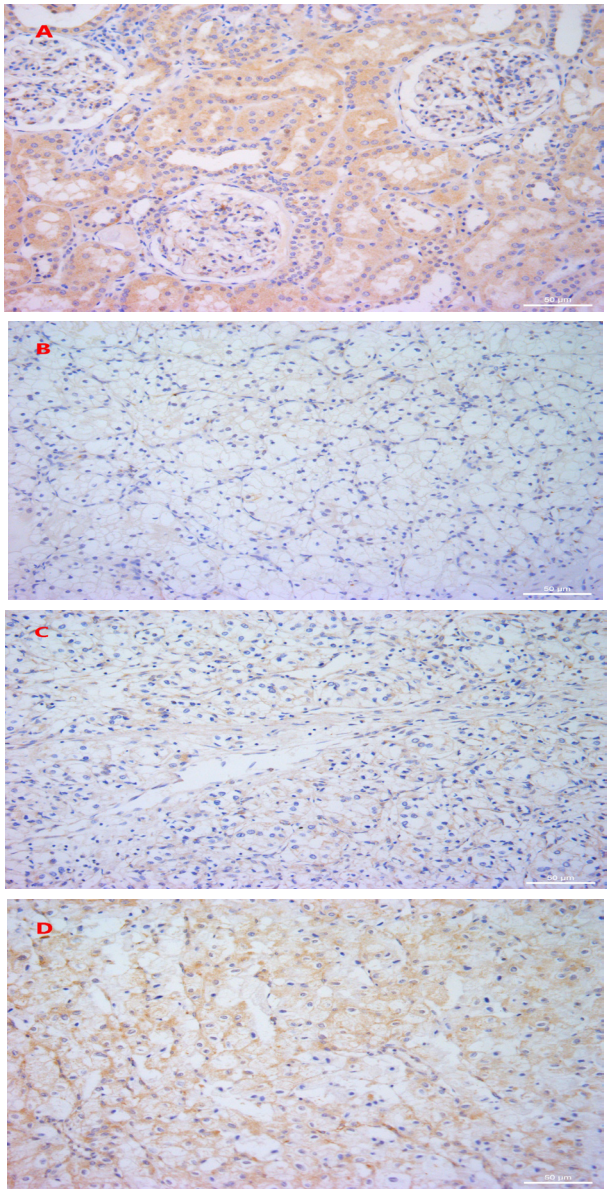

Figure 2. Immunohistochemistry Analysis of the Expression of RALYL Protein in ccRCC. (A): Normal sample showed high expression in epithelial cells' cytoplasm of renal tubule. (200X) (B): Negative or weak RALYL staining in tumour tissue (200X). (C): Moderate RALYL staining in ccRCC (200X). (D): Strong RALYL staining in tumour cells (200X)

N stage (χ^2=18.489, p<0.001) and M stage (χ^2=5.466, p=0.019), while connections with age (χ^2=0.85, p=0.357) and gender (χ^2=0.881, p=0.348) weren't found in ccRCC tissues.

Survival analysis

To investigate the prognostic value of RALYL expression in ccRCC, we used Kaplan-Meier analysis and the log-rank test to assess the relationships between RALYL protein expression in ccRCC and prognosis information. We found that the level of RALYL expression correlated with the overall survival of ccRCC patients. People with lower level of RALYL expression had poorer survival rates than those with higher level. The group of low expression RALYL patients' means survival time was 59.74 months and the medians survival time was 61 months, but the high expression group's means and

Table 1. Association of RALYL with Clinic-pathologic Characteristics in ccRCC Patients (n=146)

Clinic pathologic characteristics	n	RALYL expression Low	High	χ^2	P
All case	146	94	52		
Age(y)				0.881	0.348
<50	58	40	18		
≥50	88	54	34		
Gender				0.85	0.357
Male	105	70	35		
Female	41	24	17		
Tumor size (cm)				5.084	0.024
<7	80	44	36		
≥7	66	50	16		
T stage				10.491	0.005
T1	78	41	37		
T2	36	27	9		
T3/T4	32	26	6		
N stage			6	18.489	<0.001
N0	109	81	28		
N+	37	13	24		
M stage				5.466	0.019
M0	121	83	38		
M1	25	11	14		

Table 2. Cox Regression Analysis for the Overall Survival Rates of ccRCC Patients. RR: Relative Risk; 95% CI: 95% Confidence Interval

Risk factors	RR	95% CI	P value
T stage	1.437	0.713-1.026	0.208
N stage	1.929	1.191-3.124	0.008
M stage	5.677	2.585-12.468	<0.001
Age	0.156	0.44-1.141	0.708
Size	0.96	0.562-1.640	0.881
Gender	0.497	0.535-1.354	0.851
RALYL expression	0.662	0.405-1.081	0.039

medians survival time was 79.745 months and 89months. The log-rank test showed the survival rates were significantly different between these two groups (Figure 3, χ^2=6.406, p=0.011).

In addition, the multivariate analysis indicated that RALYL expression (p=0.039), N stage (p=0.008) and distant metastasis (p<0.001) were independent prognosis factors for the overall survival of ccRCC patients (Table 2).

Discussion

Clear cell renal cell carcinoma accounted for 2% of all cancers, which increased 1.5%-5.9% each year around the world (Mccridie 1994; Chow et al., 2010). At present the main methods for renal cell carcinoma is surgical operation, radiotherapy and chemotherapy, but unfortunately ccRCC is not sensitive to radiotherapy and chemotherapy (VAziri et al., 2009; Wu et al., 2011; Yang et al., 2011). In clinical, about 30% of patients have distant metastases when they are diagnosed for the first time. Therefore, early diagnosis and early treatment is very important, and hunting for specific molecular biomarkers of ccRCC is essential for the diagnosis and treatment.

A lot of research have been done aiming at some molecular therapeutic targets, such as p53 (VAziri et al.,

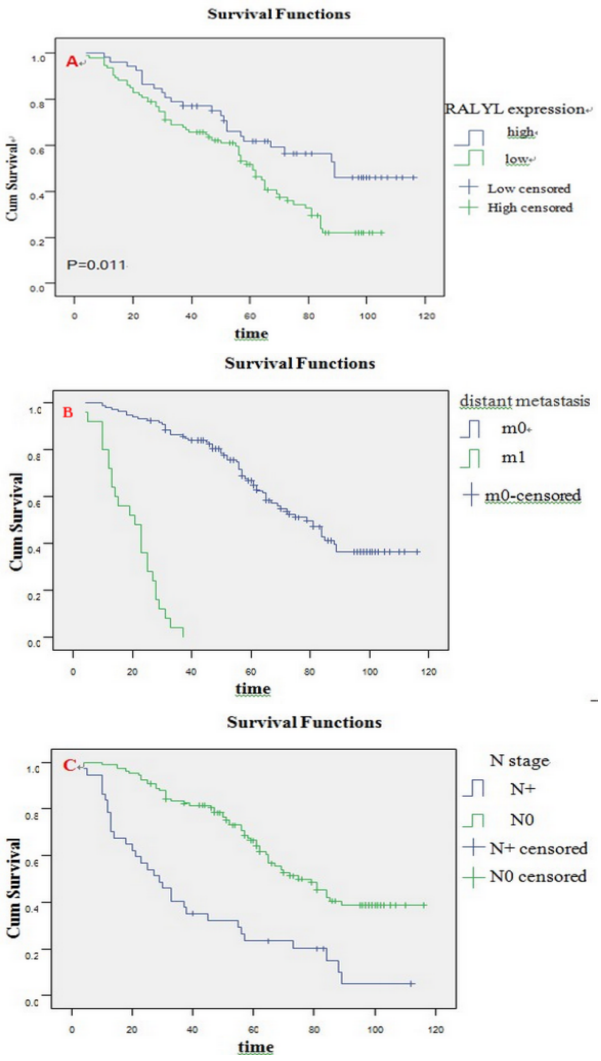

Figure 3. (A) The figure showed the Kaplan-Meier estimates for group with high expression of RALYL and group with low expression of RALYL. Mean survival times for two groups of high and low expression of RALYL were months 79.745 and 59.74 months, and the median survival times were 89 and 61 months. The log-rank test revealed that the survival rate of the group with low expression of RALYL was poorer than that of the group with high expression of RALYL (χ2=6.406, p=0.011). (B) Distant metastasis; (C) N stage. The longest follow-up time is 116 months

2009), vascular endothelial growth factor (VEGF)(Yang et al., 2003; Soulitizis et al., 2006; Patard et al., 2009), Ki67 (proliferation) (Kroeze et al., 2010) and hypoxia inducible factor (HIF) (Grandinetti et al., 2007; Baldewijns et al., 2010; Song et al., 2011), and some of the molecular therapeutic have been approved for therapeutic use or undergoing preclinical or clinical evaluation (Banks et al., 2007; Grandinetti et al., 2007; Huang et al., 2008). However the treatment effect is not obvious and the molecular mechanisms of the initiation and progression of the ccRCC are still unclear (Levi et al., 2008).

RALY RNA binding protein-like (RALYL) belongs to RALY subfamily, and it contains 10 exons and 1 RRM (RNA recognition motif) domain so as to combine to the RNA. The microarray expression date showed that RALYL high expression in the normal adrenal, kidney and brain (Shyamsundar et al., 2005), while the gene low expression correlates with mental disorder (Lee et

al., 2007), Parkinson's disease (Moran et al., 2006), brain cancer (Maris et al., 2008), adrenal cancer (Giordano et al., 2009) and kidney cancer (Higgins et al., 2003). Furthermore, this gene is linked to VHL and UBC by using Affinity Capture-MS. In this paper, we focus on the relationships between the expression of RALYL and the ccRCC patients' clinical characteristics, hunting for the reason of RALYL down regulation in ccRCC, and try to explore the gene influence on the clinical prognosis.

Our studies demonstrate that RALYL were low expression in both mRNA and protein levels in ccRCC samples compared to adjacent normal renal tissues. What's more, immunohistochemistry analysis showed that RALYL protein had low expression in ccRCCs and it had high expression in the adjacent normal tissues. We found that RALYL low expression in human clinical ccRCC samples.

The TNM stage of ccRCC was closely related to its prognosis (Levi et al., 2008). We found that the decreased expression of RALYL was correlated with tumour size, T stage, N stage and M stage. According to Kaplan-Meier analysis, RALYL protein expression in ccRCC was correlated with patients' overall survival. Patients with lower RALYL expression had a shorter survival time. The log-rank test revealed that the survival rate of the group with lower expression of RALYL was poorer than that of the group with higher expression of RALYL. In our study, we also found that RALYL expression, N stage and distant metastasis were independent prognosis factors for the overall survival of ccRCC patients by using the Cox regression analysis. Thus, our findings indicate that there were significant correlations between the RALYL expression level and clinic-pathological parameters and the gene may be a potential prognostic marker and therapeutic target in ccRCC.

Our study was a single hospital-based, retrospective study. It should be pointed out that unmeasured differences may exist and may distort the study results. A multi-centres or community-based prospective study with more extensive collection of potential confounders is required. In addition, according to the above mentioned consequence, RALYL may be a potential therapeutic target potential prognostic marker in ccRCC, and this needs more study.

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