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Original Article

Decreased expression of VPS33B correlates with disease progression and unfavorable prognosis in renal cell carcinoma

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Abstract: The aim of this study was to examine correlation between low cytoplasmic expression of VPS33B and clinicopathologic features of renal cell carcinoma (RCC). In this study, ninety RCC patients ranging from years 2006 to 2012 were reviewed. VPS33B expression in tumor tissues and adjacent normal tissues was examined using immunohistochemistry (IHC) and association of VPS33B expression with RCC patient clinicopathologic parameters was evaluated. Final staining scores of 0-5 and 6-7 were respectively considered to be low and high expression. Immunohistochemical analysis confirmed that VPS33B protein expression was predominantly localized in cytoplasm of both RCC and adjacent normal tissues. Lower cytoplasmic VPS33B expression was observed in RCC compared to normal cells (P = 0.007). In addition, cytoplasmic VPS33B protein levels in tumor tissues were correlated with T stage (T1 vs. T2 vs. T3) (*P* = 0.038), stage (I-II vs. III-IV) (*P* = 0.035), and renal vein invasion (*P* = 0.039) of RCC patients. Lower RCC cytoplasmic VPS33B expression had a significantly shorter disease free survival (DFS) compared to the higher expression group (*P* = 0.030). Multivariate analysis suggested that low cytoplasmic VPS33B expression was an independent predictor for DFS of RCC patients. (*P* = 0.030). Our results suggest that low cytoplasmic VPS33B expression is a potential unfavorable prognostic factor for progression and prognosis of RCC.

Keywords: Renal cell carcinoma, VPS33B, immunohistochemistry

Introduction

Renal cell carcinoma (RCC) is the most common type of renal cancer. It is a heterogeneous tumor originating from the kidney, accounting for ~3% of malignancies in adults [1-4]. Due to tumor recurrence or distant metastasis, the prognosis of advanced RCC was reported to be 5-10% 5-year survival rate [5, 6]. Since RCC has no characteristic symptoms, about 30% of patients are detected with metastatic symptoms. Although nephrectomy is the first choice for patients with RCC, almost all of these patients finally relapse or show distant metastasis because of resistance to targeted radiotherapy and therapeutic drugs [7, 8]. Thus, it is important to find a biomarker of RCC for early diagnosis and targeted therapy based on mechanisms of RCC tumorigenesis.

Vacuolar protein sorting 33B (VPS33B) protein is extensively expressed in human tissues, which solubles N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) binding [9]. Further, VPS33B encodes Vps33, a homolog of the class C yeast vacuolar protein sorting gene, and plays a role in regulation of SNARE-dependent membrane fusion, which may be involved in the pathogenesis of Hermansky-Pudlak syndrome-related diseases and arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome [10]. Additionally, a recent study demonstrated that VPS33B is dephosphorylated by secreted Mtb protein tyrosine phosphatase PtpA to promote innate immunity [11, 12]. VPS33B is also found to affect Rab protein interaction and collagen modification and lead to Autosomal Recessive Keratoderma-Ichthyosis-Deafness (ARKID) [13]. Further, Hanley J et al. found that VPS33B has a key role in es-

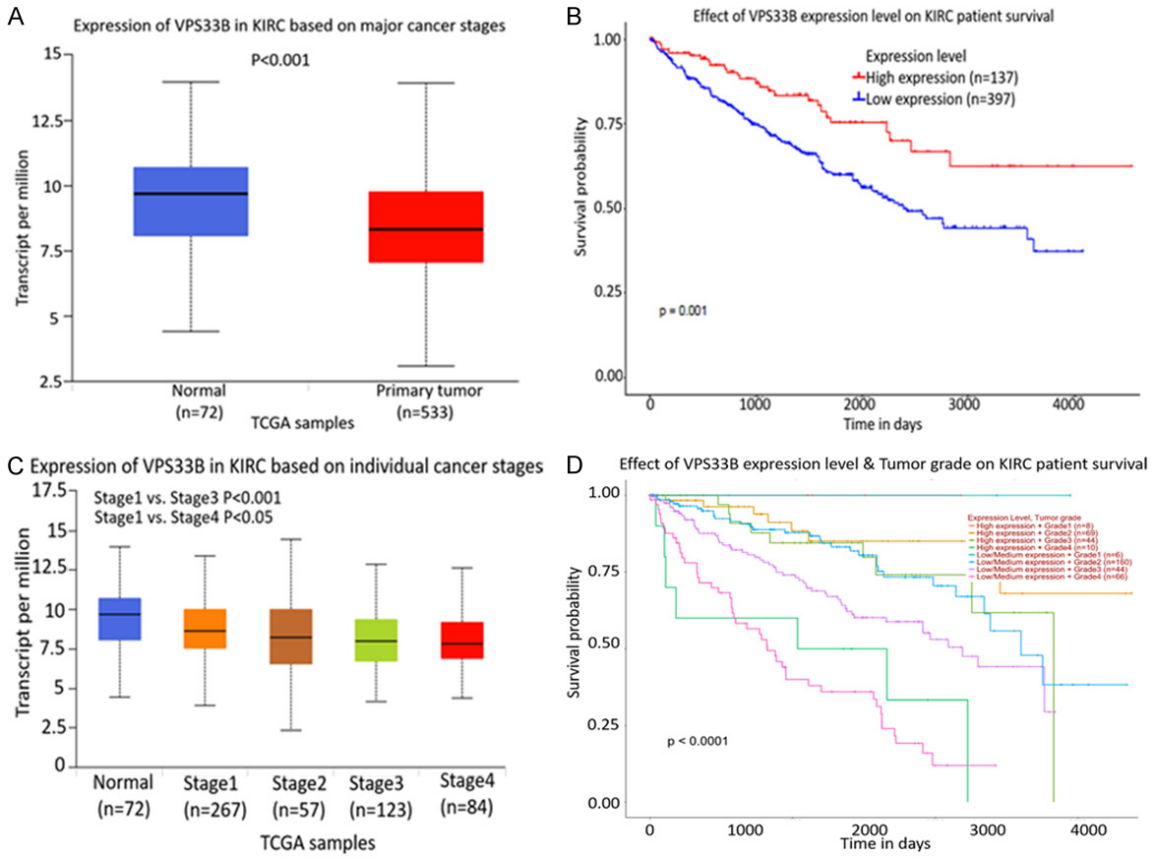

Figure 1. VPS33B may be an antitumor gene in renal cell carcinoma. A. Expression of VPS33B was detected in tumor and normal tissue of RCC patients (P<0.001). B. Low VPS33B expression was correlated to the shorter survival time for RCC patients (P = 0.001). C. Expression of VPS33B in RCC tissues based on individual stages. D. Effect of VPS33B expression & tumor grade in RCC patient survival (P<0.0001).

tablishing structural and functional aspects of hepatocyte polarity and may be a target for gene replacement therapy [14]. In leukemogenesis, VPS33B regulates exosomal autocrine signaling to mediate hematopoiesis [15]. Although several reports have demonstrated that VPS33B is involved in the pathogenesis of some diseases, its role in regulation of tumors is not yet clear.

In order to clarify the role of VPS33B in tumorigenesis of RCC, we measured correlation between VP33B protein expression and clinicopathologic features, including survival. We found that VPS33B protein expression levels were lower in RCC tissues than in peritumoral tissues. Furthermore, lower expression of VPS33B was related with poor prognosis of RCC. Our results suggest that decreased cytoplasmic expression of VPS33B is a potential unfavorable factor in progression and prognosis of RCC.

Materials and methods

Sample collection

A tissue array including 150 paired paraffin-embedded RCC and 30 peritumoral tissues were purchased from Superchip Biological Technology Co., Ltd, Shanghai, China. All patients with RCC underwent surgery from 2008 to 2010 in Taizhou Hospital of Zhejiang Province. Patient ages ranged from 24 to 83 years. Clinical follow up time of patients ranged from 4 to 90 months. Prior consent from patients and approval from the Ethics Committees of Taizhou Hospital of Zhejiang Province were obtained for research purposes. All specimens had confirmed pathological diagnosis and were staged according to the International Society of Urological Pathology (ISUP) on Cancer (AJCC) (2016) staging system for RCC.

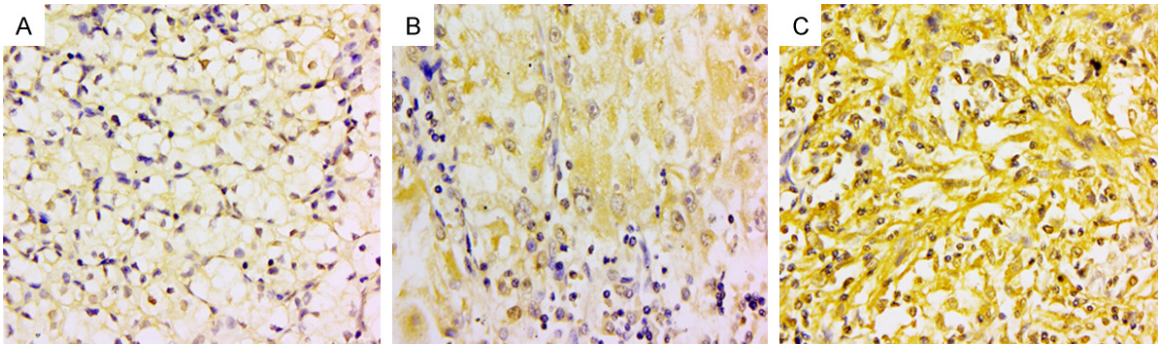

Figure 2. VPS33B expression in renal cell carcinoma tissues. Negative (A) expression of VPS33B was detected in RCC tissue with stage III (400×), positive (B) expression of VPS33B was detected in cytoplasmic of RCC tissue with stage II (400×), strong (C) expression of VPS33B was detected in nuclei and cytoplasmic of RCC tissue with stage I (400×).

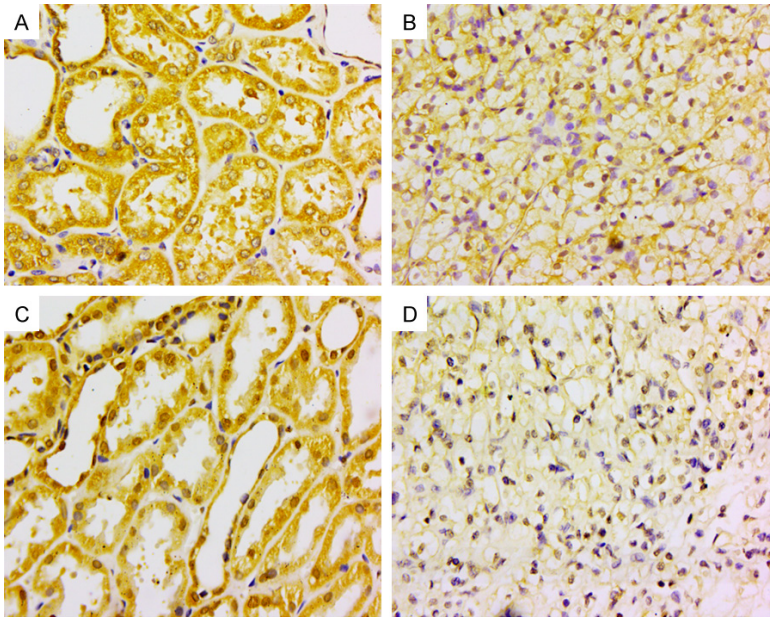

Figure 3. VPS33B expression in renal cell carcinoma and peritumoral renal of two RCC patients. High (A) and low (B) expression of VPS33B was demonstrated in RCC patient with stage II (400×). High (C) and low (D) expression of VPS33B was demonstrated in RCC patient with stage III (400×).

Immunohistochemistry

A tissue array with 150 paraffin-embedded RCC and 30 peritumoral tissues was deparaffinized in 100% xylene and subsequently rehydrated in descending ethanol series (100%, 90%, 80% and 70% ethanol) and water. Antigen retrieval was performed by immersing the tissues in 10 nM citrate buffer at 100°C for 15 minutes. The sections were washed three-times. Endogenous peroxidase activity was blocked with peroxidase blocking reagent containing 3% hydrogen peroxide and horse serum, followed by incubation with rabbit anti-human VPS-33B antibody (1:100) (Proteintech, 12195-1-AP, USA) overnight at 4°C. The tissue array was then incubated with biotin-labelled goat anti-mouse/rabbit antibody at room temperature for 1 hour and subsequently incubated with streptavidin-conjugated horseradish peroxidase (HRP) (Maixin Inc, China) for 10 minutes. The tissue array was developed with 3,3-diaminobenzidine (DAB) chromogen solution in DAB buffer substrate and then counterstained with hematoxylin and mounted in neutral gum. Finally, we analyzed the staining of tissue array using a bright-field microscope.

Evaluation of staining

The tissue array, immunohistochemically stained for VPS33B, was reviewed and cytoplasmic staining scored separately by two pathologists blinded to clinical parameters. Staining intensity was scored as described previously [16]. The extent of staining was scored by applying a semi-quantitative system ranging from negative to strong as follows: 0 = negative, 1 = weak, 2 = moderate, and 3 = strong. The percentage of positive cells was categorized was scored on a scale of 0-4 according to the positive tumor cells as follows: 1: less than 25% positive cells, 2: 25 to 50% positive cells, 3: 51 to 75% posi-

Table 1. Protein expression of VPS33B between RCC and peritumoral normal samples

Group	Cases (n)	Protein expression (n)		*P* value
		Low expression	High expression	
Cancer	30	24 (80.0%)	6 (20.0%)	0.007
Normal	30	14 (46.7%)	16 (53.3%)	

RCC, Renal cell carcinoma; Normal, peritumoral normal samples. *χ^2 test was applied to access the expression of VPS33B between RCC and peritumoral normal samples.

Table 2. Correlation between clinicopathologic characteristics and cytoplasmic expression of VPS33B protein in RCC

Characteristics	N	VPS33B expression		*P*
		High	Low	
Age				
<50	39	24 (65.8%)	15 (34.2%)	0.466
≥50	111	71 (63.5%)	40 (36.5%)	
Gender				
Male	74	63 (58.9%)	44 (41.1%)	0.074
Female	16	32 (74.4%)	11 (25.6%)	
T stage				
T1	122	83 (68.0%)	39 (32.0%)	0.038
T2	17	8 (47.1%)	9 (52.9%)	
T3	11	4 (36.4%)	7 (63.6%)	
Primary tumor stage				
I/II	103	71 (68.9%)	32 (31.1%)	0.035
III/IV	47	24 (51.1%)	23 (48.9%)	
Size (cm^3)				
<100	99	63 (63.6%)	36 (36.4%)	0.781
100-150	30	20 (66.7%)	10 (33.3%)	
>150	21	12 (57.1%)	9 (42.9%)	
Renal vein invasion				
Absent	74	88 (61.5%)	55 (38.5%)	0.039
Present	16	7 (100%)	0 (0%)	

RCC, Renal cell carcinoma; *χ^2 test was applied to access the associations between VPS33B expression and the clinicopathological parameters.

tive cells, and 4: more than 75% positive cells. The sum of the staining-intensity and staining-extent scores was used as final staining scores for VPS33B (0-7). For statistical analysis, final staining scores of 0-5 and 6-7 were respectively considered to be low and high expression.

Statistical analysis

All statistical analyses were performed using SPSS 19.0 software. χ^2 test was used to verify the relationship between levels of VPS33B expression and clinicopathologic characteristics. Survival curves were plotted using the Kaplan-Meier method and compared using log-rank test. Significance of various variables in survival was analyzed using multivariate Cox proportional hazards model. A value of *P* less than 0.05 was considered statistically significant.

Results

VPS33B may be an antitumor gene in RCC

Through searching the UALCAN database, whose statistical analysis is based on TCGA data, we found that VPS33B mRNA levels in tumor tissues were lower than normal tissues (**Figure 1A**). Patients with elevated expression of VPS33B had higher survival probability than those with low expression, according to data analysis (**Figure 1B**). As for VPS33B expression in RCC based on individual cancer stage, tissue with stage I had higher VPS33B expression than stage III ($P< 0.001$) and IV ($P<0.05$) (**Figure 1C**). In **Figure 1D**, there is a significant difference in patient survival between stage III with high expression and stage III with low/medium expression, as well as between stage IV with high expression and stage IV with low/medium expression. Thus, we speculated that VPS33B protein may be an antitumor gene in the pathogenesis of RCC.

Immunohistochemical analysis of VPS33B protein expression in RCC and peritumoral tissues

We measured expression levels and cellular localization of VPS33B protein on a tissue array containing 150 paraffin-embedded RCC and 30 peritumoral samples. Specific VPS33B protein staining was mostly detected in the cytoplasm of malignant cells while in the cytoplasmic and nuclei of peritumoral cells (**Figures 2** and **3**). In **Figure 2**, VPS33B cytoplasmic expression was significantly strong in RCC tissue with stage I compared to negative expression in RCC tissue with stage II and III. In **Figure 3**, we found that VPS33B cytoplasmic expres-

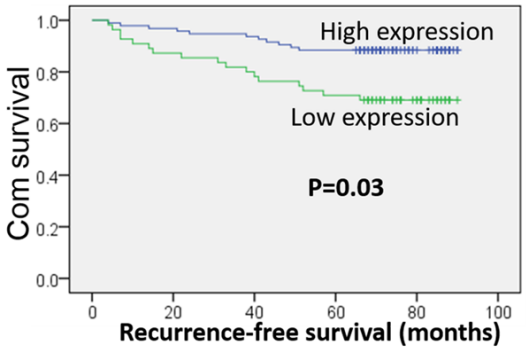

Figure 4. Cytoplasmic expression of VPS33B was an unfavorable factor for recurrence-free survival in RCC. Low expression of VPS33B protein was correlated with shorter recurrence-free survival time for RCC patients (P = 0.030).

sion was stronger in peritumoral tissue than in RCC tissue of these two patients. We observed that VPS33B protein was highly expressed in 53.3% (16/30) of peritumoral samples compared to elevated expressed VPS33B expression in only 20% (6/30) of RCC samples (*P* = 0.007) (**Table 1**).

Association between clinicopathological characteristics and VPS33B expression in RCC patients

As shown in **Table 2**, we observed that tumor VPS33B cytoplasmic expression inversely correlated with T stage (T1 vs. T2 vs. T3) (*P* = 0.038), stage (I-II vs. III-IV) (*P* = 0.035), and renal vein invasion (*P* = 0.039) in 150 RCC cases. However, we did not find significant correlation between tumor VPS33B expression and patient age, gender, and tumor size.

VPS33B expression is positively associated with recurrence-free survival of RCC

To investigate the prognostic value of tumor VPS33B expression for RCC, we measured association between levels of VPS33B expression and patient survival using Kaplan-Meier analysis with log-rank test. By analyzing prognosis information of 150 RCC cases, we observed that levels of cytoplasmic VPS33B expression were significantly correlated with recurrence-free survival. Patients with low expression of VPS33B had worse recurrence-free survival than those with high expression (*P* = 0.030) (**Figure 4**).

VPS33B expression is positively associated with recurrence-free survival of RCC patients based on T stage and primary tumor stage

We further analyzed correlation between VPS33B expression and survival prognosis for RCC patients by strata analysis against T stage and primary tumor stage. Further, tumor VPS33B protein expression was associated with survival time for patients in stages III-IV (P = 0.027) (**Figure 4**). However, we did not observe association between overall survival time and VPS33B expression levels for patients in stage T1, T2-T3, and clinical stage I-II (**Figure 5**). In these groups, patients with low expression of VPS33B had worse recurrence-free survival than those with high expression.

Cytoplasmic expression of VPS33B is an independent prognosis factor of recurrence-free survival in RCC patients

Univariate analysis showed that T stage and tumor size were significantly associated with recurrence-free survival of RCC patients (*P* = 0.002, *P* = 0.031 and *P* = 0.031, respectively). To determine the potential of VPS33B cytoplasmic expression as an independent prognostic factor for RCC, we performed multivariate analysis of VPS33B protein expression levels adjusted for T stage of RCC patients (*P* = 0.048) and tumor VPS33B expression (*P* = 0.030). These results showed that levels of VPS33B expression was an independent prognostic factor of recurrence-free survival for RCC (**Table 3**).

Discussion

Renal cell carcinoma (RCC) is one of the 10 most common cancer diagnoses globally, accounting for about 90% of all kidney tumors derived from renal tubular epithelial cells [17]. Although there are several treatments available for local RCC, nearly half of patients with RCC experience disease recurrence after chemotherapy, hormone treatment, and radiotherapy. Surgery remains the most effective method [18, 19]. According to 2004 WHO for classification and modification of the International Society of Urological Pathology (ISUP) Vancouver Classification, RCC was classified to three important subtypes which were clear cell RCC, papillary RCC, and chromophobe RCC, based on specific histopathological and genetic characteristics [20, 21]. Therefore, it is impor-

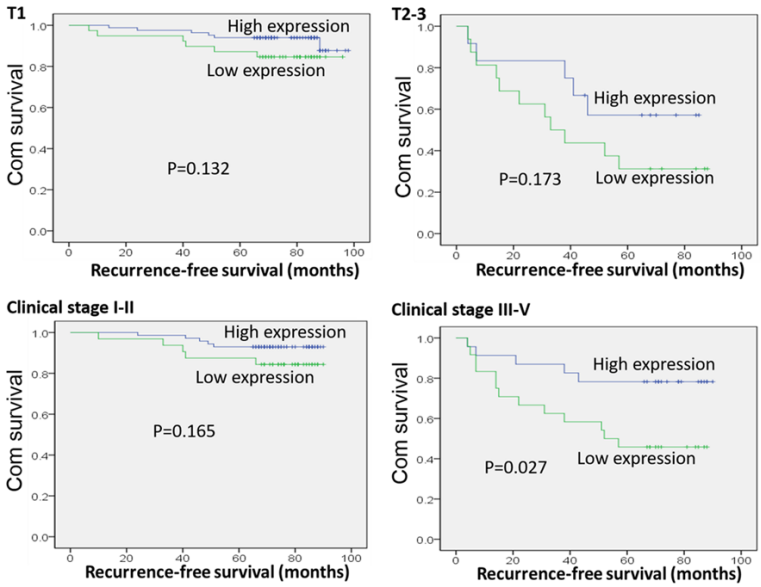

Figure 5. Correlation of VPS33B expression with recurrence-free survival time for RCC patients stratified by T stage and clinical stage. Patients with a higher expression of VPS33B protein had a longer recurrence-free survival time in the RCC patients with stage III-IV (P = 0.027).

tant to discover a valuable biomarker for prognosis of RCC. The molecular mechanisms of RCC invasion and metastasis, however, are not yet fully understood.

Up until now, the role of VPS33B in the pathogenesis of tumors has remained unclear. Ours is the first study to explore correlation between VPS33B expression and clinicopathological features and prognosis of RCC. According to UALCAN database, we predicted that VPS33B expression is significantly higher expressed in RCC samples than renal samples. Further, patients with high VPS33B expression might have higher survival probability. There is a significant difference between patients with stage I and patients with stage III/IV. In order to verify our prediction, we measured VPS33B expression in RCC patients by immunohistochemistry of RCC tissue array. Interestingly, we found that VPS33B protein expression in RCC samples was significantly lower than peritumoral renal samples. This result provides evidence to support that VPS33B protein is an antitumor protein for RCC. We then analyzed association between VPS33B expression levels and clinical features of RCC patients. Although cytoplasmic expression of VPS33B levels was not associated with most clinical features, such as age, gender and tumor size, it was inversely correlated with T stage, primary tumor stage, and renal vein invasion. These data suggest that VPS33B may be involved in proliferation and metastasis of RCC.

In this study, we observed that cytoplasmic expression of VPS33B protein in RCC was positively correlated with patient recurrence-free survival time. Our results show that patients with low expression of VPS33B had significantly worse recurrence-free survival time than those with high expression. Furthermore, we also presented evidence for a trend of significantly shorter overall survival in patients with low cytoplasmic expression of VPS33B compared with high expression. These data hint that low cytoplasmic expression of VPS33B is a relevant biomarker for RCC prognosis, especially for recurrence-free survival, and further support the role of VPS33B as a potential tumor suppressor inhibiting RCC pathogenesis.

In addition, survival prognosis was assessed by stratification analysis against different T classification and clinical stage. We observed that VPS33B protein expression was only positively associated with survival time for RCC patients in stage III-IV. Patients with high VPS33B expression had better prognosis than those of negative expression. These results hint that VPS33B expression is a potential biomarker for evaluating prognosis of NPC patients with stage III-IV.

Finally, we analyzed the possibility of VPS33B cytoplasmic expression as an independent prognostic factor for RCC. Based on univariate analysis, RCC patient recurrence-free survival is inversely related to T stage and tumor size but positively correlated with VPS33B cytoplasmic expression. Multivariate analysis showed that VPS33B cytoplasmic expression was an independent predictor of recurrence-free survival for RCC patients, regardless of patient disease status.

Table 3. Summary of univariate and multivariate Cox regression analysis of recurrence-free survival

	Univariate analysis			Multivariate analysis		
	P	HR	95% CI	*P*	HR	95% CI
Age						
<50 versus ≥50 years	0.890	1.026	0.711-1.480			
Gender						
Male versus Female	0.712	0.935	0.655-1.335			
T stage						
T1 versus T2 versus T3	0.002	1.519	1.162-1.984	0.048	1.469	0.995-2.168
Primary tumor stage						
I/II versus III/IV	0.151	1.289	0.912-1.823			
Size (cm^3)						
>100 versus 100-150 versus <150	0.031	1.275	1.022-1.589	0.786	1.044	0.766-1.424
Renal vein invasion						
Absent versus Present	0.506	0.773	0.361-1.653			
RCC VPS33B expression						
High expression versus Low expression	0.031	0.690	0.493-0.967	0.030	0.689	0.491-0.965

In summary, our study demonstrates that decreased cytoplasmic expression of VPS33B may be involved in clinical progression and poor prognosis of RCC patients. Due to our limited sample size, however, further studies are needed to clarify these findings and explore the role of VPS33B as a reliable prognostic predictor for HCC patients.

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Disclosure of conflict of interest

None.

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