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Aberrant expression of vasculogenic mimicry, PRRX1, and CIP2A in clear cell renal cell carcinoma and its clinicopathological significance

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Abstract

Vasculogenic mimicry (VM) involves a tubular structure with a basement membrane that is similar to and communicates with vessels but functions independent of blood vessels to nourish tumor cells, promote tumor progression, invasion, and metastasis, with reduced 5-year survival rates. Tumor cell proliferation, invasion, and metastasis are promoted by the epithelial-mesenchymal transition (EMT). Paired-related homeobox 1 (PRRX1), a newly discovered EMT inducer, has been shown to correlate with metastasis and prognosis in diverse cancer types. Cancerous inhibitor of protein phosphatase 2A (CIP2A) was initially recognized as an oncoprotein. In this study, we aimed to investigate the expression and clinical significance of the EMT markers PRRX1, CIP2A and VM in clear cell renal cell carcinoma (CCRCC) and their respective associations with clinicopathological parameters and survival.

Expression of PRRX1, CIP2A and VM in whole CCRCC tissues from 110 patients was analyzed by immunohistochemical and histochemical staining. Fisher's exact test or the chi square test was used to assess associations with positive or negative staining of these markers and clinicopathological characteristics.

Positive expression of CIP2A and VM presence was significantly higher and that of PRRX1 was significantly lower in CCRCC tissues than in corresponding normal tissues. Furthermore, positive expression of CIP2A and VM was significantly associated with tumor grade, size, lymph node metastasis (LNM) stage, and tumor node metastasis (TNM) stage and inversely associated with overall survival time (OST). Moreover, levels of PRRX1 were negatively associated with tumor grade, size, LNM stage, and TNM stage. The PRRX1 subgroup had a significantly longer OST time than did the PRRX1 subgroup. In multivariate analysis, high VM and CIP2A, tumor grade, LNM stage, TNM stage, and low PRRX1 levels were identified as potential independent prognostic factors for OST in CCRCC patients.

VM and expression of CIP2A and PRRX1 represent promising biomarkers for metastasis and prognosis and potential therapeutic targets in CCRCC.

Abbreviations: AJCC = American Joint Committee on Cancer, CCRCC = clear cell renal cell carcinoma, CIP2A = cancerous inhibitor of protein phosphatase 2A, CSC = cancer stem cell, DM = distant metastasis, ECM = extracellular matrix, EMT = epithelial-mesenchymal transition, HPF = high-power field, LNM = lymph node metastasis, OST = overall survival time, PAS = periodic acid-Schiff, PBS = phosphate-buffered saline, PP2A = protein phosphatase 2A, PRRX1 = paired-related homeobox 1, TNM = tumor-node-metastasis, VM = vasculogenic mimicry, WHO = World Health Organization.

Keywords: CIP2A, clear cell renal cell carcinoma, EMT, PRRX1, VM

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All clinical samples were solicited and obtained after receiving written consent from the patient, and the study was approved by the Ethical Committee of Bengbu Medical College and performed in accordance with the ethical guidelines of the Declaration of Helsinki (No. BBMUEC201718).

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1. Introduction

Kidney cancer is classified as the 16th most common cause of death from cancer worldwide,[1] and the majority of kidney cancers (70%) are classified as clear cell renal cell carcinoma (CCRCC).[2] Despite nephrectomy with curative intent, ~30% of CCRCC patients with localized disease eventually develop metastasis; therefore, identification of new early diagnostic tools and therapeutic methods for CCRCC is urgently needed.

Some researchers have found that when endothelium-dependent angiogenesis is not sufficient to support the rapid growth of tumor tissue, tumor cells themselves form a vessel-like, tubular structure with a basement membrane that communicates with blood vessels and acts as an angiogenesis-independent component of the tumor's microcirculation or microenvironment, a process known as vasculogenic mimicry (VM).[3,4] VM involves a lumen-like structure that provides nutrients and blood and promotes metastasis.[5] There are three main structures in VM: stem cell-like tumor cells, the extracellular matrix (ECM), and lumen-like structures connected to the circulatory system.[6] VM has been observed in a variety of malignant tumors, such as breast cancer, prostate cancer, liver cancer, nonsmall cell lung, and malignant glioma. Furthermore, many studies have shown that patients with cancer-related VM are prone to metastasis and have a poor prognosis.[5–8,27]

The epithelial mesenchymal transition (EMT) is a process in which cells lose their epithelial status as well as apicobasal polarity sustained by cell–cell adhesion molecules and gain mesenchymal traits. EMT is a dynamic process that converts epithelial cancer cells to dedifferentiated cells with additional mesenchymal properties. This transition entails up- and down-regulation of different proteins responsible for profound cellular reorganization resulting in the acquisition of enhanced migratory and invasive properties.[9,10] By facilitating tumor cell invasion and dissemination to distant organs, EMT has emerged as a key regulator of metastasis.

Paired-related homeobox 1 (PRRX1) is a novel EMT inducer, and its expression is associated with metastasis and prognosis in multiple tumors, participating in cancer progression in two different manners. Specifically, PRRX1 overexpression induces EMT in tumor cells, and it is also an indicator of a poor prognosis in gastric cancer and colorectal cancer.[11,12] Moreover, tumor cells acquire cancer stem cell (CSC)-like properties due to loss of PRRX1 expression, which results in distant metastasis and indicates a poor prognosis in breast cancer and hepatocellular carcinoma.[13] The function of PRRX1 in CCRCC, however, has not yet been elucidated.

Cancerous inhibitor of protein phosphatase 2A (CIP2A) is a recently identified endogenous protein phosphatase 2A (PP2A) inhibitor in human malignancies.[14] Overexpression of CIP2A has been detected in multiple malignancies, such as gastric cancer, breast cancer, prostate cancer, lung cancer, papillary thyroid carcinoma and head and neck squamous cell carcinoma,[15,16,17] and the clinical relevance of CIP2A overexpression suggests its use as a prognostic marker in cancer patients. Indeed, CIP2A plays an important role in the occurrence and development of various malignant tumors.

Overall, studies on VM, PRRX1 and CIP2A in relation to metastasis and prognosis have indicated that these biomarkers influence tumor development. Nonetheless, associations between VM, PRRX1 and CIP2A in CCRCC have not yet been extensively reported. The purpose of this study was to explore the hypothesis that these biomarkers are mutually associated with metastasis and prognosis in CCRCC.

2. Materials and methods

2.1. Specimens

A total of 110 CCRCC tissues and surrounding "normal" nephron tissues were collected from the Department of Pathology of the First Affiliated Hospital of Bengbu Medical College (China) from January 2010 to December 2012. All patients underwent radical resection and lymph node dissection (patients who underwent any preoperative anticancer therapy were excluded). The "normal" nephron tissues were obtained from the same patients and from surrounding nephron tissues at least 5 cm away from the cancer edge. Complete demographic, pathological, and follow-up data (at 6-month intervals by mobile phone and social applications) were available for all patients. The overall survival time (OST) was calculated from the date of surgery to the date of death or December 2018 (mean OS: 12 months, range: 96 months). Written consent was obtained from all patients. The study was approved by the Ethics Committee of Bengbu Medical College and performed in accordance with the guidelines of the Declaration of Helsinki. Tumor-node-metastasis (TNM) was evaluated in accordance with the 2010 edition of the American Joint Committee on Cancer (AJCC). Tumor differentiation was assessed in accordance with World Health Organization (WHO) standards. Patient characteristics are shown in Table 1.

Table 1

Patient characteristics.

Patients characteristics	Frequency (n)	Percentage (%)
Age (years)		
<60	61	55.5
≥60	49	44.5
Sex		
Male	57	51.8
Female	53	48.2
Smoking status		
No	72	65.5
Yes	38	34.5
Location		
Left	61	55.5
Right	49	44.5
Size (cm)		
<7.0	65	59.1
≥7.0	45	40.9
Vascular invasion		
No	88	80.0
Yes	22	20.0
Grade		
G1	38	34.5
G2	47	42.7
G3	19	17.3
G4	6	5.5
Lymph-node-metastasis		
No	91	82.7
Yes	19	17.3
TNM stage		
I	56	50.9
II	28	25.5
III	26	23.6

2.2. Immunohistochemistry

Immunohistochemical staining was carried out in accordance with the instructions of EliVision™ plus Detection Kit (Lab Vision, Fremont, CA). All CCRCC and corresponding "normal" nephron tissues were fixed in 10% buffered formalin and then embedded in paraffin, and continuous 4-μm-thick sections were cut. The samples were deparaffinized using routine methods and dehydrated using xylene and alcohol. Methanol containing 3% H_2O_2 solution was used to block endogenous peroxidase activity, and citrate buffer was used to retrieve antigens. All sections were then washed several times with phosphate-buffered saline (PBS) and blocked with goat serum at room temperature for 30 min. After washing with PBS, all sections were incubated with a mouse monoclonal antibody against human PRRX1 (OriGene, Rockville, MD) or CIP2A (Abcam, San Francisco, CA) at 37°C for 1 h. Periodic-acid-Schiff (PAS)-CD34 dual staining was used to identify endothelial cells in the glycosylated basement membranes of vessels, including vessel-like (VM) structures. Finally, all sections were counterstained with hematoxylin, dehydrated, air-dried, and mounted.

2.3. Evaluation of staining

All staining results were evaluated semiquantitatively by two experienced pathologists who were blinded to the patients' clinical information and follow-up data. To avoid potential intratumoral heterogeneity of antibody expression, we analyzed ten representative high-power-fields (HPF) from different areas of each CCRCC slide. The experimental results were scored according to the intensity (no staining, 0; weak staining, 1; moderate staining, and 2; strong staining, 3) and extent (<11% positive cells, 1; 11–50% positive cells, 2; 51–75% positive cells, 3; and >75% positive cells, 4) of staining.[8,27,28,29] Final scores were obtained by multiplying the intensity and extent scores, which ranged from 0–12. Final scores ≥3 were considered positive. A modified Yue and Chen method was used to assess VM in the CCRCC tissues and control tissues.[18] For tissue sections that were positive for all three factors (VM, PRRX1, and CIP2A), the average value of the final score of each tissue section was used.

2.4. Statistical analysis

All data were analyzed using SPSS 24.0 software (IBM, New York, NY). Relationships between clinicopathological indices and VM, PRRX1 and CIP2A were analyzed using Fisher's exact test or the chi-square test. Association between VM, PRRX1, and CIP2A was evaluated using Spearman's correlation test. The effects of VM, PRRX1 and CIP2A on survival were analyzed by univariate and multivariate analyses. Univariate OST analysis was carried out using the Kaplan–Meier method with the log-rank test, and multivariate OST analysis using the Cox regression model. $P < .05$ was considered indicative of statistically significant differences.

3. Results

3.1. Associations between VM, PRRX1, and CIP2A in the cancer tissues of patients and clinicopathological characteristics

To assess the effects of VM, PRRX1, and CIP2A in CCRCC, we immunohistochemically detected these factors in both CCRCC and corresponding normal renal tissue specimens. Clinicopathological characteristics were compared to these experimental data.

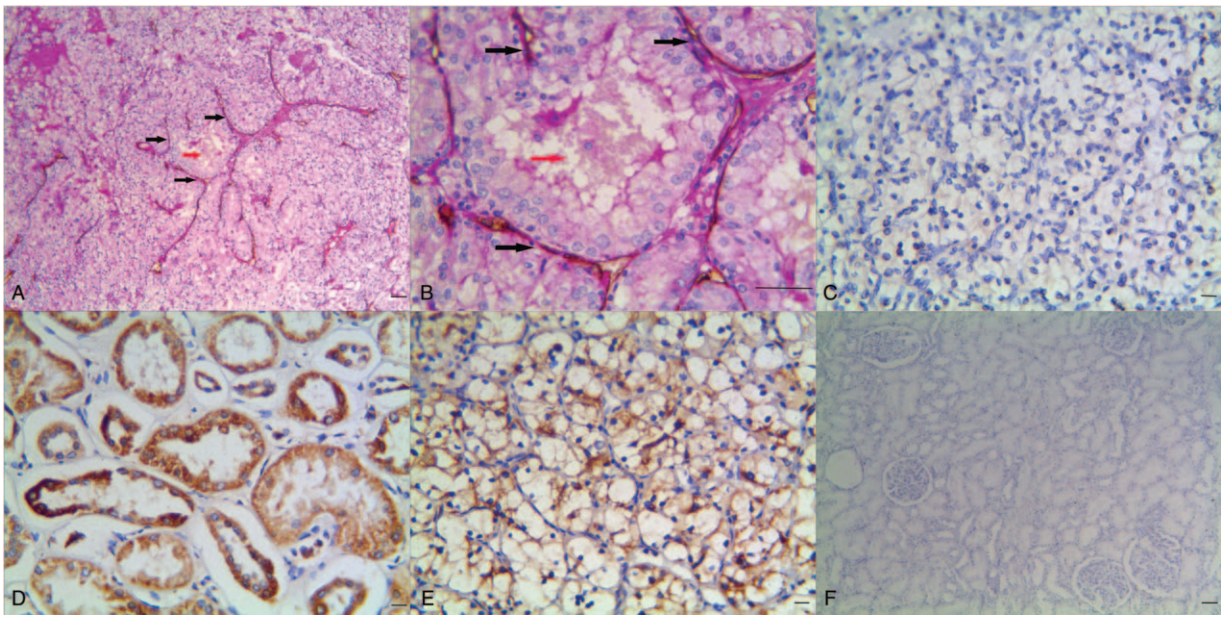

Figure 1. Immunostaining of VM, PRRX1 and CIP2A in CCRCC or the control tissue. (A) Positive staining of VM structure in the CCRCC tissue (100 magnification, bar = 100 μm, black arrow is microvessel, red arrow is VM structure). (B) Positive staining of VM in the CCRCC tissue (400 magnification, bar = 100 μm, black arrow is microvessel, red arrow is VM structure). (C) Negative staining of PRRX1 in the cytoplasm of cancer cells (100 magnification, bar = 100 μm). (D) Positive staining of PRRX1 in the adjacent renal tubular tissue (400 magnification, bar = 100 μm). (E) Positive staining of CIP2A in the cytoplasm of the cancer cells (100 magnification, bar = 100 μm). (F): Negative staining of CIP2A in the control tissue of the CCRCC (100 magnification, bar = 100 μm).

Table 2

Association between VM and expression of PRRX1, CIP2A and clinicopathological characteristics of clear cell renal cell carcinoma (CCRCC).

	VM			PRRX1			CIP2A		
Variable	**Negative**	**Positive**	***P***	**Negative**	**Positive**	***P***	**Negative**	**Positive**	***P***
Age (years)			.680			.080			.848
<60	36	25		35	26		31	30	
≥60	27	22		36	13		24	25	
Sex			.891						.849
Male	33	24		36	21		28	29	
Female	30	23		35	18		27	26	
Smoking status			.616			.825			.109
No	40	32		47	25		40	32	
Yes	23	15		24	14		15	23	
Location			.424			.582			.848
Left	37	24		38	23		31	30	
Right	26	23		33	16		24	25	
Size (cm)			<.001			<.001			<.001
<7.0	48	17		33	32		46	19	
≥7.0	15	30		38	7		9	36	
Vascular invasion			<.001			.002			<.001
No	62	26		50	38		53	35	
Yes	1	21		21	1		2	20	
Grade			<.001			<.001			<.001
G1	31	7		11	27		34	4	
G2	27	20		38	9		17	30	
G3	5	14		16	3		4	15	
G4	0	6		6	0		0	6	
Lymph-node-metastasis			<.001			.006			<.001
No	62	29		53	38		55	36	
Yes	1	18		18	1		0	19	
TNM stage			<.001			.002			<.001
I	46	10		24	32		45	11	
II	16	12		22	6		8	20	
III	1	25		25	1		2	24	

The rate of VM positivity (small vessel structure, which is a lumen-like structure in CCRCC; the lumen was PAS-positive but CD34-negative; the VM structural pattern included, e.g., linear, tubular, network aspects) was significantly higher in the CCRCC specimens (42.7%, 47/110) than in the corresponding normal tissues (0%, 0/110, $P<.001$; Fig. 1A and B). Moreover, the rate of VM structure development in CCRCC was positively related to tumor pathological grade, size, vascular invasion, lymph node metastasis (LNM) stage, and TNM stage but not to patient age, sex, smoking status or location (Table 2).

PRRX1 expression was lower in CCRCC tissues (64.5%, 71/110) than in control tissues (10.2%, 11/110; $P<.001$; Fig. 1C and D). Lack of PRRX1 expression was significantly associated with tumor grade, size, vascular invasion, LNM stage, and TNM stage. No correlation was found between PRRX1 expression and patient sex, age, smoking status or location (Table 2).

Similar to VM and PRRX1, CIP2A expression was significantly higher in CCRCC tissues (55.0%, 55/110) than in control kidney tissues (5.5%, 6/110; $P<.001$; Fig. 1E and F). CIP2A expression in CCRCC was related to tumor grade, size, vascular invasion, LNM stage and TNM stage but not to patient sex, age, smoking status or location (Table 2).

3.2. Univariate and multivariate analyses

Follow-up data suggested that OST was significantly shorter in CCRCC patients with VM+ specimens (48.62 ± 16.362 months) than in those with VM- specimens (71.25 ± 13.183 months; log-rank = 47.583, $P<.001$; Fig. 2A). Similarly, the OST of PRRX1- patients (54.06 ± 16.941 months) was significantly shorter than that of PRRX1+ patients (75.28 ± 11.978 months; log-rank = 31.322, $P<.001$; Fig. 2B). The OST of CIP2A +patients (50.02 ± 15.849 months) was significantly shorter than that of CIP2A- patients (73.15 ± 12.626 months; log-rank = 55.886, $P<.001$; Fig. 2C). In univariate analysis, the OST time was significantly related to clinicopathological information, including tumor size (log-rank = 37.409, $P<.001$, Fig. 2D), grade (log-rank = 86.590, $P<.001$, Fig. 2E), vascular invasion (log-rank = 86.598, $P<.001$, Fig. 2F), LNM stage (log-rank = 84.67, $P<.001$, Fig. 2G) and TNM stage (log-rank = 126.649, $P<.001$, Fig. 2H) (Table 3).

Moreover, multivariate analysis demonstrated that VM+, CIP2A+, PRRX1- specimens, tumor grade, TNM stage, and LNM were independent prognostic factors for CCRCC (Table 4).

3.3. Correlation among VM and expression of CIP2A and PRRX1 in CCRCC

Spearman correlation coefficient analysis indicated negative correlations between PRRX1+ expression and VM ($r=-0.333$, $P<.001$) and CIP2A ($r=-0.513$, $P<.001$), though expression of CIP2A was positively associated with VM development ($r=0.349$, $P<.001$) (Table 5).

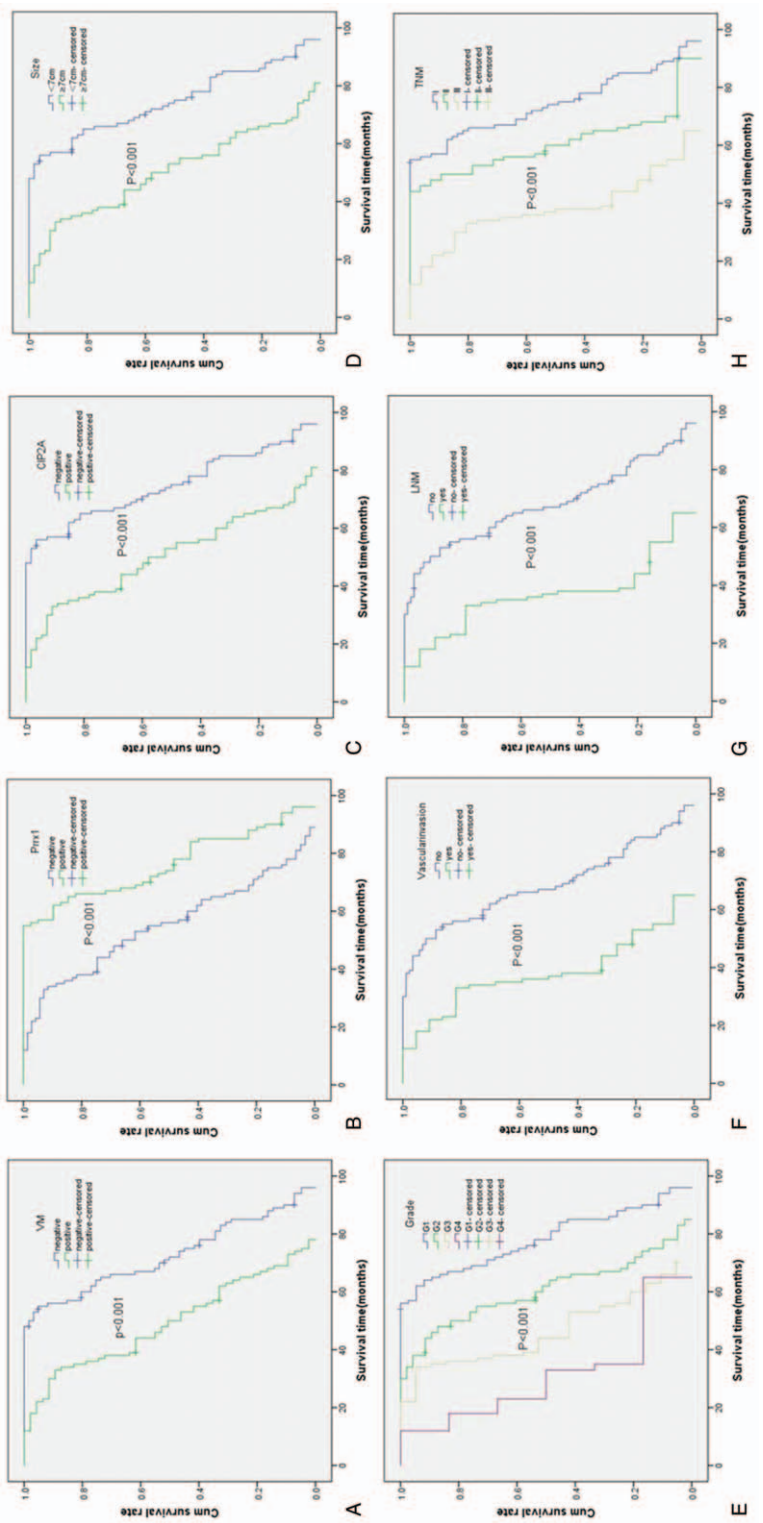

Figure 2. Kaplan–Meier analysis of the survival rate of patients with CCRCC. The y-axis represents the percentage of patients; the x-axis, their survival in months. (A) Overall survival of all patients in relation to VM (log-rank = 47.583, $P < .001$). (B) Overall survival of all patients in relation to PRRX1 expression (log-rank = 31.322, $P < .001$). (C) Overall survival of all patients in relation to CIP2A expression (log-rank = 55.886, $P < .001$); In (A–C) analyses, the green line represents patients with positive VM, or PRRX1, or CIP2A, and the blue line representing the negative VM, or PRRX1, or CIP2A. (D) Overall survival of all patients in relation to tumor size (log-rank = 37.409, $P < .001$,the blue line represents patients with tumor size less than 7.0 cm, the green line represents patients with tumor size more than 7.0 cm). (E) Overall survival of all patients in relation to tumor grade (log-rank = 86.590, $P < .001$, the blue line represents patients with grade 1 group, the green line represents patients with grade 2 group, the brown line represents patients with grade 3 group, the purple line represents patients with grade 4 group). (F) Overall survival of all patients in relation to vascular invasion (log-rank = 86.598, $P < .001$, the blue line represents patients with no group, the green line represents patients with yes group. (G) Overall survival of all patients in relation to LNM (log-rank = 84.670, $P < .001$, the blue line represents patients with no group, the green line represents patients with yes group. (H) Overall survival of all patients in relation to TNM stage (log-rank = 126.649, $P < .001$, the blue line represents patient with I stage group, the green line represents patients with II stage group, the brown line represents patients with III stage group).

Table 3

Results of univariate analyses of overall survival (OS) time.

Variables	n	Mean OS (mo)	Log-rank	*P* value
VM			47.583	<.001
Negative	63	71.25±13.182		
Positive	47	48.62±16.362		
PRRX1			31.322	<.001
Negative	71	54.06±16.941		
Positive	39	75.28±11.978		
CIP2A			55.886	<.001
Negative	55	73.15±12.626		
Positive	55	50.02±15.849		
Age (years)			1.783	.182
<60	61	61.72±20.059		
≥60	49	61.41±16.235		
Sex			0.238	.626
Male	57	60.61±17.814		
Female	53	62.62±19.115		
Location			1.032	.310
Left	61	62.82±19.234		
Right	49	60.04±17.366		
Smoking status			0.234	.629
No	72	61.31±18.359		
Yes	38	62.11±18.698		
Size (cm)			37.409	<.001
<7.0	65	70.20±14.396		
≥7.0	45	49.13±16.388		
Vascular invasion			86.598	<.001
No	88	67.65±14.216		
Yes	22	37.32±12.171		
Grade			86.590	<.001
G1	38	77.24±11.535		
G2	47	59.02±12.729		
G3	19	46.26±13.008		
G4	6	31.00±18.815		
LNM			84.670	<.001
No	91	66.88±14.676		
Yes	19	36.21±12.282		
TNM stage			126.649	<.001
I	56	73.89±11.574		
II	28	59.43±9.551		
III	26	37.38±11.335		

4. Discussion

CCRCC represents the most aggressive subtype of kidney cancer and poses a serious threat to human health.[19] However, the clinical prognosis of CCRCC is influenced by progression, covering a complex network of gene interactions. As biomarkers for early detection and follow-up of the disease are not currently available, it is necessary to investigate the pathogenic mechanism of the molecular field of renal malignancy and the comprehensive utilization of biological markers for clinical diagnosis and treatment.

Table 4

Results of multivariate analyses of overall survival (OS) time.

Variables	B	SE	*P*	RR	95% CI
TNM-stage	1.124	0.408	0.006	3.078	1.383–6.849
VM	0.919	0.268	0.001	2.506	1.431–4.390
PRRX1	-0.715	0.292	0.014	0.489	0.276–0.867
CIP2A	0.852	0.301	0.005	2.344	1.300–4.225

A large number of experimental studies have confirmed that activation of EMT can promote the invasion and migration of tumor cells. In addition, many molecules have been confirmed to be involved in EMT and to participate in this process through a certain signal transduction pathway.[20,21] EMT can promote the formation of VM through different signaling pathways, with related transcription factors including Twist1, ZEB1, Snail, and Slug.[22–25] EMT can also induce the formation of VM in tumor cells under hypoxia, which has a synergistic effect and is closely related to the biological behavior of tumor invasiveness and increased death rates for tumor patients.[26] In our research, we found that VM was positively correlated with CCRCC grade, tumor size, LNM stage, and TNM stage. Moreover, Kaplan-Meier survival analysis indicated that VM+ CCRCC patients had a significantly shorter OST than did VM- patients. Compared with malignant tumor patients without VM, patients with VM had a worse prognosis. Our study is consistent with previous studies.[4,8,27,28]

Expression of the transcription factor PRRX1 as a transcription factor is related to embryonic limb development, vascular differentiation and skeletal muscle development,[30,31] and it has been suggested that transforming growth factor-b and microRNA regulate expression of PRRX1.[32–34] However, details are lacking and further analysis is required to uncover the signaling network regulating PRRX1. In this study, we observed that the level of PRRX1 expression was lower in CCRCC tissues than in adjacent tissues. Furthermore, low expression was associated with tumor progression and poor prognosis in CCRCC, as based on an analysis of clinicopathological variables, and the OST of PRRX1- patients was significantly shorter than that of PRRX1+ patients. Our results are consistent with those from previous studies.[35–37] According to our results, we speculate that PRRX1 likely acts as a bidirectional regulatory factor in diverse cancer types and that PRRX1 may act as a tumor suppressor in CCRCC.

CIP2A inhibits the activity of PP2A and thus maintains the malignant phenotype of tumor cells and plays an important role in the occurrence, development and biological behavior of tumor cells. CIP2A (cancerous Inhibitor of PP2A) is an important oncogene. PP2A is a potent tumor inhibitor that acts on a variety of carcinogenic transcription factors, including MYC, beta-catenin, AKT and BCL2 dephosphorylation and degradation,[38,39] and CIP2A has been shown to decrease cancer cell viability and anchorage-independent growth and to induce apoptosis.[40] In our study, we found that CIP2A was significantly overexpressed in the majority of CCRCC tissues compared with control tissues. CIP2A expression was positively related to tumor grade, tumor size, LNM stage, and TNM stage. Moreover, Kaplan-Meier survival curve analysis demonstrated that CIP2A+ CCRCC patients had a significantly shorter OST than did CIP2A- patients. Our results are similar to those from other studies.[41–43]

In conclusion, our research data show that VM, PRRX1 and CIP2A can be used as a biological reference index for the clinical prognosis of renal clear cell carcinoma. It is acknowledged that adhesion molecules and other factors that modulate the EMT process can regulate VM formation. PRRX1 and CIP2A are major EMT inducers that play a predominate role in tumors progress via several signaling pathways and may become therapeutic targets for intervention in CCRCC. Nonetheless, further study is required to clarify the mechanism involved. Our present research results provide some reference data for the diagnosis and prognosis of renal cell carcinoma. Due to the limited number of clinical samples and the uncertainty of follow-

Table 5

Correlation among VM, expression of PRRX1 and CIP2A in CCRCC.

Variables	PRRX1				VM				CIP2A			
	Negative	Positive	*r*	*P*	Negative	Positive	*r*	*P*	Negative	Positive	*r*	*P*
VM			−0.333	<.001								
Negative	32	31										
Positive	29	8										
PRRX1											−0.513	<.001
Negative									22	49		
Positive									33	6		
CIP2A							0.349	<.001				
Negative					41	14						
Positive					22	33						

up results as well as the relatively simple experimental content, a large number of cytological and animal experiments are required to verify the use of PRRX1, CIP2A, and VM as effective markers in renal cell carcinoma.

5. Conclusions

We examined the roles of VM, PRRX1 and CIP2A in CCRCC. Low PRRX1 expression combined with high VM and CIP2A was associated with metastasis and a poor prognosis in CCRCC. Furthermore, VM, PRRX1 and CIP2A might serve as valuable biomarkers in CCRCC, and the comprehensive detection of VM, PRRX1 and CIP2A may be valuable for indicating prognosis in CCRCC. It has certain clinical significance for the treatment of CCRCC.

Author contributions

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Writing – review & editing: Shiwu Wu.

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