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Original Article

Prognostic significance of ubiquinol-cytochrome c reductase hinge protein expression in patients with clear cell renal cell carcinoma

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Abstract: Ubiquinol-cytochrome c reductase hinge protein (UQCRH), as a connecter between cytochrome c_1 with cytochrome c in complex III of respiratory chain, is top-ranked hypermethylated gene in clear cell renal cell carcinoma (ccRCC). This study aims to evaluate the impact of UQCRH on recurrence and survival of 424 ccRCC patients enrolled retrospectively from a single institution after surgical resection using immunohistochemistry method. UQCRH was specifically downregulated in ccRCC, compared with papillary and chromophobe RCC. Moreover, patients with low UQCRH were prone to possess high T stage and TNM stage and associated with poor survival and early recurrence. UQCRH remained an independent favorable prognosticator for OS (Hazard rate [HR]: 0.510, 95% CI: 0.328-0.795, p=0.003) and RFS (HR: 0.506, 95% CI: 0.334-0.767, p=0.001) adjusting with other well-established factors using backward Cox model. Furthermore, in stratified subgroups, patients with low UQCRH had an increased risk of recurrence (HR: 0.452, 95% CI: 0.261-0.783, p=0.005) and mortality (HR: 0.386, 95% CI: 0.205-0.726, p=0.003) in subgroup of early TNM stage. Taken together, UQCRH is a potential independent favorable prognostic factor for recurrence and survival of patients with ccRCC after nephrectomy.

Keywords: Clear cell renal cell carcinoma, ubiquinol-cytochrome c reductase hinge protein, prognostic biomarker, overall survival, recurrence-free survival

Introduction

With the widespread use of abdominal imaging, incidental diagnoses of renal cell carcinoma (RCC) became frequent findings; however, during the same period, the mortality rates of RCC were climbing steadily [1]. Moreover, although ~60% patients with RCC were diagnosed at early stage with a low risk of cancer-specific death, ~30% of them would recur after surgery with poor 5-year survival [2]. Taken together, the enigmatic nature history of RCC underscores the requirement to continue efforts to broaden our understanding of the factors that predict disease progression, specially the growing number of patients diagnosed with low-risk RCC.

Clear-cell renal cell carcinoma (ccRCC) accounts major type of RCC, characterized by inactivation of the *VHL* gene and resistance to both chemotherapy and conventional radiation therapy [3]. Among the genetic changes underlying ccRCC, ubiquinol-cytochrome c reductase hinge protein (*UQCRH*) has been found hypermethylation in 36% of the tumors, top-ranked gene by inverse correlation between gene and DNA methylation, suggesting its role in ccRCC might be a key tumor suppressor gene [4]. UQCRH is highly conserved from yeast to human, represents mitochondrial ubiquinol cytochrome c oxidoreductase complex, complex III of the respiratory chain, and works as a connecter with cytochrome c_1 and cytochrome *c* [5]. The research based on yeast implied that it might exert a regulatory of complex III and of association with cytochrome *c* [6, 7]. Considered UQCRH function and subcellular location, more experiments were focused to demonstrate its

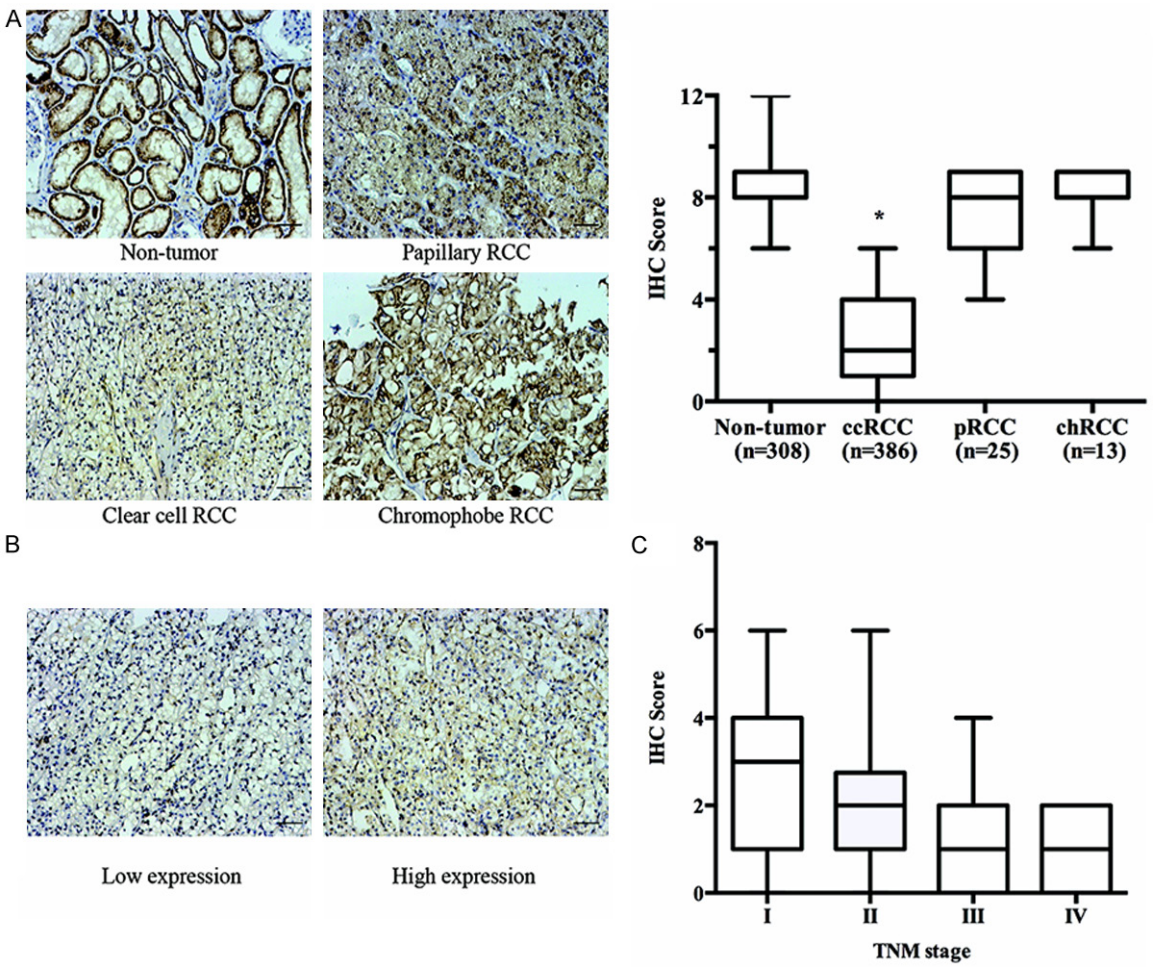

Figure 1. UQCRH immunohistochemical expression in renal cell carcinoma (RCC) specimens. A. Representative UQCRH immunohistochemical (IHC) images of non-tumor, clear cell RCC, papillary RCC and chromophobe RCC specimens and the quantitative result of the IHC scores. B. Representative IHC images of differential levels of UQCRH intensity in ccRCC specimens. C. The IHC scores of UQCRH in differential TNM stages of ccRCC specimens. Scale bar: 50 μm.

biology functions. Previously, *UQCRH* has been suggested to be a tumor suppressor in some cancer cell lines [8]; in contrast, it is overexpressed in some cancers [9]. However, these studies were not linked to RCC. Hence, the value of UQCRH is worth further exploration, especially its potential prognostic value in ccRCC progress.

Here, we retrospectively analyzed UQCRH expression of the specimens coming from a single institution using immunohistochemical analysis and correlation with clinicopathologic characteristics and clinic outcomes. Further step was taken to evaluate prognostic values of UQCRH expression in Cox regression models.

Patients and methods

Clinical specimens

A total of 424 patients who underwent radical or partial nephrectomy for RCC at Zhongshan Hospital, Fudan University, Shanghai, China, were enrolled in this study, constituted by 386 patients with ccRCC, 25 patients with papillary RCC (pRCC) and 13 patients with chromophobe RCC (chRCC). To guarantee the consecution of patients, ccRCC patients were separated into two groups. Specimens of group A (n=191) were obtained from January 2003 to December 2004, and specimens of group B (n=195) were obtained from January to December 2008. The database of patients includes baseline clinico-

Table 1. Correlation between UQCRH expression and patients characteristics

Characteristic	Group A (n=191)				Group B (n=195)			
	Patients	UQCRH expression			Patients	UQCRH expression		
	Total (%)	Low (n=71)	High (n=120)	*P*	Total (%)	Low (n=68)	High (n=127)	*P*
Age (years)†				0.835				0.416
Mean	54.78	54.56	54.92		54.85	55.75	54.37	
Median	54	53	54.5		55	56	55	
IQR	47-62	47-64.5	47.5-62		48-61	48-63	47-61	
Gender				0.186				0.261
Male	133 (70)	54	79		144 (74)	54	90	
Female	58 (30)	17	41		51 (26)	14	37	
Tumor size (cm)†,‡				0.465				0.821
Mean	4.63	4.81	4.52		4.45	4.63	4.36	
Median	4	4	4		4	4	4	
IQR	3-6	3-6	2.5-6		3-5.5	2.5-5.9	3-5.5	
pT stage				0.007*				0.007*
1	116 (61)	33	83		138 (71)	45	93	
2	18 (9)	8	10		19 (10)	2	17	
3	57 (30)	30	27		36 (18)	20	16	
4	0 (0)	0	0		2 (1)	1	1	
Lymph node status§				1.000				0.345
Negative	189 (99)	70	119		190 (97)	65	125	
Positive	2 (1)	1	1		5 (3)	3	2	
Distant metastasis§				0.146				1.000
Negative	187 (98)	68	119		194 (99)	68	126	
Positive	4 (2)	3	1		1 (1)	0	1	
TNM stage				0.012*				0.013*
I	116 (61)	33	83		137 (70)	45	92	
II	18 (9)	8	10		18 (9)	2	16	
III	53 (28)	27	26		37 (19)	20	17	
IV	4 (2)	3	1		3 (2)	1	2	
Fuhrman grade				0.579				0.483
1	31 (16)	10	21		47 (24)	19	28	
2	84 (44)	35	49		87 (45)	30	57	
3	54 (28)	20	34		39 (20)	10	29	
4	22 (12)	6	16		22 (11)	9	13	
Necrosis				0.684				0.612
Absent	147 (77)	53	94		160 (82)	54	106	
Present	44 (23)	18	26		35 (18)	14	21	
ECOG-PS				0.422				0.586
0	160 (84)	57	103		163 (84)	55	108	
≥1	31 (16)	14	17		32 (16)	13	19	

IQR: Interquartile range; ECOG-PS, Eastern Cooperative Oncology Group performance status. †, The results are modeled as continuous variables. *, $P < 0.05$ is considered statistically significant. §, The results are calculated by Fisher's exact test. ‡, The results are calculated by Mann-Whitey test.

pathologic characteristics and follow-up outcomes. TNM stage was resigned according to the American Joint Committee on Cancer 2010 TNM classification. The primary endpoint was overall survival (OS) with recurrence-free survival (RFS) as a secondary endpoint. The patients with tumor metastasis at the time of surgery were excluded from analysis of tumor recurrence. OS and RFS were calculated from the day of surgery to the day of death and recurrence, respectively, or to the data of the last follow-up. The patients were excluded if larger area of necrotic and hemorrhagic influencing the obtainment of representative area in samples or receiving preoperative neoadjuvant therapy. Ethical approval was granted by the

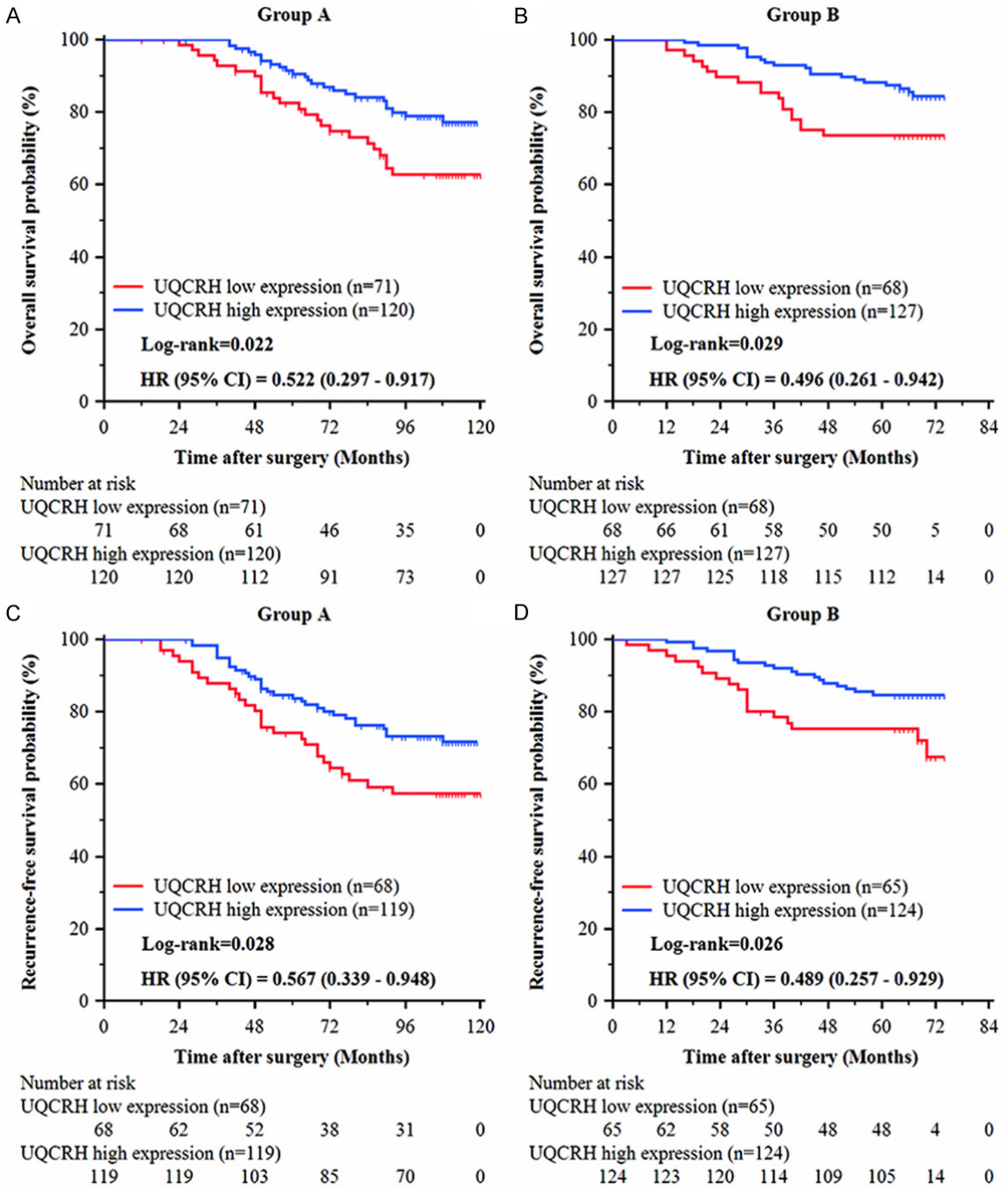

Figure 2. Estimated OS and RFS following surgery dichotomized by UQCRH expression for ccRCC patients in group A and B. Kaplan-Meier analysis of OS dichotomized by UQCRH expression in group A (A) and in group B (B). Kaplan-Meier analysis of RFS dichotomized by UQCRH expression in group A (C) and group B (D).

research medical ethics committee of Fudan University.

Tissue microarray and immunohistochemistry

Tissue microarrays were constructed as previously described [10]. Primary anti-UQCRH antibody (1:200; Sigma-Aldrich Corp, MO) was performed for immunohistochemistry staining. The primary antibody was omitted for the negative controls. The staining of each specimen was assessed by one experienced pathologist blinded to the clinical data using semi-quantitative immunoreactivity score (IRS) system, deriving from the multiplication of intensity of immunohistochemical staining (0, no staining; 1, weak;

2, moderate; and 3, strong) and percentage of tumor cells (0, 0~25%; 1, 25~50%, 3, 50~75% and 4, 75~100%) ranges from 0 to 12. The average score over the two cores from each case was used for further analysis. Less than medium value was considered as low expression.

Statistical analysis

Both groups are analyzed equivalently. The *t* tests were used to compare immunohistochemical scores among patients with different types of RCC. Clinicopathologic data were compared between patients with UQCRH-low and UQCRH-high tumors using *t* tests, Mann-Whitey test, Chi-square tests and Fisher's exact tests as appropriate. Age and tumor size were modeled as continuous variables. In addition, OS and RFS was estimated by Kaplan-Meier method and analyzed by log-rank test. Subsequently, UQCRH expression was further evaluated in multivariable models adjusting for well-known prognostic variables via backward method. Statistical analysis was preformed with MedCalc software (version 12.7.0.0; MedCalc, Mariakerke, Belgium). All tests were two sided and *P* values < 0.05 were considered statistically significant.

Results

UQCRH immunohistochemical expression

UQCRH expression was evaluated by immunohistochemical staining analysis in 424 RCC specimens and 308 unpaired non-tumor specimens, respectively. As shown in **Figure 1**, expression of UQCRH was significantly lower in ccRCC than non-tumor and other two types of RCC (papillary and chromophobe type), respectively (**Figure 1A**). UQCRH showed variable intensity in ccRCC tissues (**Figure 1B**). In addition, with the disease progression, the expression of UQCRH was steadily declining (**Figure 1C**). Taken together, the downregulation of UQCRH might be specific event in the progress of ccRCC.

Association of UQCRH with clinical and pathologic characteristics

According to the IRS criterion, 71 (37.2%) and 68 (34.9%) were grouped as UQCRH low-expression in group A and group B, respectively. The clinical characteristics dichotomized by UQCRH were listed in **Table 1**. The specimens with high pT stage tended to expression low-expression of UQCRH (p=0.007 in group A and p=0.007 in group B). Accordingly, the negative correlation between UQCRH and TNM stage was found (p=0.012 in group A and p=0.013 in group B). There was no difference in age, gender, tumor size, pN stage, pM stage, and Eastern Cooperative Oncology Group performance status (ECOG-PS) between the patients dichotomized by UQCRH (**Table 1**).

Prognostic value of UQCRH for clinical outcomes of ccRCC patients

At last follow-up, a mean duration of OS was 90.6 months (median=106 months; range 12-120 months) and RFS was 85.9 months (median=103 months; range 12-120 months) in group A, and a mean duration of OS was 62.2 months (median=67 months; range 12-74 months) and RFS was 61.0 months (median=67 months; range 3-74 months) in group B. Patients with UQCRH low-expression were more likely to experience death (log-rank p=0.022; Hazard ratio [HR]=0.522; 95% confidence interval [CI]=0.297-0.917; p=0.024 in group A and log-rank p=0.029; HR=0.496; 95% CI=0.261-0.942; p=0.033 in group B) (**Figure 2A** and **2B**). In addition, low-expression UQCRH was an adverse factor for RFS (log-rank p=0.028; HR=0.567; 95% CI=0.339-0.948; p=0.031 in group A and log-rank p=0.026; HR=0.489; 95% CI=0.257-0.929; p=0.030 in group B) (**Figure 2C** and **2D**).

Multivariate analysis of UQCRH with OS and RFS

To include more clinicopathologic factors into multivariate model, two groups were combined for further evaluation. To assess the robustness value of UQCRH, multivariate cox regression analysis using backward method was performed to derive risk assessment correlated to OS and RFS with the clinicopathologic characteristics. Along with well-established prognosticators, UQCRH remained an independent prognostic factor for OS (HR=0.510; 95% CI=0.328-0.795; p=0.003) and RFS (HR=0.506; 95% CI=0.334-0.767; p=0.001) (**Figure 3**).

Impact of UQCRH on OS and RFS stratified by TNM stage

As UQCRH was significantly related with TNM stage, further step was taken to investigate the

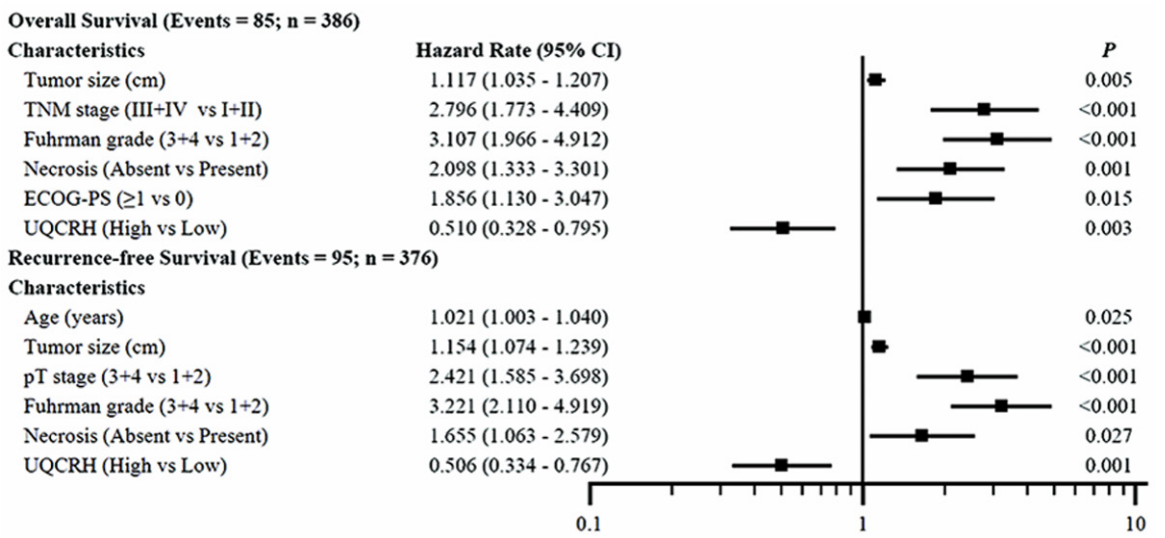

Figure 3. Multivariable Cox regression models associated of UQCRH expression with ccRCC patients OS and RFS. Multivariable Cox model using backward method associated UQCRH expression and other well-established variables with ccRCC patients' OS and RFS.

impact of UQCRH on OS and RFS of patients stratified by TNM stage. In subgroup of low TNM stage, the patients with UQCRH low-expression had a worse survival than these with high-expression (log-rank p=0.002; HR=0.386; 95% CI=0.205-0.726; p=0.003) (**Figure 4A** and **4E**). Meanwhile, in subgroup of low pT stage, the patients with UQCRH low-expression suffered more recurrence than these with high-expression (log-rank p=0.004; HR=0.405; 95% CI= 0.214-0.766; p=0.006) (**Figure 4C** and **4E**). However, UQCRH did not significantly affect OS in advanced TNM stage and RFS in advanced pT stage (**Figure 4B** and **4D**). After adjusting for age, UQCRH retain a favorable factor in OS (HR=0.452; 95% CI=0.261-0.783; p=0.005) in subgroup of low TNM stage and RFS (HR=0.472; 95% CI=0.271-0.820; p=0.008) in subgroup of low pT stage (**Figure 4E**).

Discussion

Inherited predisposition to RCC has been suggested to arise from genes relevant to administrating cellular metabolism and mitochondrial oxidative phosphorylation, making RCC a model for the role of an oncologic-metabolic shift, leading to malignancy [11]. Moreover, the metabolic characterization could evidence a cancer-specific bioenergetics signature depending on the history of tumor, which composed by the activated oncogenes and microenvironments [12]. Although many metabolic genes have been shown dysregulated in ccRCC, which are correlated with patients' survival, the prognostic function of UQCRH, as a top ranked gene by hypermethylation, is unknown [4]. Here, this is the first study to identify the potential prognostic value of UQCRH in ccRCC. Our work suggested that significant downregulation of UQCRH could be a specific event in ccRCC, as dysregulation of UQCRH expression was not seen in other two common types of RCC (pRCC and chRCC) (**Figure 1A**). Admittedly, the number of patients with pRCC and chRCC were petty small in our study; more patients with different RCC types are pursued to prove whether downregulation of UQCRH is a specific event in ccRCC, not in other types of RCC.

Pervious study reported that low mitochondrial respiratory chain contents were related with tumor aggressiveness in renal cell carcinoma [13]. In our study, the relationship between UQCRH expression and metastasis was failed to been observed (**Table 1**), considered the population of patients with metastasis enrolled in our study was small. However, our study suggested that UQCRH expression is steadily declining in the progress of ccRCC (**Figure 1B**) and it is an independently favorable factor for these patients' death and recurrence (**Figure 3**). Further study with larger population of

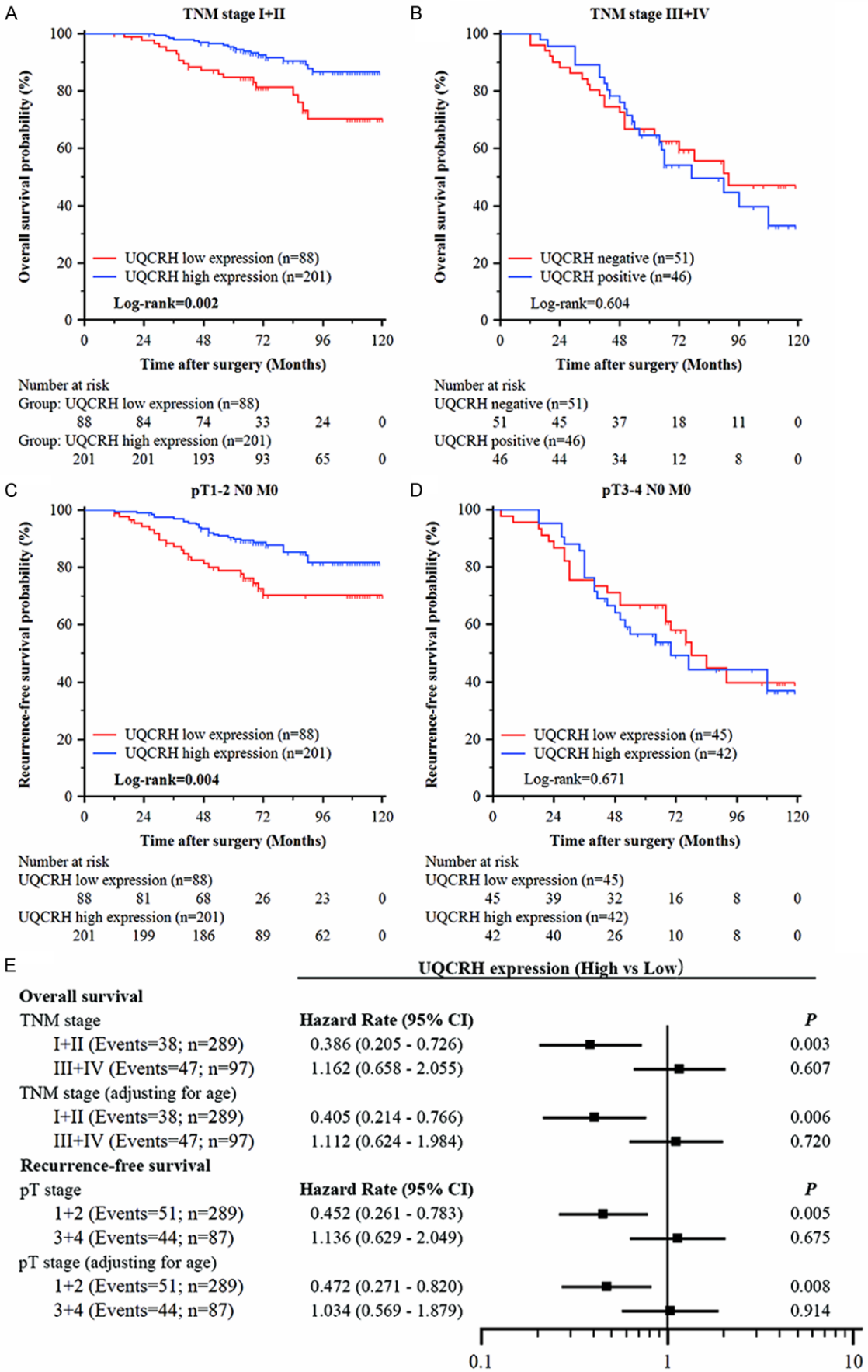
A
TNM stage I+II
Overall survival probability (%)
UQCRH low expression (n=88)
UQCRH high expression (n=201)
Log-rank=0.002
Time after surgery (Months)
Number at risk
Group: UQCRH low expression (n=88)
88 84 74 33 24 0
Group: UQCRH high expression (n=201)
201 201 193 93 65 0
B
TNM stage III+IV
Overall survival probability (%)
UQCRH negative (n=51)
UQCRH positive (n=46)
Log-rank=0.604
Time after surgery (Months)
Number at risk
UQCRH negative (n=51)
51 45 37 18 11 0
UQCRH positive (n=46)
46 44 34 12 8 0
C
pT1-2 N0 M0
Recurrence-free survival probability (%)
UQCRH low expression (n=88)
UQCRH high expression (n=201)
Log-rank=0.004
Time after surgery (Months)
Number at risk
UQCRH low expression (n=88)
88 81 68 26 23 0
UQCRH high expression (n=201)
201 199 186 89 62 0
D
pT3-4 N0 M0
Recurrence-free survival probability (%)
UQCRH low expression (n=45)
UQCRH high expression (n=42)
Log-rank=0.671
Time after surgery (Months)
Number at risk
UQCRH low expression (n=45)
45 39 32 16 8 0
UQCRH high expression (n=42)
42 40 26 10 8 0
E
UQCRH expression (High vs Low)
Overall survival
TNM stage
Hazard Rate (95% CI)
P
I+II (Events=38; n=289) 0.386 (0.205 - 0.726) 0.003
III+IV (Events=47; n=97) 1.162 (0.658 - 2.055) 0.607
TNM stage (adjusting for age)
I+II (Events=38; n=289) 0.405 (0.214 - 0.766) 0.006
III+IV (Events=47; n=97) 1.112 (0.624 - 1.984) 0.720
Recurrence-free survival
pT stage
Hazard Rate (95% CI)
P
1+2 (Events=51; n=289) 0.452 (0.261 - 0.783) 0.005
3+4 (Events=44; n=87) 1.136 (0.629 - 2.049) 0.675
pT stage (adjusting for age)
1+2 (Events=51; n=289) 0.472 (0.271 - 0.820) 0.008
3+4 (Events=44; n=87) 1.034 (0.569 - 1.879) 0.914
0.1
1
10

Figure 4. Association of UQCRH expression with OS and RFS in patients dichotomized by TNM stages. Association of UQCRH expression with OS at subgroup of low TNM stage (A) and advanced TNM stage (B). Association of UQCRH expression with RFS at subgroup of low pT stages (C) and advanced pT stages (D). (E) Cox analysis of UQCRH expression with OS and RFS alone and after adjusting age in differential subgroup of TNM stage, respectively.

patients with metastasis ccRCC is warranted to exploit the relationship between UQCRH and metastasis.

In ccRCC, the inactivation of VHL leads to deregulate the control of hypoxia-inducible factor (HIF), then contributing to create a pseudohypoxia environment; to response this situation, the cells would enhance expression of glycolytic enzymes and concurrently downregulate mitochondrial respiration via HIF signaling pathway [14]. Further studies suggested that HIF could reduce oxidative phosphorylation (OXPHOS) via inhibiting mitochondrial biogenesis and respiratory chain activity [15, 16]. Meanwhile, the reduced respiration rate happening in hypoxia favors the release of reactive oxygen species (ROS) by mitochondrial complex III, which contribute to HIF stabilization and induction of Bcl-2 [17]. Moreover, unlike complex I and II, complex III could release ROS into the mitochondrial intermembrane space and, subsequently, into the cytosol [18]. These ROS might serve as signaling molecules to communicate with other cellular compartments to influence the tumor microenvironment [19, 20]. Admittedly, the detailed regulation mechanism of UQCRH and its influence for complex III function in ccRCC progress is unknown. Considered its role in regulating complex III in yeast, the further work is pursued to exploit the potential biological function of UQCRH in ccRCC and other human cancer.

There are several limitations of our study that warrant further discussion. Chief among these is the population of our specimens is small and not continuous. As failed to access the specimens between 2005-2007, we had to divide specimens into two groups to ensure all samples were continuous firstly. After the results observed in two groups were coincident, the groups were combined for further multivariate and stratified analyses. Additional limitations include the retrospective nature, specimens comes from the same institution, and the focus only on one variable in the OXPHOS contents. Hence, the conclusion should be validated by larger-population, multi-institutions and prospective. And the further studies about prognostic values of the other contents of OXPHOS on predicting clinic outcomes or treatment response of ccRCC patients would be under consideration.

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Disclosure of conflict of interest

None

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