

## Original Article

# Histone deacetylase 10 suppresses proliferation and invasion by inhibiting the phosphorylation of $\beta$ -catenin and serves as an independent prognostic factor for human clear cell renal cell carcinoma

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Received December 16, 2014; Accepted December 31, 2014; Epub March 15, 2015; Published March 30, 2015

**Abstract:** Objective: Histone deacetylase (HDAC) is a tumor suppressor gene in various carcinomas; however, the effect of HDAC10 on human renal cell carcinoma (RCC) remains unknown. In the current study we analyzed the expression and function of HDAC10 in human clear cell RCC. Methods: RCC tissues from 145 patients who underwent radical nephrectomies were evaluated. HDAC10 protein and mRNA expression was examined by immunohistochemistry and quantitative RT-PCR, respectively. HDAC10 expression was increased by stable transfection with a vector containing full-length cDNA of HDAC10, and HDAC10 expression was decreased by siRNA in two RCC cell lines. Proliferation analysis of RCC cells *in vitro* was investigated using the WST-1 assay, and the invasion assay was performed using a 24-well Transwell chamber. The phosphorylation of  $\beta$ -catenin induced by HDAC10 was evaluated by Western blot. Results: HDAC10 expression in RCC tissues was significantly down-regulated compared to normal kidney tissues. Moreover, the low level of HDAC10 expression was uniformly associated with advanced clinical stage, larger tumor diameter, higher pathologic grade, and metastatic RCC. In addition, decreased expression of HDAC10 significantly prompted the proliferation and invasion of RCC cells *in vitro*. Although HDAC10 did not regulate the expression of  $\beta$ -catenin, HDAC10 suppressed the phosphorylation of  $\beta$ -catenin in RCC cells. Conclusions: HDAC10 expression is suppressed in human clear cell RCC and is involved in development and metastasis of RCC. The findings herein suggest that HDAC10 is an independent predictive factor for RCC prognosis, and restoring HDAC10 expression may be a new therapeutic strategy for advanced RCC.

**Keywords:** Renal cell carcinoma, HDAC10, proliferation, invasion, prognosis

## Introduction

Human renal cell carcinoma (RCC) is the most lethal genitourinary malignancy involving the adult kidney. With an incidence of 5-10 per 100,000, RCC accounts for 2%-3% of all tumors [1]. Clear cell RCC is the most common subtype of human RCC and is responsible for 75% of all cases [2]. Although localized RCC is regarded as a surgical disease, 40% of patients who present with localized RCC will develop advanced RCC [3]. Because RCC is highly resistant to chemotherapy and radiotherapy, no treatment has been shown to be effective for advanced RCC [4]. Recently, a number of genes have been investigated as predictors of survival and as therapeutic targets for human RCC [5, 6]. Nevertheless, the detailed molecular mech-

anism underlying the metastasis and development of RCC remains unknown.

Histone deacetylase (HDAC) can remove the acetyl group from N-acetyllysine on histones [7], and plays an important role in modifying transcription of the chromatin structural genes [8, 9]. The HDAC family consists of 18 proteins, which are divided into classes I-IV based on homology and structure. Eleven of the HDAC family members belong to classes I, II, and IV, and are referred to as classic HDACs; 7 HDAC family members belong to class III and are referred to as sirtuins [10]. Aberrant expression of HDACs has been reported to be involved in carcinogenesis and progression of various tumors [11-14]. HDAC10 is a member of the class II HDACs; HDAC10 has one deacetylase

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**Table 1.** Characteristics of RCC patients and HDAC10 expression detected by immunohistochemistry and quantitative RT-PCR

Parameters	Total	HDAC10 mRNA	p	HDAC10 protein				p
				-	+	++	+++	
RCC	145	2.69±0.24		45	66	23	11	
Normal kidney	145	6.78±0.61	< 0.05	6	13	74	52	< 0.05
Gender								
Male	85	2.70±0.21		25	41	13	6	
Female	60	2.66±0.22	> 0.05	20	25	10	5	> 0.05
Age (years)								
< 60	74	2.73±0.23		24	33	12	5	
≥ 60	71	2.67±0.22	> 0.05	21	33	11	6	> 0.05
Tumor size								
< 7 cm	81	3.56±0.32		21	32	19	9	
≥ 7 cm	64	1.58±0.13	< 0.05	24	34	4	2	< 0.05
Histological grade								
G1	65	3.71±0.32		14	28	14	9	
G2	44	2.09±0.17		16	20	6	2	
G3	36	1.58±0.13	< 0.05	15	18	3	0	< 0.05
Tumor stage								
I	82	3.56±0.32		21	33	19	9	
II	29	1.93±0.15		11	14	3	1	
III	18	1.45±0.11		5	11	1	1	
IV	16	0.96±0.08	< 0.05	8	8	0	0	< 0.05
Metastasis								
M <sub>0</sub>	115	2.99±0.23		34	48	22	11	
M <sub>1</sub>	30	1.34±0.11	< 0.05	11	18	1	0	< 0.05

domain and one additional catalytically-inactive, leucine-rich domain [15]. HDAC10 has been reported to suppress the accumulation of reactive oxygen species [16] and relieve repression on the melanogenic program [12]. HDAC10 also plays an important role in homologous recombination [17]. A recent study indicated that expression of HDAC10 is significantly decreased in gastric cancer tissues as compared with adjacent tissues in gastric cancer, and HDAC10 is an independent prognostic factor for gastric cancer patients [18]. Another study showed that the level of HDAC10 expression was significantly lower in patients with cervical squamous cell carcinoma and lymph node metastasis compared to patients without lymph node metastasis, and that HDAC10 plays a critical role in suppression of cervical cancer metastasis [19]. Indeed, no study has analyzed the correlation between HDAC10 expression and prognosis of human RCC, and the function of HDAC10 in RCC is still largely unknown.

In the current study we determined the expression of HDAC10 and analyzed its effect on proliferation, invasion, and prognosis in human RCC. Our re-sults indicated that HDAC10 expression is significantly down-regulated in RCC tissue compared with normal kidney tissues, and decreased HDAC10 expression suppresses the proliferation and invasion of RCC cells by inhibiting phosphorylation of  $\beta$ -catenin. Moreover, overexpression of HDAC10 was shown to be associated with a poor prognosis in patients with RCC. These findings suggest that loss of HDAC10 activity may affect the carcinogenesis and development of

RCC, and that HDAC10 could serve as a prognostic factor for human RCC. In addition, restoring HDAC10 activity may be a novel therapeutic strategy by which to treat RCC.

### Materials and methods

#### *Patients and tissue samples*

One hundred forty-five primary RCC tissues and adjacent normal kidney tissues were obtained from patients who underwent radical nephrectomies in the Department of Urology of the First Affiliated Hospital of Kunming Medical University between 2001 and 2008. The current study was approved by the Ethics Committee of the First Affiliated Hospital of Kunming Medical University and RCC patients were followed clinically until November 2013. None of the RCC patients received chemotherapy or radiotherapy before surgery. The histologic cell type of the RCC specimens was evaluated by experienced pathologists and all specimens used in this

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study were confirmed to be clear cell renal carcinoma. The clinical tumor stages were classified according to the TNM classification system and the pathologic grades were classified according to the Fuhrman grading system. The clinicopathologic characteristics of the tumors are presented in **Table 1**.

All RCC tissues and corresponding normal kidney tissues were formalin-fixed and paraffin-embedded. Tissue samples were also kept in liquid nitrogen until mRNA or protein extraction.

### *Cell culture*

Two RCC cell lines (ACHN and Caki-2) were purchased from the American Type Culture Collection (ATCC; USA) and used in this study. All RCC cell lines were cultured in RPMI-1640 medium with 10% fetal bovine serum (Gibco, Glasgow, Scotland), 50 µg/ml of streptomycin, and 50 U/ml of penicillin.

All RCC cell lines were cultured as monolayers in 10-cm plastic dishes and incubated in a sterile incubator maintained at 37°C with 5% CO<sub>2</sub>.

### *Immunohistochemistry*

Paraffin slices (4 µm) of RCC and normal kidney tissues were deparaffinized with xylene and rehydrated with graded alcohol, then all slices were blocked with 10% rabbit serum for 30 min and endogenous peroxidase activity was suppressed with 0.3% hydrogen peroxide for 30 min. Subsequently, the tissue slices were incubated with anti-HDAC10 (H3413) for 4 h, triple-washed with Tris-buffered saline, and the slices were incubated continuously with biotinylated anti-rabbit antibody (DAKO, Glostrup, Denmark) overnight. Determinations of the immunohistochemistry were carried out using a streptavidin-biotin complex system and the staining was examined with a light microscope. Immunostaining of HDAC10 was semi-quantitatively evaluated for positive intensity as follows: negative, -; weak, +; moderate, ++; and strong, +++.

### *Fractionation and Western blot*

Protein from RCC cells was extracted with lysis buffer containing 0.2 mmol/L Na<sub>3</sub>VO<sub>4</sub>, 1 mmol/L dithiothreitol, 50 mmol/L NaF, and 5.7 µg/mL of aprotinin. The protein concentration was determined using the Bradford dye-binding protein assay (Bio-Rad, Richmond, CA, USA), and

SDS-PAGE was performed. Antibodies to β-catenin and phospho-β-catenin (Ser33/37) were purchased from Cell Signaling Technology and anti-β-actin monoclonal antibody (Abcam, Cambridge, UK) was used as an internal control. The immune complexes were visible using an enhanced chemiluminescence (ECL) system (Amersham, Aylesbury, UK).

### *Quantitative RT-PCR*

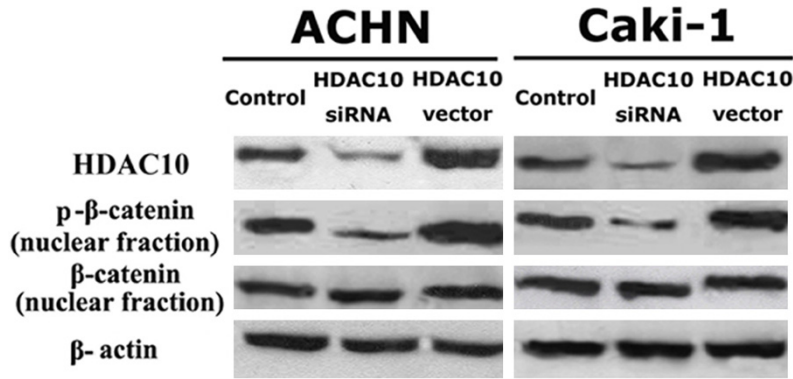
The primer sequences of human HDAC10 were designed using Primer Premier 5.0 and GAPDH was used as an internal control. The total RNA of the cells was extracted using a Quick Prep-mRNA purification kit (GE Healthcare, Buckinghamshire, UK). Total RNA was reverse-transcribed with a synthesis kit (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instruction. Quantitative RT-PCR was performed using LC FastStart DNA Master SYBR Green (Roche, Indianapolis, IN, USA) and data was collected and analyzed with Light Cycler (Roche).

### *siRNAi and vector transfection*

Oligonucleotide sequences for HDAC10 were designed using siDirect software, and the sequences of two siRNAs are as follows: CGGAGUCAGUGUGCAUGACAGUAC; and UCACUGCACUUGGAAGCUCCUGU. Briefly, ACHN and Caki-1 cells were cultured with complete medium until cell confluence reached 50%, then RCC cells were transiently transfected with siRNA oligonucleotides with lipofectamine 2000 (Invitrogen, CA, USA). After 48 h, the cells transfected with siRNA were harvested for Western blot analysis to determine the transfection efficiency. RCC cells were also stably transfected with a pFLAG-CMV vector containing full-length cDNA of HDAC10 by lipofectamine 2000. The monoclonal RCC cell line expressing a high level of HDAC10 was selected with G418 and the transfection efficiency with this vector was determined by Western blot analysis.

### *Proliferation assay of RCC cells*

The proliferative ability of RCC cells was assessed using the WST-1 assay, according to the manufacturer's instructions. ACHN and Caki-1 cells were plated into 96-well plates after being transfected with siHDAC10 or HDAC10 vector; 4×10<sup>3</sup>/well RCC cells were cultured with 3 replicates. The cells were treated with 10 µl WST-1 (Roche, Penzberg, Germany) after incubation



**Figure 1.** HDAC10 suppresses the phosphorylation of  $\beta$ -catenin in RCC cells. HDAC10 vector was stably transfected into RCC cell lines and HDAC10 expression was also decreased using siRNA; all transfections were confirmed by Western blot analysis.

for 24, 48, and 72 h, then the incubation was continued for another 2 h. Finally, the absorbance was measured at 450 nm using a micro-culture plate reader (Immunoreader, Tokyo, Japan); 3 independent experiments were performed.

#### *Invasion assay of RCC cells*

A RCC cell invasion assay was performed using a 24-well Transwell chamber, which was pre-coated with 100  $\mu$ g of Matrigel. ACHN and Caki-1 cells transfected with siHDAC10 or HDAC10 vector were collected and re-suspended in serum-free medium at a concentration of  $5 \times 10^4$  cells/ml, then 400- $\mu$ l cell suspensions were added to the upper chamber and the bottom chamber was filled with 500  $\mu$ l of culture medium containing 10% FBS. After incubation for 48 h in a sterile incubator maintained at 37°C with 5% CO<sub>2</sub>, the non-invaded RCC cells on the upper membrane surface were removed with a cotton tip and the cells that passed through the filter were fixed and stained with Hoechst 33342. The number of invaded cells was counted in 10 randomly selected high power fields under a fluorescence microscope.

#### *Statistical analysis*

Statistical analyses were performed using SPSS (version 19.0; SPSS, Inc., Chicago, IL, USA). All experiments were performed in triplicate. The data were analyzed using an independent two-tailed t test and represented as the mean  $\pm$  standard deviation. Categorical data were analyzed using the two-sided chi-square test to

determine the correlation between HDAC10 expression and clinical characteristics. Overall survival was analyzed using the Kaplan-Meier method. Values of  $P < 0.05$  were considered statistically significant.

#### **Results**

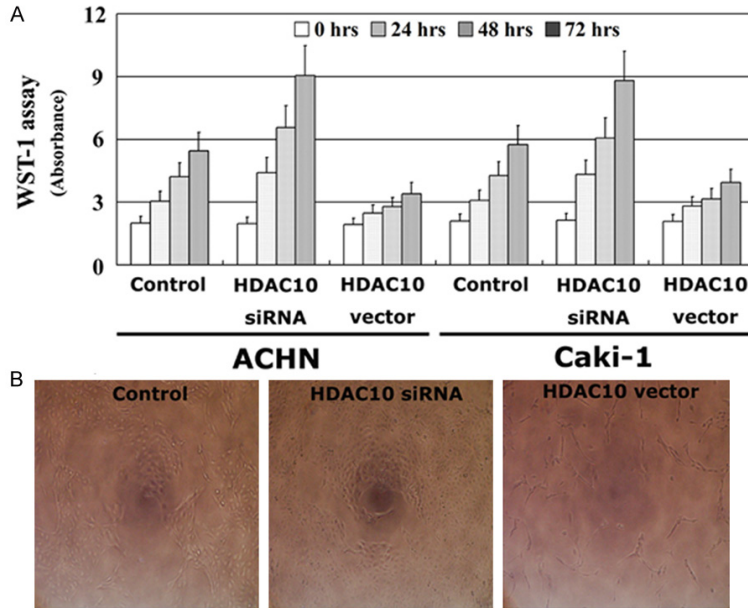
##### *Clinical characteristics of RCC patients*

Data were obtained from 85 males and 60 females. The patient ages ranged from 47-78 y (median, 63 y). The tumor sizes ranged from 1.4-15.3 cm (median, 3.7 cm). Among 145 RCC patients, 82, 29, 18, and 16 were stage I-IV, respectively. Sixty-five, 44, and 36 patients had Fuhrman grade 1-3, respectively. One hundred fifteen patients had localized RCC and 30 patients had lymphatic or distant metastases.

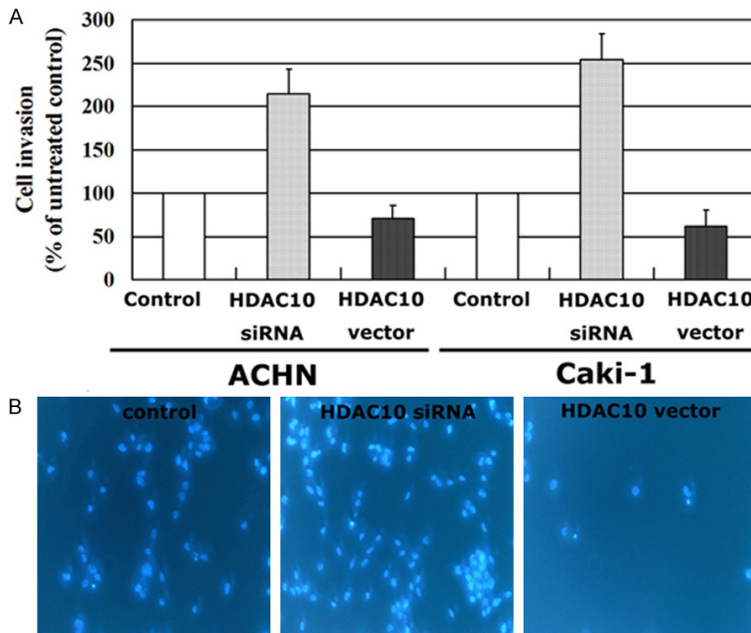
##### *HDAC10 expression in RCC*

To elucidate the role of HDAC10 in RCC, qRT-PCR and immunohistochemistry were performed to determine HDAC10 levels in 145 pairs of RCC tissues and adjacent normal kidney tissues. The expression of HDAC10 protein was determined by immunohistochemistry; HDAC10 was shown to be highly localized in the cytoplasm and nucleus. Specifically, HDAC10 expression was significantly decreased in RCC compared to normal kidney tissues; HDAC10 expression was detected in 100 of 145 (69%) RCC tissue specimens, 45.5% of which stained weakly. In contrast, the expression of HDAC10 protein expression was detected in 139 of 145 (95.9%) normal kidney tissue specimens, 86.9% of exhibited median or strong HDAC10 expression. We also analyzed the correlation between HDAC10 expression and clinical characteristics. The expression of HDAC10 protein was significantly reduced in patients with large tumor sizes, high tumor stages and pathologic grades, and metastatic tumors. In contrast, gender and age did not have a significant correlation with HDAC10 expression (**Table 1**). In addition, HDAC10 expression in human normal kidney tissues and RCC tissues was also detected by quantitative RT-PCR and similar findings were found. These results suggested

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**Figure 2.** HDAC10 suppresses the proliferation of RCC cells. The proliferative ability *in vitro* was detected using the WST-1 assay (A), the results of ACHN were also shown (B,  $\times 200$ ). All experiments were performed in triplicate and the error bar represents the SD.



**Figure 3.** HDAC10 suppresses the invasion of RCC cells. The invasive ability *in vitro* was detected using a 24-well Transwell chamber (A), the results of ACHN were also shown by Hoechst 33342 staining (B,  $\times 200$ ). All experiments were performed in triplicate and the error bar represents the SD.

that decreased HDAC10 expression may be involved in the carcinogenesis and development of human RCC.

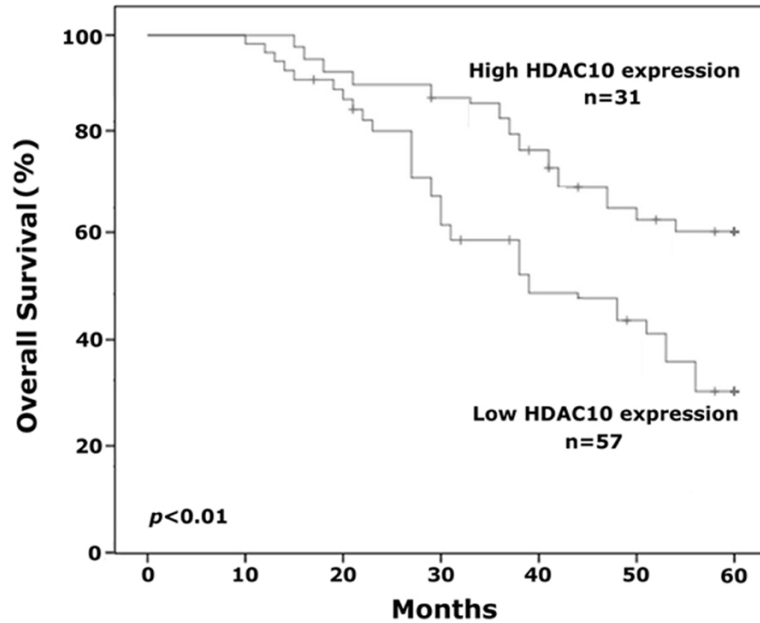
### *HDAC10 suppresses the proliferation of RCC cells*

The effect of HDAC10 on the proliferation of RCC cells was analyzed using the WST-1 assay. A vector containing the full length cDNA for HDAC10 was transfected into ACHN and Caki-1 cell lines, and HDAC10 expression was also decreased using siRNA. After all transfections, HDAC10 expression was detected by Western blot analysis. HDAC10 expression was significantly increased by the HDAC10 vector insert and decreased by siRNA in two RCC cell lines (**Figure 1**). Thus, RCC cells expressing a high level of HDAC10 have significantly decreased proliferative ability compared to untreated cells. In contrast, RCC cells expressing a low level of HDAC10 have a higher proliferative ability (**Figure 2**). These findings suggest that HDAC10 can suppress the proliferation of RCC cells.

### *HDAC10 suppresses the invasion of RCC cells*

Metastasis is a major cause of mortality in patients with RCC, and cell invasion plays an important role in this process. The effects of HDAC10 on the invasion of RCC cells were also investigated in the current study. The expression of HDAC10 in two TCC cell lines was decreased by siRNA technology, and HDAC10 expression was stably increased by transfection with a vector containing the full length cDNA of HDAC10. HDAC10 expression was evaluated using Western blot analysis (**Figure 1**). As shown in **Figure 3**, RCC cells with a high level of HDAC10 expression had a decreased ability of invasion compared to untreated controls. In contrast, RCC cells with a

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**Figure 4.** Kaplan-Meier analysis was performed to analyze the correlation between HDAC10 expression and overall survival of patients with RCC. Decreased expression of HDAC10 was associated with a poor prognosis and HDAC10 is an independent factor for predicting the prognosis of human RCC.

low level of HDAC10 expression due to siRNA treatment have a significantly increased ability of invasion. These findings indicate that decreased HDAC10 expression can increase the invasion of RCC cells, and that HDAC10 plays an important role in the metastasis of RCC.

### *HDAC10 decreased $\beta$ -catenin activity in RCC cells*

To clarify how the  $\beta$ -catenin pathway is involved in HDAC10-induced biological function in RCC cells, the phosphorylation of  $\beta$ -catenin was evaluated. HDAC10 expression was increased by transfection with a pcDEF3 vector containing the full length cDNA for HDAC10, and HDAC10 expression was also decreased by siRNA in ACHN and Caki-1 cell lines. Although HDAC10 expression did not regulate the expression of nuclear  $\beta$ -catenin protein in two RCC cell lines, increased expression of HDAC10 suppressed the phosphorylation of nuclear  $\beta$ -catenin based on Western blot analysis. In contrast, decreased expression of HDAC10 enhanced the phosphorylation of nuclear  $\beta$ -catenin (**Figure 1**). These findings suggest that phosphorylation of nuclear  $\beta$ -catenin is negatively regulated by HDAC10 activity and the  $\beta$ -catenin pathway plays an important role in

HDAC10-induced anti-proliferation and anti-invasion in RCC cells.

### *Prognostic significance of HDAC10 expression in RCC*

Because a correlation between HDAC10 expression and clinical stage and histologic grade was demonstrated in this study, we also investigated whether or not HDAC10 could be a prognostic factor for human RCC. The correlation between HDAC10 expression and overall survival in RCC patients was calculated by Kaplan-Meier analysis. Our results suggest that overall survival is significantly different between the high and low HDAC10 expression groups. After 5 years of clinical follow-up, 18 of 57 (31.6%) RCC patients in whom

immunohistochemical staining displayed a low level of HDAC10 expression (- and +) were alive and disease-free. In contrast, 20 of 31 (64.5%) RCC patients in whom staining displayed a high level of HDAC10 expression (++ and +++) were alive and disease-free. These findings suggest that HDAC10 expression may be an independent factor in predicting the prognosis of human RCC.

## **Discussion**

Although RCC at early stage is usually regarded as localized disease, some patients can develop metastatic RCC and the prognosis of advanced RCC is extremely poor. Indeed, the current treatments available for advanced RCC are not effective [20]. Because advanced RCC is insensitive to chemotherapy, and no agents are available to eliminate this disease, novel gene targets for the diagnosis, prognosis, and treatment of patients with advanced RCC are needed.

In recent years it has been well-established that histone acetylation in the promoter regions of some tumor suppressor genes is aberrant in various malignancies and some members of the HDAC family play an important role in carci-

nogenesis [21]. HDACs can serve as a signal to regulate the expression of downstream genes by modifying the structure of chromatin and binding of transcription factors to the DNA region [22]. HDAC members are divided into 4 classes according to enzymatic activity, molecular structure, expression, and localization, as follows: class I (HDAC1, 2, 3, and 8); class IIa (HDAC4, 5, 7, and 9); class IIb (HDAC6 and 10); and class IV (HDAC11) [23]. Class I HDACs have been reported to enhance cell cycles and cancer cell proliferation [24]. A high level of HDAC2 expression predicts an unfavorable prognosis in gallbladder cancer [25]. HDAC2 and HDAC3 are also significantly up-regulated in subgroups of malignancies with aggressive features [26]. It has been reported that expression of HDAC10 is significantly decreased in gastric cancer and HDAC10 is an independent prognostic factor for this disease [18]. Moreover, another study indicated that HDAC10 suppresses the expression of matrix metalloproteinase 2 and 9, thus inhibiting invasion and metastasis of cervical cancer cells [19]. Although several studies have confirmed that HDAC10 acts as a cancer suppressor gene in human tumors, the role of HDAC10 in human RCC remains unknown. Thus, the effect of HDAC10 expression on the growth, metastasis, and prognosis in RCC needs further evaluation.

This is the first study involving the functions of HDAC10 in human RCC. We demonstrated that HDAC10 expression is significantly decreased in RCC tissues compared to normal kidney tissues by immunohistochemistry and quantitative RT-PCR. In addition, the level of HDAC10 expression was significantly associated with tumor size, clinical stage, pathologic grade, and tumor metastasis. We further analyzed the effect of HDAC10 on the proliferation and invasion of RCC cells, and showed that decreased expression of HDAC10 could significantly prompt the proliferation and invasion of RCC cells *in vitro*. These findings suggest that decreased HDAC10 expression is involved in the carcinogenesis and development of human RCC. In addition, the correlation between HDAC10 expression and overall survival of human RCC was also investigated by the Kaplan-Meier analysis in this study, which indicated that a decreased level of HDAC10 expression could be regarded as a valuable factor for prediction

of poor prognosis in patients with RCC, and that HDAC10 could serve as an independent prognostic factor and guide the follow-up schedule in RCC patients (Figure 4).

Although the expression and functions of HDAC10 in RCC have been investigated in our study, the underlying molecular mechanism of HDAC10 in RCC remains unclear and should be further assessed.  $\beta$ -catenin serves as a co-activator for lymphoid enhancer-binding factor 1 (LEF1) and may be a key transcription activator in the Wnt signaling pathway [27]. LEF1 contains a DNA binding domain near its C terminus and a domain at the N terminus that binds  $\beta$ -catenin [28]. Upon Wnt stimulation,  $\beta$ -catenin could activate Wnt-responsive target genes. Alterations in the  $\beta$ -catenin signaling pathway have been reported in several tumors [29], and dysregulation of  $\beta$ -catenin is an important phenomenon in carcinogenesis and an independent predictor in patients with RCC [30-32]. Our study suggests that although HDAC10 did not regulate the expression of  $\beta$ -catenin in the nucleus, HDAC10 decreased the phosphorylation of nuclear  $\beta$ -catenin in RCC cells. Thus, the  $\beta$ -catenin pathway is a potential target for HDAC10, and  $\beta$ -catenin plays an important role in HDAC10-induced anti-proliferation and anti-invasion of RCC cells.

In summary, our study revealed that HDAC10 expression is significantly suppressed in human RCC and may be involved in the development and metastasis of this disease. These findings suggest that RCC patients with a low level of HDAC10 expression may be vulnerable to develop metastases of RCC, and HDAC10 may be an independent factor to predict the prognosis of patients with RCC. Moreover, our study also suggested that restoring HDAC10 expression may prove to be a novel therapeutic treatment for patients with advanced RCC.

### Acknowledgements

This study don't have any grant supported. WenXing Fan is responsible for data collection, Jie Huang is responsible for data analysis and Hua Xiao is responsible for manuscript preparation.

### Disclosure of conflict of interest

None.

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