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Original Article

HDAC9 associates with distant metastasis and predicts poor prognosis in clear cell renal cell cancer

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Abstract: Accumulating evidence demonstrated various HDACs implicated in the development progression of clear cell renal cell cancer (ccRCC). However, the function of HDAC9 in ccRCC had not been fully illustrated. Here we investigated the expression and clinical significance of HDAC9 in a cohort of ccRCC patients by immunohistochemistry. The results indicated that HDAC9 expression was specially localized in the nucleus of all ccRCC tissues, as well as adjacent tissues. In compared with paired para-carcinoma tissues, HDAC9 expression was down-regulated in 82.8% of the ccRCC, while 9.2% was up-regulated and 8.0% was unaltered. Similarly, HDAC9 mRNA expression was down-regulated in ccRCC in Oncomine data analysis. The HDAC9 expression was correlated with M stage ($r = 0.332$, $P = 0.002$). Survival analyses presented that ccRCC patients with up-regulated HDAC9 expression hadinferior outcome; conversely, down-regulated HDAC9 expression might contribute to good prognosis (25.0% vs 57.1% vs 72.2%, $P = 0.002$). Subsequent multivariate analyses demonstrated that HDAC9 expression independently associated with poor prognosis of ccRCC patients ($P = 0.002$). Overall, our results firstly revealed the oncogenic function of HDAC9 expression in ccRCC, and suggested HDAC9 to be a potential predict prognosis factor.

Keywords: Renal clear cell carcinoma, tissue microarray, immunohistochemistry, HDAC9, prognosis

Introduction

There are 66,800 new estimated kidney cancer cases and 23,400 cases dead of kidney cancer in China [1, 2]. Clear cell renal cell carcinoma (ccRCC) was the most common kidney cancer. Despite the advance management for ccRCC, the mortality for advanced ccRCC was extremely high attributing to multi-drug resistant property [3, 4]. Therefore, discovering new diagnostic and therapeutic molecular markers for ccRCC was urgent for developing new advanced management for ccRCC.

Numerous reports demonstrated that histone deacetylases (HDACs) was implicated in various normal and abnormal biological functions by remodeling chromatin and regulating downstream gene expression [5]. According to different structure, HDACs were divided into four classes (I, II, III and IV) [6]. Dysregulated expression of different HDACs had been observed in ccRCC, including HDAC1, 2, 3 (class I), 4, 5, 6 and 10 (class II) [7-10]. Most of the investigated HDACs were involved in the carcinogenic progression of ccRCC, such as HDAC1, 2, 3 and 6 [7, 9]. In contrast, HDAC10 acted as a tumor suppressor by inhibiting phosphorylation of β-catenin [10]. HDAC9 belongs to class II HDACs and shows conflicting function in the progress of different cancers [11-17]. Most reports presented the oncogenic function of HDAC9 such as osteosarcoma [14]. Conversely, the tumor suppressor role of HDAC9 was also been demonstrated in lung cancer [17]. To date, the function of HDAC9 in ccRCC had not been fully investigated.

In this study, we aim to investigate the expression of HDAC9 in a cohort of ccRCC patients by immunohistochemistry, and analysis the prognostic significance of HDAC9, in order to reveal the potential function of HDAC9 expression in ccRCC.

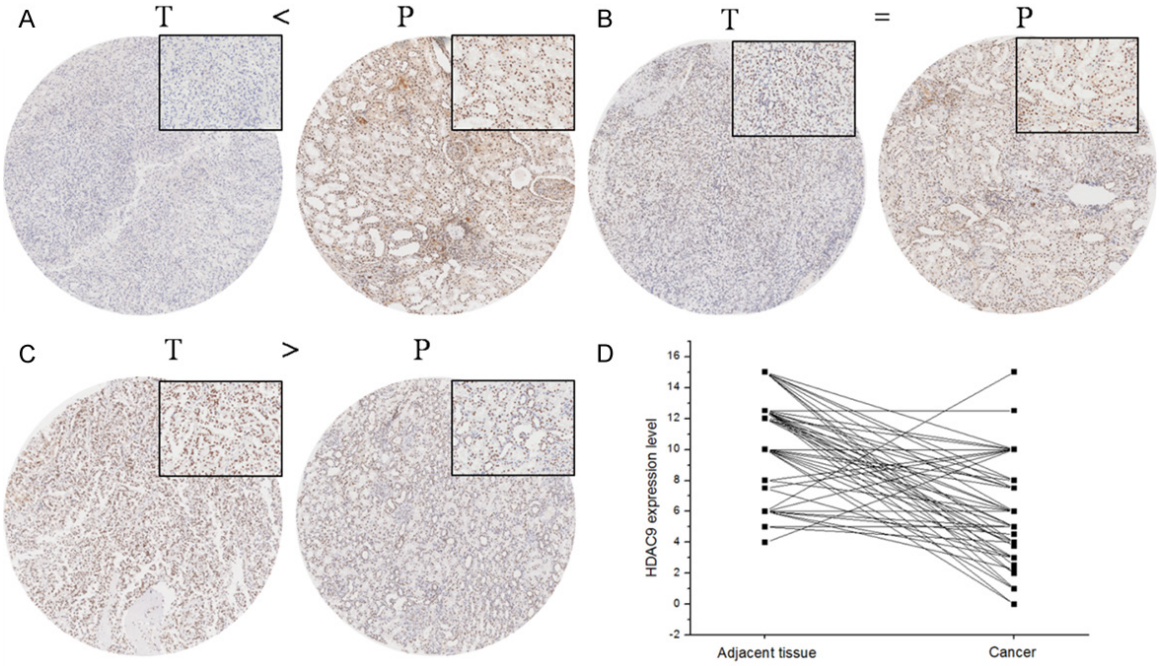

Figure 1. The representative immunohistochemistry images of HDAC9 expression: HDAC9 was specifically resided in nucleus of all specimens. A. T < P; B. T = P; C. T > P; D. HDAC9 expression level in paired cancer and adjacent tissues (Magnification times: ×200).

Table 1. Expression of HDAC9 in HCC tissues and adjacent carcinoma tissues

Histology	No. of patients	Expression (Mean ± Std. Deviation)	*P*-value
ccRCCs	89	5.58 ± 3.00	0.000
Adjacent carcinoma	88	10.74 ± 2.83	

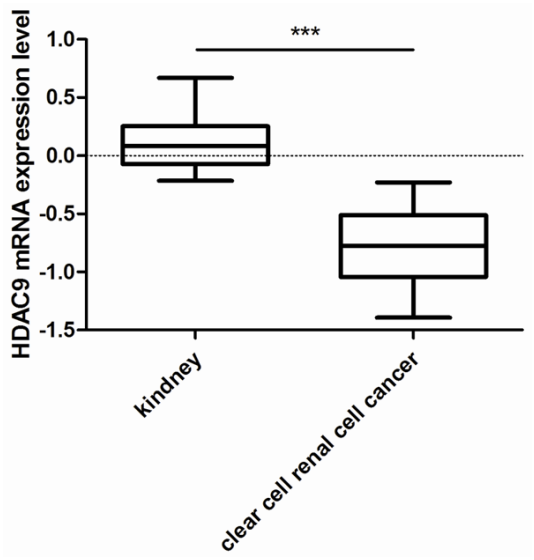

Figure 2. Oncomine analysis of HDAC9 mRNA expression in ccRCC. ***$P < 0.001$.

Materials and methods

Patients information

The ccRCC tissue microarray (TMA) (HKid-CRC-180Sur-01) that contained 90 paired ccRCC paraffin sections and adjacent carcinoma tissues (1.5 cm away from the carcinoma) was obtained from Shanghai Outdo Biotech Co., Ltd. (SOBC).

The 90 ccRCC patients contained 51 males and 39 females with a median age of 59 (ranged from 29 to 82). All the patients were diagnosed as ccRCC by pathologist and received no extra therapy before surgery from July 2006 to February 2008. The size of tumor varied from 2 cm to 14 cm. There are 60 cases belonged to clinical stage I, 18 were stage II, 4 were stage III and 2 were stage IV. 6 patients' clinical stage was missing. All the patients were followed up until September 2013 or death. During this follow up time, 59 patients were died of ccRCC with a median survival time of 59 months, while other 31 patients were still alive.

Immunohistochemistry

The deparaffinage of TMA was performed by xylene and graded alcohol. Then tissue slices were incubated with EDTA at 60°C to retrieve

Table 2. Correlation between HDAC9 expression and clinical characters of the patients with ccRCC

Clinical characters	HDAC9 expression			Correlation coefficient	*P* value
	T < P (72 cases)	T = P (7 cases)	T > P (8 cases)		
Gender				0.097	0.372
Male	44	2	5		
Female	28	5	3		
Age				-0.112	0.302
≤ 60	38	4	6		
> 60	34	3	2		
Tumor size				0.185	0.087
≤ 5 cm	41	3	2		
> 5 cm	31	4	6		
Tumor differentiation				0.161	0.135
I	28	4	0		
II	35	2	5		
III	9	1	3		
T stage				0.144	0.195
T1	53	6	3		
T2	14	0	3		
T3	2	1	1		
Lost	3	0	1		
N stage				-0.048	0.661
N0	70	6	8		
N1	1	0	0		
Lost	1	1			
M stage				0.332	0.002
M0	72	6	7		
M1	0	1	1		
cTNM stage				0.147	0.190
1	51	5	3		
2	14	0	3		
3	3	0	0		
4	0	1	1		
Lost	4	1	1		

Abbreviations and Note: T, renal cell carcinoma tissues; P, para-carcinoma tissues; T > P, the HDAC9 expression in ccRCC tissues was higher than that in para-carcinoma tissues; T = P, the expression level was not altered in carcinoma tissues compared with para-carcinoma tissues; T < P, the expression level was down-regulated in tumor tissues.

antigen and blocked with goat serum. Later, the primary antibody anti-HDAC9 (1:1200, Abcam, ab109446) was added to the tissue slices and the TMA was incubated at 4°C overnight. Lastly, sections were incubated with HRP (horse radish peroxidase) labeled antibody (DAKO, K8000) for 30 minutes, visualized by diaminobenzidine (DAB) system and hematoxylin redying. The staining intense scored as follows: negative for 0, '+' for 1, '++' for 2 and '+++' for 3. Similarly, the positive rate scored as bellow: negative for 0, '1%-20%' for 1, '21%-40%' for 2, '41%-60%' for 3, '61%-80%' for 4, '81%-100%' 5. The expression level of HDAC9 was evaluated by the score of staining intense multiply by positive rate.

Statistical analysis

The difference between HDAC9 expression in HCC tissues and adjacent tissues was evaluated by Wilcoxon Signed Ranks Test. Spearman rank correlation coefficient and Two-tailed test were used to evaluate the correlation between HDAC9 expression and clinical characters. Kaplan-Meier method and log-rank test were used to analysis the association between overall survival and HDAC9 expression, clinical index. Subsequently, all the potential predict factors were involved in COX multivariate regression survival analysis. All statistical analyses were conducted using SPSS 17.0 software. $P < 0.05$ was considered significantly.

Results

Aberrant HDAC9 expression in ccRCC tissues

To detect the HDAC9 expression in ccRCC, we tested the HDAC9 expression in 90 ccRCC tissues when compared with adjacent tissues by immunohistochemistry. Eliminating 1 tumor off-point and 2 para-carcinoma off-point, there are 87 paired ccRCC tissues and adjacent carcinoma tissues. As the immunohistochemistry results shown in **Figure 1A-C**, HDAC9 expression was localized in nuclear of all specimens. However, no HDAC9 cytoplasm or membrane

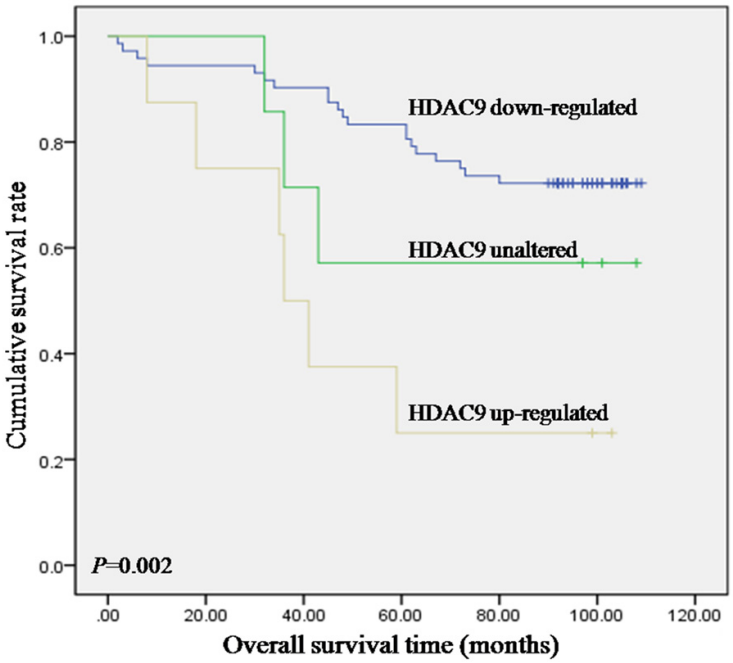

Figure 3. Survival analyses dependent on different HDAC9 expression in ccRCC. T > P, up-regulated; T = P, unaltered; T < P, down-regulated. T: ccRCC tissue; P: para-carcinoma. *P* values were calculated by log-rank test.

Table 3. Univariate and multivariate analysis of factors associated with survival in ccRCC

Factors	Univariate analysis	Multivariate analysis			
	P-value	Df	*P*-value	Exp(B)	95.0% CI
HDAC9 expression	0.002	1	.017	1.920	1.123-3.284
Gender	0.578	-	-	-	-
Age	0.016	1	.027	2.571	1.112-5.945
Tumor size	0.081	-	-	-	-
Tumor differentiation	0.000	1	.030	2.120	1.076-4.174
T stage	0.000	1	.044	2.284	1.023-5.102
N stage	0.000	1	.026	15.561	1.400-172.986
M stage	0.000	1	.633	1.659	0.208-13.228
Clinical stage	0.000	0a	-	-	-

Abbreviations and Note: Df, degree of freedom; 95% CI, 95% confidence interval; HR, Hazard ratio; Cox proportional hazards regression model. a Degree of freedom reduced because of constant or linearly dependent covariates; Constant or Linearly Dependent Covariates cTNM = T + 2*N + M.

expression was observed in ccRCC as well as para-carcinoma tissues. Statistical analysis revealed that HDAC9 expression was significantly down-regulated in ccRCC (5.58 + 3.00 vs 10.74 + 2.83, $P < 0.001$, **Table 1**). The HDAC9 expression in most of the ccRCC tissues was down-regulated when compared to paired para-carcinoma tissues, high to 82.76% (72 out of 87) (**Figure 1A**); only 8 cases showed up-regulated HDAC9 expression in ccRCC tissues (**Figure 1C**); the expression of HDAC9 in the rest 7 cases was unaltered (**Figure 1B**). The dysregulated HDAC9 expression was shown in **Figure 1D**. The ccRCC patients were divided into three subgroups according to different HDAC9 expression status: T > P, up-regulated; T = P, unaltered; T < P, down-regulated. T: ccRCC tissue; P: para-carcinoma tissues.

Down-regulating HDAC9 mRNA expression in ccRCC

The HDAC9 mRNA expression in ccRCC was obtained from the Oncomine data analysis uploading by Jones and colledgues [18]. The mRNA expression of HDAC9 in 23 ccRCC tissues was down regulated 1.864 fold in campared with kidney tissues ($P = 2.46e-14$). The results were shown in **Figure 2**.

HDAC9 expression correlated with distant metastasis

As the down-regulating expression of HDAC9 observed in ccRCC, the correlation between HDAC9 expression and clinical characters of ccRCC patients were further analyzed to reveal the potential function of HDAC9 in ccRCC. The results indicated that HDAC9 expression was only related to M (distant metastasis) stage ($r = 0.332$, $P = 0.002$), but showed no statistically significant association with gender ($P = 0.372$), age ($P = 0.302$), tumor size ($P = 0.087$), tumor differentiation ($P = 0.135$), T (tumor invasion) stage ($P = 0.195$), N (regional lymph node metastasis) stage ($P = 0.661$) or clinical stage ($P = 0.190$). The detailed results were shown in **Table 2**.

Correlation between HDAC9 expression and prognosis of ccRCC carcinoma patients

As the results showed in **Figure 3**, elevated HDAC9 expression in ccRCC tissues associated with poor prognosis in compared with unaltered HDAC9 expression. Conversely, down-regulated HDAC9 expression predicted a better prognosis in ccRCC (25.0% vs 57.1% vs 72.2%, $P = 0.002$). The cumulative survival rate of ccRCC patients was differed from different HDAC9 expression level. In the subgroup of up-regulated HDAC9 expression, the survival rate was low to 25% with a mean survival time of 49.88 months, while for the unaltered and down-regulated HDAC was 57.1% (mean survival time, 73.43 months), 72.2% (mean survival time, 84.25 months) respectively.

Additionally, age ($P = 0.016$), tumor differentiation ($P = 0.000$), T stage ($P = 0.000$), N stage ($P = 0.000$), M stage ($P = 0.000$) and clinical stage ($P = 0.000$) were all associated with overall survival of ccRCC patients (see **Table 3**).

Subsequent multivariate analysis revealed that, HDAC9 expression was an independent prognostic marker in ccRCC ($P = 0.017$). Otherwise, age ($P = 0.027$), tumor differentiation ($P = 0.030$), T stage ($P = 0.044$) and N stage ($P = 0.026$) were also independent prognostic markers (see **Table 3**).

Discussion

The conflicting function of HDAC9 had been observed in different types of cancer. To our knowledge, the function of HDAC9 in ccRCC was not fully understood. Therefore, we investigated the clinical and prognosis significance in a cohort of ccRCC patients.

HDAC9 expression was specially localized in the nucleus of all ccRCC tissues, as well as adjacent tissues. In compared with paired para-carcinoma tissues, HDAC9 expression was down-regulated in 82.8% of the ccRCC, while 9.2% for up-regulated and 8.0% for unaltered. The change status of HDAC9 expression was correlated with M stage ($r = 0.332$, $P = 0.002$). Survival analyses presented that ccRCC patients with up-regulated HDAC9 expression had inferior outcome; conversely, down-regulated HDAC9 expression might contribute to good prognosis (25.0% vs 57.1% vs 72.2%, $P = 0.002$). Subsequent multivariate analyses demonstrated that HDAC9 expression independently associated with prognosis of ccRCC patients ($P = 0.002$). All these results suggested that the aberrant expression of HDAC9 was involved in the development of ccRCC, and possibly acted as a tumor promoter in ccRCC. Up-regulated HDAC9 expression might be a promote factor for metastasis progression of ccRCC, and thus correlated with an inferior outcome.

HDACs expression in cancers has been investigated in a series of studies previously. Interestingly, the expression of class II HDACs was down-regulated in most of ccRCC tissues, involving HDAC4, HDAC5, HDAC6 and HDAC10 [9, 10], in consistent with the HDAC9 down-regulated expression observed in this study. However, the class II HDACs' function in ccRCC was not consistent, even opposite. For example, HDAC6 improved the tumor cells invasion and migration and acted as a tumor promoter [9]; conversely, HDAC10 play a pivotal role as a tumor suppressor in ccRCC [10]. The alternation of HDAC9 expression was observed in most of the ccRCC tissues in this study, although most of which was down-regulated. Nevertheless, whether up- or down-regulated, aberrant HDAC9 expression affected the outcome of ccRCC patients. Consistently, HDAC9 expression up-regulated associated with determines of poor outcome, while HDAC9 down-regulated expression seemed to be a great impulse of advanced prognosis. Considering the intense link between HDAC9 expression and poor prognosis, as well as M stage, we could speculate that those tumors with up-regulated HDAC9 expression have a potential to be more metastatic in the clinical setting.

The regulatory mechanism of HDAC9 in ccRCC was poorly understood. One possible hypothesis that might illustrate the oncogenic function of HDAC9 in ccRCC was depending on the association between HDAC9 and hypoxia-inducible factor (HIF-1α) [19]. HIF-1α was a master regulator involved in the oncogenic progression of various cancers [20, 21]. The proteolysis of HIF-1α was regulated by von HippelLindan (VHL), a tumor suppressor which is common deregulated in around 70% ccRCC [22-25]. Previous studies investigated various HDACs function on HIF-1α, involved in HDAC1, 3, 4, 5,

6, however only HDAC4 and HDAC6 could stabilize HIF-1α in kidney tumor cells [8, 9, 26]. Latterly, HDAC9 was proved to be implicated in the enhancement of HIF-1α translation in liver cancer cells, which might be an indirect evidence supporting the assumption that HDAC9 effected HIF-1α function in ccRCC [19]. Based on these findings, we hypothesis that HDAC9 might promote the metastasis of ccRCC by regulating the oncogenic function of HIF-1α.

Overall, our results firstly revealed the oncogenic function of HDAC9 expression in ccRCC, and suggested HDAC9 to be a potential predict prognosis factor. Further study focusing on the regulatory mechanism of HDAC9 in ccRCC should be done by knocking down/overexpressing *HDAC9* in kidney tumor cells to support our notion that HDAC9 was an oncogene in ccRCC.

Disclosure of conflict of interest

None.

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