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Original Article

EHD2, a novel HIF target gene, is a promising biomarker in clear cell renal cell carcinoma

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Abstract: Objectives: The aim of the present study was to determine the clinical value of a novel hypoxia-inducible factor (HIF) target EH domain-containing protein 2 (EHD2) for predicting the outcome of patients with clear cell renal cell carcinoma (ccRCC). Materials and methods: GEPIA public database was searched to determine a possible association between HIF2A and EHD protein family members, and kidney renal clear cell carcinoma data were used to find the expression profile of EHD proteins in ccRCC samples. A tissue microarray from 70 ccRCC samples was used for immunohistochemical analysis to determine the specific expression pattern of EHD2 in ccRCC samples. In addition, univariate and multivariate analyses were performed to assess the utility of EHD2 as an independent prognostic factor for ccRCC. Results: EHD protein family members were all found to be significantly correlated with HIF2A expression in ccRCC. However, EHD2 was the only protein that was observed to be overexpressed in ccRCC cancer tissues compared with normal tissues. EHD2 and HIF2A mRNA expression levels were found to be higher in cancer tissues compared with those in adjacent normal tissue according to reverse transcription-quantitative PCR analysis. Among the 70 patients with ccRCC, EHD2 was overexpressed in 52.8% (37/70). Subsequently, EHD2 was found to be significantly associated with both overall survival (P=0.016) and disease-free survival (P=0.029). Furthermore, by multivariate analysis, EHD2 was an independent prognostic factor for patients with ccRCC. Conclusion: EHD2 is a novel HIF target, based on a relatively large sample of EHD2 research in patients with ccRCC. Furthermore, our study provided evidence that EHD2 can serve as a promising biomarker for predicting ccRCC outcome.

Keywords: EHD2, prognostic value, ccRCC

Introduction

Renal cell carcinoma (RCC) is one of the most lethal urological cancers worldwide. According to the Global Cancer Statistics, in 2020 the incidence and mortality from RCC were 431,288 and 179,368, respectively [1]. As a result of developments in imaging technology and physical examination technique, the incidence of RCC has been on the increase over the past decade. In addition, a large proportion of patients with RCC are first diagnosed already at the advanced tumor stages [2, 3]. Clear cell RCC (ccRCC) is the most common pathological subtype of RCC, accounting for ~75% of all patients with RCC [4]. Surgery is an important means of treatment for patients with RCC. Several studies have recently found that the different pathologic types of RCC are likely driven by different oncogenes, and they also have different histologic characteristics, distinct clinical development and treatment response profiles [5]. Therefore, it remains of importance to explore novel effective molecular markers for evaluating the prognosis of ccRCC, in addition to developing novel therapeutic targets.

The von-Hippel Lindau tumor suppressor-hypoxia-inducible factor (HIF) signaling pathway is one of the most important oncogenic pathways in ccRCC. To date, >800 genes have been found to be targets of this pathway according results from a previous large-scale chromatin-immunoprecipitation-sequencing study [6]. Zhang *et al* [7] subsequently reported a novel HIF transcriptional target, EH-domain containing protein 2 (EHD2), which was found to drive membrane ruffle formation and macropinocytosis [8] in hypoxic hepatocellular carcinoma (HCC) cells. Specifically, EHD2 inhibition was demonstrated to prevent the development of HCC in mice, resulting in the proposal that the HIF/EHD2 axis is a viable therapeutic approach for HCC.

EHD2 is a member of EH domain-containing protein family, which contains four proteins (EHD1-4). These all serve important roles in nucleotide-dependent membrane remodeling and membrane transport [9, 10]. A previous study found that EHD2 overexpression can inhibit the migration and invasion of liver cancer cells by interacting with E-cadherin [11]. In addition, in colorectal cancer cells, overexpression of EHD2 was shown to inhibit cell proliferation while promoting apoptosis and cell cycle arrest [12]. Another study previously reported that EHD2 can reduce lung adenocarcinoma cell migration and invasion by inhibiting epithelial-mesenchymal transition [13]. The aforementioned findings from previous studies suggest that EHD2 functions as a tumor suppressor gene. However, a report exists finding EHD2 overexpression to enhance the proliferation, invasion and migration of ccRCC cells, though these findings were derived from clinical data and prognosis analysis [14]. Accumulating evidence in renal cancer showed that HIF2α might act as a key factor for cancer promotion, and HIF1α acts as a tumor suppressor [15, 16], therefore, we speculate whether the expression of EHD2 is related to HIF2α in RCC.

In the present study, a tissue microarray (TMA) consisting of 70 ccRCC and adjacent tissues was used for immunohistochemistry (IHC). In addition, patient information and prognosis analysis were performed. Subsequently, a public database was used to find the expression profile of EHD proteins in ccRCC before differences in the different pathological types of ccRCC were assessed. We provided the solid evidence that the oncogenic value of EHD2 in ccRCC.

Patients and methods

Patients and samples

This study enrolled 70 ccRCC patients, including 55 men and 15 women. The clinicopathologic characteristics, including age, tumor size, TNM stage and Fuhrman grade, in addition to follow-up information, were all collected. These were all patients who underwent radical nephrectomy at Putuo Hospital between January 2008 to December 2015 with ccRCC pathology confirmed. Patients who were either not followed up or had their follow-up information missing from our hospital were excluded. Furthermore, another five paired fresh ccRCC samples were collected for reverse transcription-quantitative PCR (RT-qPCR) analysis. The overall survival (OS) and disease-free survival (DFS) rates were measured according to the clinical data collected. The present study was approved by the Ethics Review Board of Putuo Hospital, Shanghai University of Traditional Chinese Medicine (Shanghai). Written informed consent for each participant was obtained.

Public database analysis

The expression levels of EHDs in kidney cancer were explored using Gene Expression Profiling Interactive Analysis (GEPIA) database (http://gepia.cancer-pku.cn) [17]. The correlation between HIF2A and EHD expression were also determined using GEPIA.

IHC

The TMA was made by using the 70 paired cancerous and para-cancerous tissues of ccRCC from Shanghai Qutdo Biotech Company (Shanghai, China). IHC analysis was performed using the streptavidin-peroxidase method (Zymed Laboratories, Inc.; Thermo Fisher Scientific, Inc.) as previously described [18]. The EHD2 antibody was purchased from Abcam (cat. no. ab222888) and diluted 1:50. The EHD2 expression level was evaluated by both the intensity and percentage of positive tumor cells. The intensity of IHC was score from 0 (negative) to 3 (strong), whereas the percentage was classified as either 1 (<25%), 2 (26-50%), 3 (51-75%) or 4 (>75%). The final IHC score were calculated by the intensity score × percentage score. Tissue scoring <6 was deemed the low EHD2 expression group, and >6 was deemed the high EHD2 expression group.

RNA extraction and RT-qPCR

The total RNA was extracted from the five paired ccRCC samples using TRIzol (Thermo Fisher Scientific, Inc.) according to the standard protocol. qPCR was performed with SYBR Green kit (Takara Bio, Inc.). The result was analyzed using the $2^{-\Delta\Delta Cq}$ method to normalize to the expression of GAPDH. The reaction system and condition of qPCR was listed as follows: 2X Universal SYBR qPCR Master Mix, 10 μl; forward primers (10 μM), 0.4 μl; reverse primers (10 μM), 0.4 μl; template cDNA, 1 μl; ddH_2O, up

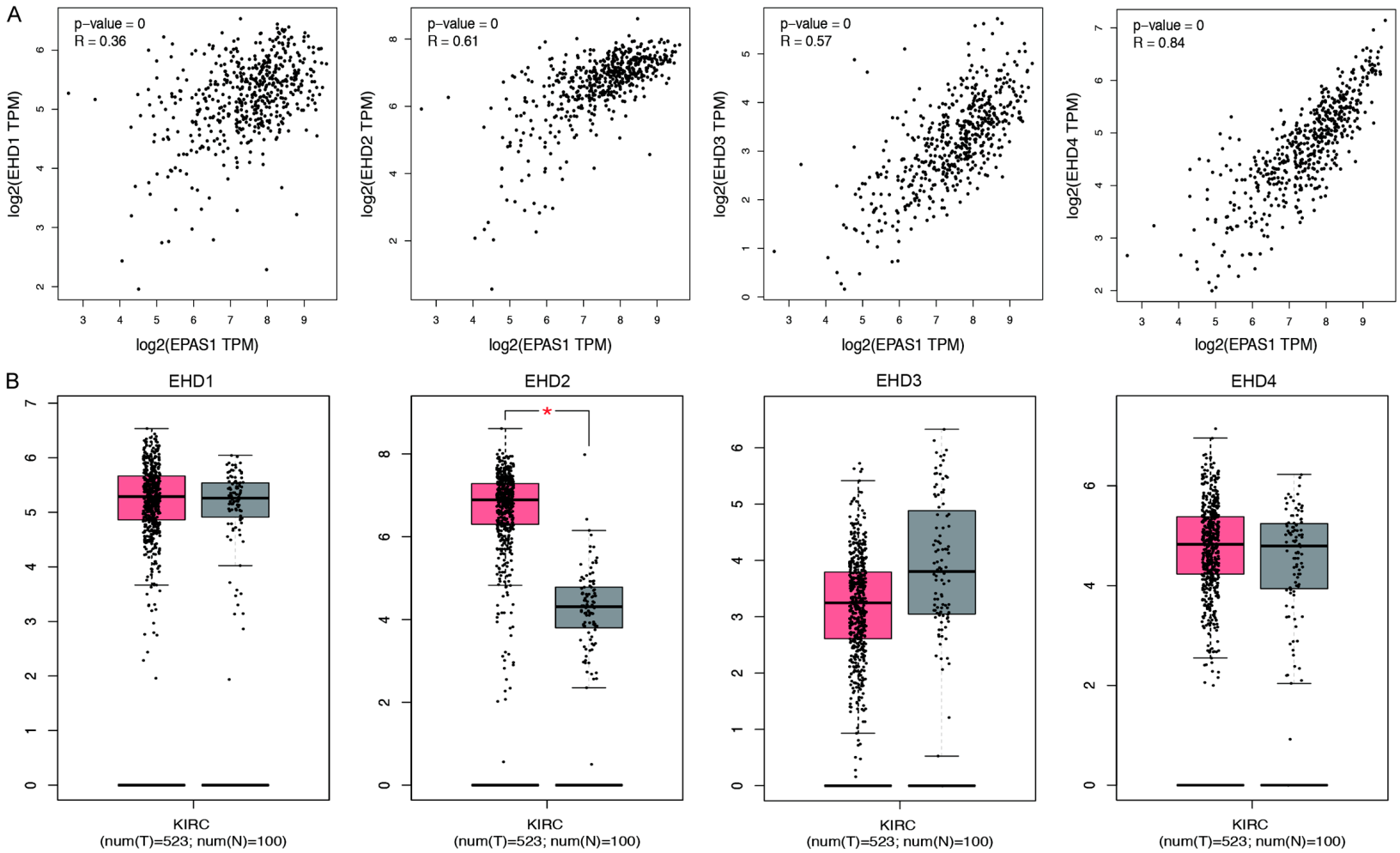

Figure 1. Expression profiles of EHD proteins in ccRCC according to the GEPIA database. A. Correlation between the four EHD proteins and endothelial PAS domain protein 1 (which encode HIF2A) from GEPIA database. B. Expression level of EHD protein family members in the clear cell renal cell carcinoma (KIRC) database. EHD, EH domain-containing protein; ccRCC, clear cell renal cell carcinoma; HIF, hypoxia inducible factor.

Table 1. Baseline characteristic

Characteristic	Patients		Tumoral EHD2 expression		
	n	%	Low	High	*P*-value
All patients	70	100	33	37	
Gender					0.976
Male	55	78.6	26	29	
Female	15	21.4	7	8	
Age (years)					0.230
≤55	32	45.7	18	14	
>55	38	54.3	15	23	
TNM stage					0.175
I+II	68	97.1	33	35	
III+IV	2	2.9	0	2	
Fuhrman grade					0.190
I+II	57	81.4	29	28	
III+IV	13	18.6	4	9	
Tumor size (cm)					0.269
≤4	39	55.7	14	11	
>4	31	44.3	19	26	

to a total volume of 20 μl. qPCR thermocycling conditions: Initial denaturation at 95°C for 5 min, followed by a three-step method of 95°C for 10 sec, 60°C for 20 sec and 72°C 20 sec for 40 cycles. The PCR primers were listed as follows: human EDH2 forward, 5'-CGGAATTCCATGTTCAGCTGGCTG-3' and reverse 5'-CGGGATCCCTCGGCGGAGCCCTT-3'; human HIF2A forward, 5'-TGATGTGGAAACGGATGA-3' and reverse 5'-ATGGGGTTTTGGGTGAA-3'; human GAPDH forward, 5'-ACAGTCAGCCGCATCTTCTT-3' and reverse 5'-GACAAGCTTCCC GTTCTCAG-3'.

Statistical analysis

The statistical analysis was performed by using SPSS statistical version 19.0 (SPSS, Inc.). For the clinical characteristics, and the normality and homogeneity of variance were both tested before, the paired Student's t-test or the Mann-Whitney test were used to compare continuous variables, while χ^2 or Fisher's exact tests were used to compare categorical variables. Survival analysis was performed by using the Kaplan-Meier method and compared using a log-rank test. Cox proportional hazards regression model was used for prognostic factor investigation. *P* value <0.05 was considered a significant difference.

Results

Expression of EHD in public database

We first searched the expression data of the EHD family of proteins in ccRCC tissues in the GEPIA database. The results showed that all EHD family of proteins were significantly correlated with HIF2A expression in ccRCC samples (**Figure 1A**). The expression profile of EHDs was then assessed using the kidney renal clear cell carcinoma (KIRC) data. We found EHD2 to be the only member of the EHD family that has its expression significantly higher in ccRCC samples compared with that in normal tissues (**Figure 1B**). These results suggest that EHD2 is a viable biomarker of ccRCC development.

EHD2 expression in patients with ccRCC

We then examined the levels of EHD2 expression in ccRCC TMA using IHC. The clinical characteristic were collected in **Table 1**. As shown in **Figure 2A**, EHD2 staining was mainly localized in the nuclei and its expression was markedly higher in cancer tissues. RT-qPCR analysis was also performed to test both EHD2 and HIF2A expression in the using fresh ccRCC samples. The mRNA expression level of EHD2 was found to be higher in tumor tissues compared with that in adjacent normal tissues (**Figure 2B**), which was also the case for the expression of HIF2A (**Figure 2C**). These results were consistent with those from the aforementioned HIF2A and EHD2 bioinformatics expression analyses.

Relationship between clinical characteristics and EHD2 expression in patients with ccRCC

The OS and DFS survival rates of 70 patients with ccRCC according to the TMA clinical data were performed using Kaplan-Meier survival analysis. The result showed that patients with higher EHD2 expression tended to have inferior prognostic outcomes (**Figure 3A**, **3B**). Specifically, the 5-year survival rate in the high EHD2 expression group was 69.14%, compared with 86.18% in the low EHD2 expression group.

The prognostic values of the various different risk factors were then determined using univariate and multivariate Cox regression analyses. As shown in **Tables 2** and **3**, univariate

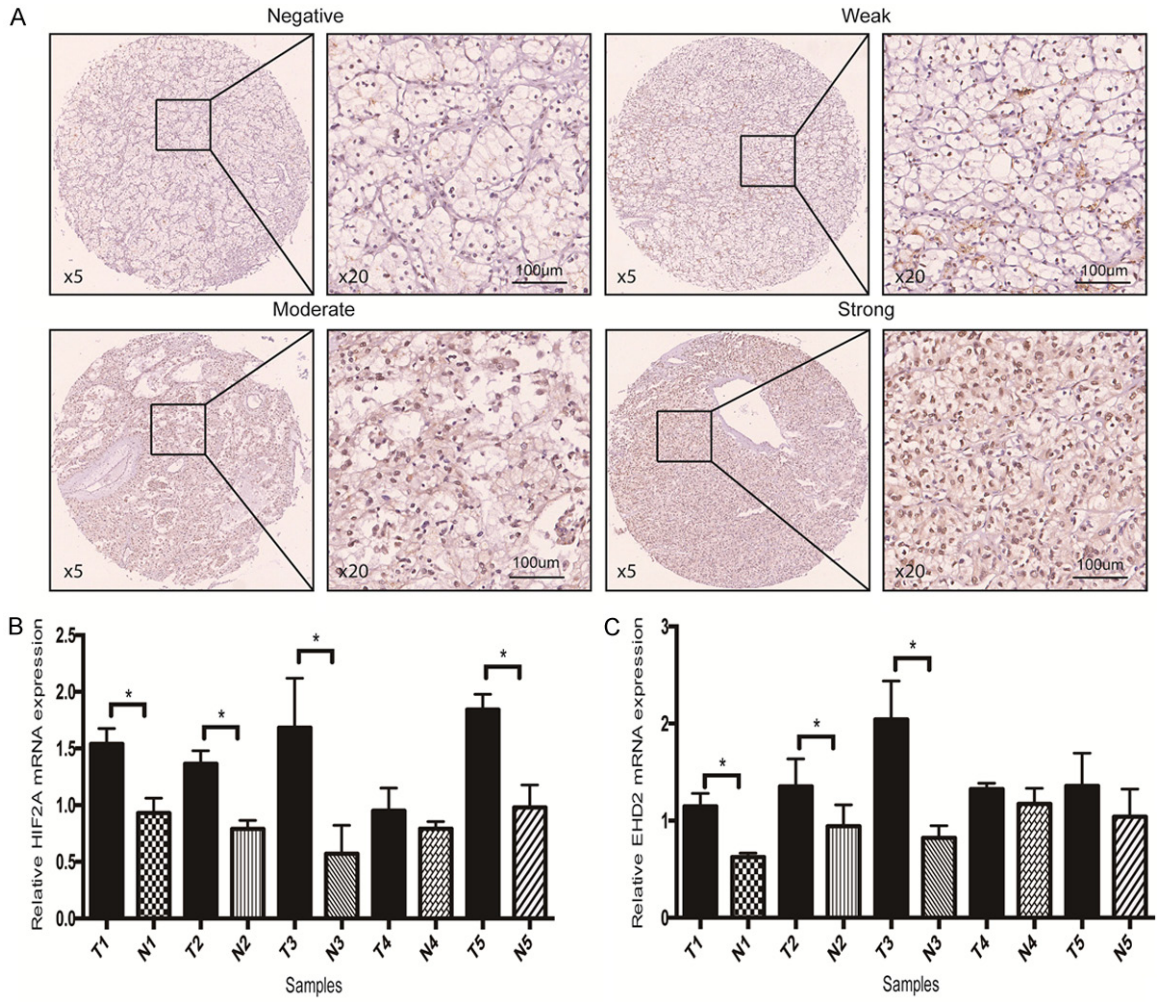

Figure 2. Expression level of EHD2 in ccRCC samples. (A) Representative images of EHD2 in ccRCC samples according to immunohistochemical analysis. (B) HIF2A and (C) EHD2 expression in fresh ccRCC tissues and normal tissues. T, tumor; N, normal. *P<0.05. EHD2, EH domain-containing protein 2; ccRCC, clear cell renal cell carcinoma; HIF, hypoxia inducible factor.

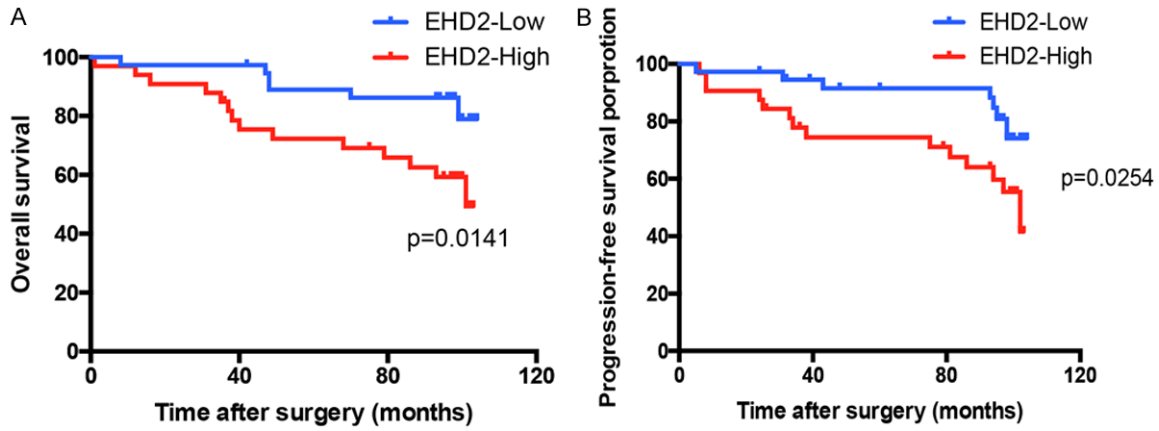

Figure 3. Kaplan-Meier survival analysis of patients with ccRCC based on EHD2 expression. A. Overall survival rate of patients with ccRCC with different EHD2 expression. B. Disease-free survival rate of patients with ccRCC with different EHD2 expression. EHD2, EH domain-containing protein 2; EHD2, EH domain-containing protein 2; ccRCC, clear cell renal cell carcinoma.

Table 2. Univariate and multivariate Cox regression analysis of OS duration in ccRCC

Variable	Univariate analysis			Multivariate analysis		
	HR	(95% CI)	p	HR	(95% CI)	p
EHD2 expression (Low vs High)	2.634	1.627-3.827	0.016*	2.173	1.253-2.839	0.042*
Age (<55 vs >55)	1.429	0.913-1.728	0.046*	1.302	0.795-1.515	0.372
TNM stage (I+II vs III+IV)	6.953	3.635-9.184	<0.001*	3.874	2.192-7.844	0.029*
Fuhrman grade (I+II vs III+IV)	5.992	2.781-8.192	<0.001*	4.878	1.192-6.919	0.059
Tumor size (<4 vs >4)	3.129	1.109-7.928	0.019*	2.693	1.343-6.918	0.039*

HR, hazard ratio; 95% CI, 95% confidence interval. *p value <0.05.

Table 3. Univariate and multivariate Cox regression analysis of DFS duration in ccRCC

Variable	Univariate analysis			Multivariate analysis		
	HR	(95% CI)	p	HR	(95% CI)	p
EHD2 expression (Low vs High)	1.445	1.092-3.536	0.029*	1.138	1.003-2.175	0.048*
Age (<55 vs >55)	2.183	1.132-3.933	0.032*	1.827	1.345-3.721	0.284
TNM stage (I+II vs III+IV)	4.162	2.972-6.328	<0.001*	2.926	1.726-5.412	0.039*
Fuhrman grade (I+II vs III+IV)	3.537	2.218-5.753	<0.001*	2.162	1.412-4.559	0.071
Tumor size (<4 vs >4)	2.658	1.883-5.177	0.028*	2.019	1.792-5.118	0.082

HR, hazard ratio; 95% CI, 95% confidence interval. *p value <0.05.

analysis revealed EHD2 expression [hazards ratio (HR), 2.634], as well as TNM stage, Fuhrman grade, and tumor size to all be significantly associated with the OS. In addition, EHD2 expression (HR, 1.145) was significantly associated with DFS. Subsequent multivariate analysis showed that EHD2 expression was an independent predictive factor of OS and DFS. These findings suggest EHD2 to be a novel prognostic factor for predicting ccRCC outcome.

Discussion

The EHDs family of proteins (EHD1-4) was previously reported to be a type of lipid membrane-activated ATPase that regulates inward and outward vesicular trafficking across the plasma membrane [6]. In particular, EHD2 was found to specifically localize to the plasma membrane caveolae, suggesting that EHD2 performs a distinct biologic function compared to its EHD relatives [19]. Specifically, EHD2 was previously found to positively regulate mechano-transduction to serve a role in the development of breast cancer [20]. Recent studies have shown that EHD2 can mediate different roles in various types of malignancies, where both obvious anti-cancer effects and oncogenic functions have been reported. EHD2 has been shown to inhibit cell proliferation in esophageal, colorectal, breast and HCC cancers. By contrast, in ccRCC, the expression of EHD2 was previously found to be elevated in cancer tissues compared to normal tissue. EHD2 knockdown was observed to significantly suppress the proliferation and invasion of ccRCC cells [11, 16]. However, there was an important limitation to this previous finding, which was a lack of prognostic survival analysis of patients with different expression levels of EHD2. Therefore, a comprehensive clinical prognostic analysis was required to verify the clinical value of EHD in patients with ccRCC.

In our current study, we first searched the GEPIA public database. The association analysis revealed that endothelial PAS domain protein 1 (which encodes HIF2A) expression was positively associated with that of the EHD family of proteins. HIF2α has been repeatedly shown to be an important oncogenic gene in ccRCC, while HIF1α appears to be a tumor suppressor as mentioned before. This suggests that EHD2 may be a downstream target of HIF2A. KIRC data analysis found that the EHD2 expression level was significantly higher in tumor samples, but the expression of other EHD proteins revealed no significant difference. We then used a standardized process to produce kidney cancer TMAs containing the 70 cancer samples and adjacent samples for IHC

analysis. Analysis of protein expression in the TMA and of mRNA expression in the ccRCC tissue samples suggests that EHD2 is highly expressed in the ccRCC tumors. Survival rate analysis revealed that patients with higher EHD2 expression had inferior OS and DFS outcomes. In addition, the univariate and multivariate Cox regression analyses confirmed that EHD2 is an independent prognostic factor in patients with ccRCC.

To conclude, results from our study further confirmed the role of EHD2 in ccRCC. By verifying previously reported experimental data, the additional clinical data presented in the present study suggest that EHD2 is a viable target for ccRCC.

Disclosure of conflict of interest

None.

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