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Original article

Implication of VHL, ERK5, and HIF-1alpha in clear cell renal cell carcinoma: Molecular basis[1]

Leticia Serrano-Oviedo, Ph.D.[a], José M. Giménez-Bachs, Ph.D., M.D.[b,*], Syongh Y. Nam-Cha, M.D.[c], Francisco J. Cimas, Ph.D.[a], Jesús García-Cano, Ph.D.[a], Ricardo Sánchez-Prieto, Ph.D.[a], Antonio S. Salinas-Sánchez, Ph.D., M.D.[b]

[a] *Unidad de Medicina Molecular, Laboratorio de Oncología, Centro Regional de Investigaciones Biomédicas, Unidad de Biomedicina CSIC—Universidad de Castilla-La Mancha, Albacete, Spain*

[b] *Servicio de Urología, Complejo Hospitalario Universitario de Albacete, Albacete, Spain*

[c] *Servicio de Anatomía Patológica, Complejo Hospitalario Universitario de Albacete, Albacete, Spain*

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Abstract

Objectives: To determine the expression status of several proteins related to *VHL* gene function and its relationship with common clinicopathological parameters.

Material and methods: Observational, analytical, cross-sectional study with 50 patients diagnosed with clear cell renal cell carcinoma. The study analyzed *VHL* mutations and hypermethylation as well as protein expression of VHL, CA-IX, HIF-1alpha, VEGF, ERK1/2, and ERK5, relating them to clinical variables.

A bivariate and multivariate descriptive logistical regression analysis was performed, using the presence of metastasis at diagnosis as dependent variable.

Results: The study identified 13 (26%) *VHL* mutations related to nuclear grade ($P = 0.036$). *VHL* hypermethylation was found in 20% of cases. VHL expression was associated with the presence of mutations ($P = 0.013$), and the absence of expression was associated with nuclear grade and the presence of metastasis ($P < 0.05$). HIF-1alpha was negative in only 5 cases. Vascular endothelial growth factor (VEGF) was positive in 31 of 47 cases and was associated with Fuhrman nuclear grade, presence of metastasis, and stage ($P < 0.05$). ERK5 expression was increased in 58% of cases and associated with the presence of metastasis and more advanced stages ($P < 0.05$). In the logistic regression analysis, the only variable remaining in the model was VEGF expression ($P = 0.014$).

Conclusions: VEGF has prognostic value in clear cell renal cell carcinoma, and ERK5 may be a new prognostic marker in this type of tumor owing to its relationship with metastasis and more advanced stages.

Keywords: Clear cell renal cell carcinoma; VHL; Signaling pathway; Biomarkers

1. Introduction

Renal tumors account for 3% of all neoplasms, and renal cell carcinoma is the most common malignant tumor in adult kidney. Various molecular abnormalities characterize the different subtypes of this neoplasm, making them different from each other and giving them various properties which can affect therapeutic response or prognosis [1].

VHL gene abnormalities are a known factor in clear cell renal cell carcinoma (ccRCC). Both the gene and its protein have tumor suppressor function, but this is based on a series of molecular mechanisms involving other proteins, which will initiate their activity depending on the amount of oxygen in the environment and on VHL functionality itself [2].

Therefore, certain molecular and environmental states will activate several proteins, such as carbonic anhydrase-IX

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[*] Corresponding author. Tel.: +34-967-597-235; fax: +34-967-243-952.
E-mail address: gbjosem@sescam.jccm.es (J.M. Giménez-Bachs).

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(CA-IX), which regulates cell proliferation in response to hypoxia. Another is hypoxia-inducible factor 1-alpha (HIF-1alpha), which mediates metabolic adaptation according to available oxygen and can transactivate other genes with protein products that increase oxygen uptake. Yet another protein is vascular endothelial growth factor (VEGF), related to neoangiogenesis. Likewise, the mitogen-activated protein kinase (MAPK) family may also play a key role in the molecular cascade activated when clear cell renal tumor genesis begins, in many cases fostered by VHL malfunction [3].

The aim of this study was to characterize the molecular state of a ccRCC series to better understand the behavior of this type of tumor and to determine if molecular abnormalities more clearly explain patient prognosis or if certain characteristics will aid the molecular diagnosis or therapeutic approaches taken.

2. Subjects, material, and method

The study was based on an observational, analytical, and cross-sectional design with 50 patients diagnosed with ccRCC between 2006 and 2011 and treated surgically.

Tumor tissue specimens and healthy renal tissue (control) were collected and stored at −80°C, and the following techniques were used for molecular analysis of the samples:

(1) *VHL gene mutational and methylation analysis*: Genomic DNA was extracted using the QIAamp DNA kit (QIAGEN). The polymerase chain reaction primers and conditions for VHL gene sequencing were those used by Patard et al. [4]. Mutations were determined via automatic DNA sequencing by capillary electrophoresis in both directions of the gene, such that only abnormalities detected in both sequencing directions in the electrophoretogram were considered mutations. The control group consisted of polymerase chain reaction products obtained from DNA taken from healthy renal parenchyma.

Sodium bisulphite conversion with 1 μg of genomic DNA was performed using an EZ-96 DNA Methylation kit (Zymo Research) according to the manufacturer's protocol and the alternative conversion protocol (a 2-temperature DNA denaturation) designed by Sequenom. Sequenom's MassARRAY platform was used to perform quantitative methylation analysis. EpiTYPER methylation was performed by the Medical Research Department of the Central Research Unit (ICU) at the University of Valencia. Tumor specimens with at least 10 of 41 methylated CpGs (26%) were considered as hypermethylated [5]. The Caki-2 and 769-P cell lines were used as positive and negative controls with nonmethylated and methylated *VHL* promoter, respectively [6].

(2) *Western blot*: Samples were processed and quantified as previously described [7]. Then 100 μg (extracellular signal-regulated kinase 5 [ERK5]) or 50 μg (CA-IX and ERK1/2) were loaded onto 6% or 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride filters and blotted against different proteins using specific antibodies. Antibody detection was achieved by enhanced chemiluminescence (ECL, Amersham). Antibodies used were CA-IX (CA-IX Antibody H-120; 1:1000 in 0.5% bovine serum albumin [BSA] in Tris-buffered saline [T-TBS], Santa Cruz Biotechnology), ERK5 (1:1000 in 5% BSA in T-TBS, Cell Signaling); ERK1/2 (1:1000 in 5% BSA in T-TBS, Santa Cruz Biotechnology) as loading control membranes were reproved against Tubulin (1:3000 in 5% milk in T-TBS, Santa Cruz Biotechnology).

(3) *Immunohistochemistry*: This technique was used to determine protein expression in the case of CA-IX, VHL, HIF-1alpha, and VEGF. All cases were reviewed and diagnosed according to the criteria of the World Health Organization classification [8] in the Pathology Department of our center. Paraffin blocks were available in all cases. Formalin-fixed paraffin-embedded tissue sections of 4 μm were cut, deparaffinized in xylene and rehydrated in a graded series of ethanol. Endogenous peroxidase was blocked with 3% H_2O_2 for 5 minutes. Slides were treated with heat-induced epitope retrieval and immunostained with monoclonal rabbit antibody CA-IX in 1:1500 dilution (Abcam); VHL in 1:200 dilution (BD Pharmingen); HIF-1alpha in 1:200 dilution (Novus); and VEGF in 1:400 dilution (Dako) and detected using the EnVision system-HRP (Dako) according to the manufacturer's instructions.

Immunohistochemical staining was done by a single pathologist blinded to the clinicopathologic variables. Tumor cells were scored as strong (+3), moderate (+2), weak (+1) (all considered to be positive expression in further analyses), and negative CA-IX expression.

The statistical analysis evaluated the presence of *VHL* gene mutations and the site and type of mutation. Additionally, the presence of *VHL* gene hypermethylation and the presence of expression (according to criteria described earlier) were analyzed by western blot or immunohistochemistry of the VHL, CA-IX, HIF-1alpha, VEGF, ERK5, and ERK1/2 proteins. Patient data were collected for sociodemographic and clinical variables, as well as Fuhrman nuclear grade and tumor stage according to the TNM classification [8]. A descriptive analysis and a bivariate analysis were performed by independence tests (Pearson χ_i^2, linear trend, Student *t*-test, and analysis of variance) applying their respective nonparametric tests in the cases indicated. A multivariate logistic regression analysis was performed using the presence of metastasis at the time of diagnosis as a dependent variable and the other independent variables as covariables, estimating the respective regression coefficients. Because this was a cross-sectional study, the ORs obtained are understood to be of prevalence.

Table 1
Clinicopathological characteristics of the patients

	N	%
Sex		
Male	34	68
Female	16	32
Clinical symptoms		
Incidental	19	38
Nonincidental	31	62
Fuhrman nuclear grade		
1	4	8
2	21	42
3	14	28
4	11	22
T stage		
pT1a	9	18
pT1b	13	26
pT2	13	26
pT3a	8	16
pT3b	7	14
M stage		
M1	19	38
Mx or M0	31	62
TNM		
I	19	38
II	10	20
III	2	4
IV	19	38

3. Results

Mean patient age was 62.2 years (standard deviation = 11.9), and 68% were men, giving a male-to-female ratio of 2:1. There were no significant age- or sex-related differences.

Table 2
Relationship between mutations and clinical characteristics of the patients

Site	Codon	Type	Fuhrman nuclear grade	pT	pM	TNM
Exon 2	136	Deletion (−1) (−A)	3	pT1b	pMx	I
Exon 1	88	Point (C ⇒ T)	1	pT1b	pMx	I
Exon 2	137	Insertion (+1) (+T)	2	pT2	pM0	II
Exon 1	112	Insertion (+1) (+A)	2	pT3a	pM1	IV
Intronic		Point (C ⇒ A)	4	pT3b	pM1	IV
Exon 2	128	Insertion (+1) (+C)	2	pT1b	pM0	I
Exon 3	175	Insertion (+1) (+T)	1	pT1a	pM0	I
Exon 1	78	Point (G ⇒ T)	2	pT1b	pM0	I
Exon 1	54	Deletion (−11)	4	pT1b	pM1	IV
Exon 1	62	Deletion (−2) (−TG)	1	pT3a	pMx	III
Exon 3	176	Deletion (−3) (−AGG)	2	pT3a	pMx	III
Exon 1	107	Deletion (−1) (del −C)	2	pT1a	pM0	I
Exon 1	88	Point (A ⇒ G)	2	pT2	pM1	IV

Table 3
Correlation between *VHL* gene mutation and protein expression by immunohistochemistry

VHL mutation	VHL expression by immunohistochemistry			*P*
	None to low	Moderate to high	Total	
No, *N*	17	20	37	0.013
%	94.4	62.5	74	
Yes, *N*	1	12	13	
%	5.6	37.5	26	
Total, *N*	18	32	50	
%	100	100	100	

In the histological study, the most common Fuhrman nuclear grade was grade 2 in 42% of cases, followed by grade 3 in 28%, and grade 4 in 22%. TNM stage was I in 38% of cases and IV in another 38% of cases. The clinicopathological characteristics are listed in Table 1.

All tumors were positive for the CA-IX protein by immunohistochemistry, but were negative in 2 cases by western blot.

VHL gene sequencing found 13 mutations in 13 (26%) patients. No statistically significant relationship was found between the presence of mutations and the usual clinicopathological parameters (sex, age, and stage) except for Fuhrman nuclear grade, as mutations were mainly detected in grade 2 tumors ($P = 0.036$). The remaining characteristics of the mutations are listed in Table 2, which shows that most were detected at exon 1 and only 1 in intron position.

The *VHL* gene methylation study revealed hypermethylation in 10 (20%) cases, but the determination was impossible in 4 (8%) patients. An association was only found between promoter methylation state and clinical picture, with this association being more common in patients who showed clinical symptoms at the time of diagnosis than in those diagnosed incidentally ($P = 0.008$). Although not statistically correlated, patients with distant metastases were more likely to have hypermethylation (20% vs. 8%).

When VHL protein expression was measured by immunohistochemistry, 32 (64%) cases showed moderate-to-high expression, which was associated with the presence of mutations, as shown in Table 3 ($P = 0.013$). VHL nonexpression correlated with higher Fuhrman grades ($P = 0.001$) and the presence of distant metastasis ($P < 0.05$). There was no significant relationship between clinicopathological factors (nuclear grade, pT, pN, pM, or TNM) and VHL expression in tumors with no mutation.

The correlations between *VHL* gene mutations, *VHL* gene hypermethylation, and VHL protein expression and the pathological factors are summarized in Table 4.

HIF-1alpha expression by immunohistochemistry was seen in most samples, although at differing degrees of intensity (Figure 1). Only 5 cases showed no expression of

Table 4
VHL status (gene and protein) and correlation with clinical parameters

	VHL gene mutation			*VHL* gene hypermethylation			VHL protein expression		
	No, *N* (%)	Yes, *N* (%)	*P*	No, *N* (%)	Yes, *N* (%)	*P*	None to low, *N* (%)	Moderate to high, *N* (%)	*P*
Fuhrman nuclear grade									
1	1 (25)	3 (75)	0.036	4(100)	0 (0)	ns	0 (0)	4 (100)	0.023
2	14 (66.7)	7 (33.3)		19 (95)	1(5)		6 (28.6)	15 (71.4)	
3	13 (92.9)	1 (7.1)		9 (64.3)	5 (35.7)		4 (8.6)	10 (71.4)	
4	9 (81.8)	2 (18.2)		8 (62.5)	3 (37.5)		8 (72.7)	3 (27.3)	
pT stage									
pT1a	7 (77.8)	2 (22.2)	ns	9 (88.9)	1 (11.1)	ns	2 (22.2)	7 (77.8)	ns
pT1b	8 (61.5)	5 (38.5)		11 (81.8)	2 (18.2)		5 (38.5)	8 (61.5)	
pT2	11 (84.6)	2 (15.4)		12 (92.3)	1 (7.7)		4 (30.8)	9 (69.2)	
pT3a	5 (62.5)	3 (37.5)		5 (71.4)	2 (28.6)		2 (25)	6 (75)	
pT3b	6 (85.7)	1 (14.3)		3 (50)	3(50)		5 (71.4)	2 (28.6)	
pM stage									
pMx	10 (71.4)	4 (28.6)	ns	13 (100)	0 (0)	ns	4 (28.6)	10 (71.4)	ns
pM0	12 (70.6)	5 (29.4)		13 (81.3)	3 (18.7)		5 (29.4)	12 (70.6)	
pM1	15 (78.9)	4 (21.1)		11 (64.7)	6 (35.3)		9 (47.4)	10 (52.6)	
TNM stage									
I	13 (68.4)	6 (31.6)	ns	15 (88.2)	2 (11.8)	ns	5 (26.3)	14(73.7)	ns
II	9 (90)	1 (10)		9 (90)	1(10)		4 (40)	6 (60)	
III	0 (0)	2 (100)		2 (100)	0 (0)		0 (0)	2 (100)	
IV	15 (78.9)	4 (21.1)		11 (64.7)	6 (35.3)		9 (47.4)	10 (52.6)	

this protein. The relation with clinicopathological factors are shown in Table 5.

VEGF expression was positive in 31 of 47 cases analyzed for this protein. No correlation was found between the expression of this protein and clinical parameters, although there was a correlation with pathological parameters, such as Fuhrman nuclear grade, presence of metastasis, and TNM stage (Table 6) ($p < 0.05$). However,

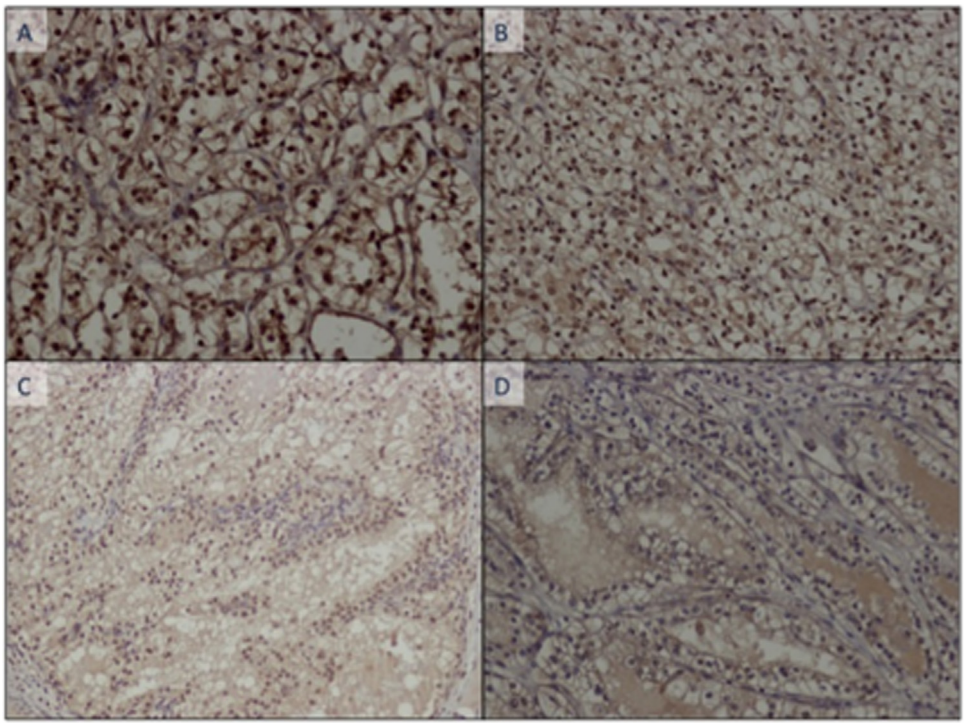

Fig. 1. HIF-1alpha expression by immunohistochemistry: (A) severe (+3); (B) moderate (+2), (C) mild (+1); and (D) negative. (Color version of figure is available online.)

Table 5
HIF-1alpha and clinicopathological factors

	HIF-1alpha by inmunohistochemistry										*P*
	Negative		<10% cells		10%–50% cells		>50% cells		Total		
	Frec.	%	Frec.	%	Frec.	%	Frec.	%	Frec.	%	
Fuhrman nuclear grade											
Grades 1 and 2	0	0	3	33.3	4	50	18	72	25	53.2	0.014
Grades 3 and 4	5	100	6	66.7	4	50	7	28	22	46.8	
Total	5	100	9	100	8	100	25	100	47	100	
pT stage											
pT1a	0	0	1	11.1	2	25	6	24	9	19.1	ns
pT1b	1	20	2	22.2	1	12.5	8	32	12	25.5	
pT2	3	60	3	33.3	1	12.5	6	24	13	27.7	
pT3a	0	0	1	11.1	2	25	4	16	7	14.9	
pT3b	1	20	2	22.2	2	25	1	4	6	12.8	
Total	5	100	9	100	8	100	25	100	47	100	
pN stage											
pNx	4	80	7	77.8	8	100	22	88	41	87.2	ns
pN0	1	20	2	22.2	0	0	3	12	6	12.8	
Total	5	100	9	100	8	100	25	100	47	100	
pM stage											
pMx	1	20	1	11.1	2	25	9	36	13	27.7	ns
pM0	1	20	3	33.3	2	25	11	44	17	36.2	
pM1	3	60	5	55.6	4	50	5	20	17	36.2	
Total	5	100	9	100	8	100	25	100	47	100	
TNM stage											
I	0	0	2	22.2	3	37.5	13	52	18	38.3	ns
II	2	40	2	22.2	1	12.5	5	20	10	21.3	
III	0	0	0	0	0	0	2	8	2	4.3	
IV	3	60	5	55.6	4	50	5	20	17	36.2	
Total	5	100	9	100	8	100	25	100	47	100	

there was no correlation between VEGF expression and *VHL* abnormality because of either methylation or the presence of mutations.

ERK5 expression was evaluated by western blot (Figure 2) and compared with the control sample from healthy parenchyma, with increased expression found in 58% of cases, whereas the rest showed equal or lower expression than the respective control. The presence of distant metastases and more advanced stage was significantly related to ERK5 overexpression ($P < 0.05$) but not with other clinicopathological parameters analyzed (Table 7). All tumors not expressing VHL by immunohistochemistry did overexpress ERK5 ($P = 0.009$), whereas promoter methylation abnormalities or the presence of VHL gene mutations were not related to ERK5 expression. ERK5 expression was stronger in 21 of 31 cases that overexpressed VEGF ($P = 0.017$).

There were no differences in ERK1/2 expression between tumor samples and healthy control 47 of cases presented similar expression and only 3 overexpressed this protein.

When logistic regression analysis was used to evaluate the effect of various independent variables on the presence of distant metastases at the time of diagnosis, the only variable remaining in the model was VEGF expression. Consequently, the presence of metastasis in patients with ccRCC was 8.27-fold (CI: 1.53–44.62) in patients who overexpress VEGF than in those with no overexpression ($P = 0.014$). The power of explanation of the model is 23.9% (Nagelkerke's $R^2 = 0.239$).

4. Discussion

The aim of this study was to establish the molecular state of the *VHL*-mediated pathway in a series of clear cell renal tumors. To do so, the status of various proteins involved in the molecular pathway of the *VHL* gene and protein was determined by western blot or immunohistochemistry.

CA-IX protein expression in this type of tumor appears to be constant and, therefore, can be considered a characteristic and exclusive factor of the clear cell subtype. Although it can be used as a diagnostic marker, its use as a prognostic factor is not clear and the issue is still under debate; nevertheless,

Table 6
Correlation between VEGF expression status and clinical parameters

	VEGF by immunohistochemistry						*P*
	Negative		Positive		Total		
	N	%	*N*	%	*N*	%	
Fuhrman nuclear grade							
Grades 1 and 2	12	48	13	52	25	100	0.031
Grades 3 and 4	4	18.2	18	81.8	22	100	
Total	16	34	31	66	47	100	
pT stage							
pT1a	3	33.3	6	66.7	9	100	ns
pT1b	5	41.7	7	58.3	12	100	
pT2	6	46.2	7	53.8	13	100	
pT3a	2	28.6	5	71.4	7	100	
pT3b	0	0	6	100	6	100	
Total	16	34	31	66	47	100	
pN stage							
pNx	14	34.1	27	65.9	41	100	ns
pN0	2	33.3	4	66.7	6	100	
Total	16	34	31	66	47	100	
pM stage							
pMx	5	38.5	8	61.5	13	100	0.037
pM0	9	52.9	8	47.1	17	100	
pM1	2	11.8	15	88.2	17	100	
Total	16	34	31	66	47	100	
TNM stage							
I	8	44.4	10	55.6	18	100	0.034
II	4	40	6	60	10	100	
III	2	100	0	0	2	100	
IV	2	11.8	15	88.2	17	100	
Total	16	34	31	66	47	100	

Table 7
Correlation between ERK5 expression and clinicopathological factors

	ERK5 expression (Western blot)						*P*
	Overexpression		No expression		Total		
	Frec.	%	Frec.	%	Frec.	%	
Fuhrman nuclear grade							
Grades 1 and 2	12	48	13	52	25	100	ns
Grades 3 and 4	17	68	8	32	25	100	
Total	29	58	21	42	50	100	
pT stage							
pT1a	5	55.6	4	44.4	9	100	ns
pT1b	6	46.2	7	53.8	13	100	
pT2	7	53.8	6	46.2	13	100	
pT3a	5	62.5	3	37.5	8	100	
pT3b	6	85.7	1	14.3	7	100	
Total	29	58	21	42	50	100	
pN stage							
pNx	25	56.8	19	43.2	44	100	n.s.
pN0	4	66.7	2	33.3	6	100	
Total	29	58	21	42	50	100	
pM stage							
pMx	8	57.1	6	42.9	14	100	0.03
pM0	6	35.3	11	64.7	17	100	
pM1	15	78.9	4	21.1	19	100	
Total	29	58	21	42	50	100	
TNM stage							
I	8	42.1	11	57.9	19	100	0.043
II	6	60	4	40	10	100	
III	0	0	2	100	2	100	
IV	15	78.9	4	21.1	19	100	
Total	29	58	21	42	50	100	

quantitation of expression is what would correlate with the various clinicopathological parameters [9–11].

The *VHL* gene is known to be altered in ccRCC, but there are differences according to the study. Gene mutation levels range from 22% to 57% according to the study [12–14], and promoter hypermethylation is around 20% [15,16]. Our study analyzed abnormalities (mutation and promoter hypermethylation), such that an abnormality in each or both can lead to inactivation of the gene and its tumor suppressor activity. However, the fact that these parameters are not related to those commonly used in clinical practice may mean that the *VHL* gene is altered in early stages of tumor development and is not related to tumor progression [12].

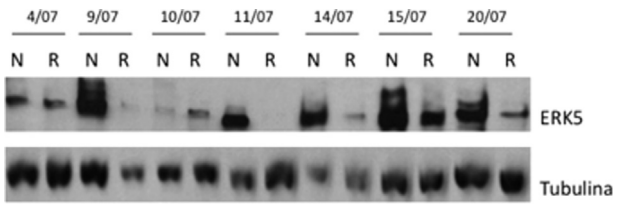

Fig. 2. ERK5 protein expression by western blot in a series of ccRCC tumors. N = tumor specimen; R = healthy renal parenchyma.

Immunohistochemistry to investigate VHL protein expression found that the presence of mutations led to greater protein expression. This could be explained by abnormal recognition of the antibody that generates positive expression (but not necessarily functionality) of the protein or by a genetic or epigenetic abnormality that affects the stability of the protein, as described in other proteins that may have increased half-life and nuclear build-up [17,18]. The fact that the absence of VHL protein expression is related to higher Fuhrman grades implies a poorer prognosis for tumors not expressing the protein, an idea reinforced by a correlation with the presence of distant metastasis, probably because of loss of the tumor suppressor function of the VHL protein [19].

If VHL protein abnormalities lead to little or no HIF-1alpha degradation, the presence of high HIF-1alpha expression will clearly be common in ccRCC, as occurs in our series. This finding is usually present in ccRCC and makes it difficult to correlate increased expression of this protein with the clinical parameters because most tumors overexpress it. Nevertheless, some studies have found

greater expression of the protein in tumors with distant metastasis [20].

One of the proteins activated by increased expression of HIF-1alpha is VEGF, a regulator, among other things, of angiogenesis. Several studies have found that overexpression of this protein is an adverse prognostic factor that correlates to the presence of distant metastases or more advanced stages [21]. In fact, this was the only variable remaining in the regression model in our study and, therefore, the protein (end of the VHL-HIF-1alpha pathway) could be a prognostic biomarker for ccRCC.

MAPK signaling pathways are one the critical mediators of cell growth and have been related to hypoxia [22]. Indeed, it has been reported a direct control of HIF-1alpha by several MAPK [23] and in addition ERK5 has been proposed as a putative target of the *VHL* signaling axis [7] known to share biological targets with HIF-1alpha [24]. Regarding ERK5 our data show prominent up-regulation in tumor samples vs. normal tissue in a high percentage of ccRCC samples, in agreement with a putative target for VHL deregulation. However, it is noteworthy that neither mutational nor methylation studies have shown a correlation with ERK5 expression. In this regard, several possibilities can be considered, including other mechanisms to explain high expression levels of ERK5, for instance deregulated tyr-kinase activity [25]. However, a relationship has been found between the ERK5 signaling pathway and the prognosis of CCRCC [7], as well as other types of tumors (e.g., colon, breast, or prostate) [26–28], such that increased expression of this protein is associated with poorer prognosis (more advanced stages and presence of metastasis), as reported in the current study. Consequently, we can consider that ERK5 expression may be useful as a prognostic and perhaps therapeutic marker.

This was not the case of ERK1/2, in spite of the high homolog in the n-terminal region, where no differences were found in expression between tumor and healthy parenchyma. Further studies, including a study of these proteins, are necessary to fully elucidate the role of this MAPK cascade.

In summary, our analysis of a series of ccRCC tumors appears to indicate that CA-IX expression is always present in this cell subtype, that genetic and epigenetic abnormalities do not consistently alter the pattern of protein expression measured by immunohistochemistry and that alterations to other proteins (such as HIF-1alpha, VEGF, and ERK5) appear predominantly, suggesting that they are implicated in the molecular pathway of the *VHL* gene, even though the VEGF protein appears to be the one most strongly related to prognosis. ERK5 is postulated as a possible future marker to assess the behavior of this type of tumor. However, these conclusions should be confirmed by larger studies that could reveal a pattern of molecular behavior potentially of use in clinical medicine.

In conclusion, the molecular characterization of tumors offers considerable possibilities to study tumor genesis and development as well as the potential response to certain therapeutic agents.

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