Hypoxia-Inducible Factor 1α in Clear Cell Renal Cell Carcinoma

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Abstract

Purpose: Hypoxia-inducible factor-1α (HIF-1α) plays an important role in tumoral adaptation to hypoxic conditions by serving as a transcription factor for several crucial proteins, including vascular endothelial growth factor and carbonic anhydrase IX (CAIX). Here, we evaluated the significance of HIF-1α in renal cell carcinoma (RCC).

Experimental Design: Immunohistochemical analysis was done on a tissue microarray constructed from paraffin-embedded primary tumor specimens from 357 patients treated by nephrectomy for RCC. Nuclear expression was evaluated by a single pathologist who was blinded to outcome. The expression levels were associated with pathologic variables and survival.

Results: HIF-1α expression was greater in RCC than in benign tissue. Clear cell RCC showed the highest expression levels. In clear cell RCC, HIF-1α was significantly correlated with markers of apoptosis (p21, p53), the mammalian target of rapamycin pathway (pAkt, p27), CXCR3, and proteins of the vascular endothelial growth factor family. HIF-1α was correlated with CAIX and CAXII in localized, but not in metastatic RCC. HIF-1α expression predicted outcome in metastatic patients: patients with high HIF-1α expression (>35%) had significantly worse survival than patients with low expression (≤35%); median survival, 13.5 versus 24.4 months, respectively ($P = 0.005$). Multivariate analysis retained HIF-1α and CAIX expression as the strongest independent prognostic factors for patients with metastatic clear cell RCC.

Conclusions: HIF-1α is an important independent prognostic factor for patients with metastatic clear cell RCC. Because HIF-1α and CAIX are independently and differentially regulated in metastatic clear cell RCC, both tumor markers can be complementary in predicting prognosis.

Renal cell carcinoma (RCC) represents the most lethal urologic malignancy, accounting for >200,000 new cases and >100,000 deaths worldwide annually (1). The incidence of RCC has been steadily increasing at a rate of 2% to 3% per year across the past several decades (2, 3).

Recent achievements in the basic sciences have led to an increased understanding of the molecular pathways underlying the various RCC subtypes. In 1995, Wang et al. (4) isolated hypoxia-inducible factor 1α (HIF-1α), which plays a central role in RCC tumorigenesis by acting as a transcription factor for several proteins that are important in tumoral adaptation to a tissue microenvironment that is low in oxygen (5). In a physiologic microenvironment, HIF-1α is rapidly degraded through the ubiquitin-proteasome pathway (6), but under hypoxic conditions, HIF-1α is stabilized and accumulates (7) due to the inactivation or absence of the von Hippel-Lindau tumor suppressor gene (*VHL*; refs. 8, 9) and via activation of other independent mechanisms such as the mammalian target of rapamycin pathway (10). HIF-1α regulates angiogenesis, tumor growth, progression, metastatic spread, and glucose metabolism by acting as a transcription factor for crucial proteins such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor receptor (EGFR), insulin-like growth factor (IGF), glucose transporters (GLUT-1), chemokine receptors (CXCR), and carbonic anhydrase IX (CAIX) and XII (CAXII; ref. 5). In addition, HIF-1α plays an important role in regulating the cell cycle and apoptosis (11, 12). Despite its unquestioned role as a central regulator of tumoral pathophysiology, very little has been attempted in exploring HIF-1α's prognostic role in RCC. In a recent Western blot–driven study among 66 patients with clear cell RCC, high HIF-1α expression was paradoxically found to be a favorable prognostic factor (13). However, in a separate study, the same group evaluated 176 tumor specimens using immunohistochemistry, and found that the survival difference between low and high HIF-1α expressors did not reach statistical significance (14). The prognostic role of HIF-1α in clear cell RCC therefore remains unclear. Moreover, a comprehensive correlative expression analysis of HIF-1α and its upstream and downstream targets has thus far not been done. In this report, we present an immunohistochemical study of HIF-1α, correlate expression levels to upstream and downstream proteins, and evaluate the prognostic role of HIF-1α in a large cohort with RCC.

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Patients and Methods

Patients. Our study cohort consisted of 357 patients who underwent nephrectomy for sporadic RCC at the University of California, Los Angeles between 1989 and 2000. Following approval by the UCLA Institutional Review Board, a retrospective study was done with outcome assessment based on chart review of clinical and pathologic data. Clinical data included age, gender, and Eastern Cooperative Oncology Group performance status (ECOG PS; ref. 15). Pathologic data included tumor-node-metastasis stage (16), histologic subtype (17), and Fuhrman grade (18). Localized RCC was defined as N_0M_0 RCC, whereas metastatic RCC was defined if regional lymph node metastasis and/or distant metastasis were present.

Tissue microarray construction. The respective formalin-fixed paraffin-embedded primary tumor specimens were obtained from the Department of Pathology, UCLA Medical Center. Three core tissue biopsies, 0.6 mm in diameter, were taken from selected morphologically representative regions of each paraffin-embedded RCC and precisely arrayed using a custom-built instrument as described previously (19). Additional core tissue biopsies were taken from morphologically benign-appearing surrounding renal parenchyma tissue for each tumor. Sections of the resulting tumor tissue microarray block, 4 μm thick, were transferred to glass slides using the paraffin sectioning aid system (adhesive-coated slides PSA-CS4x, adhesive tape, UV lamp; Instrumedics, Inc.) to support the cohesion of 0.6 mm array elements.

Immunohistochemistry and evaluation of expression. The sections were heated at 56°C for 30 min, deparaffinized with xylene, and rehydrated with a descending series of ethanol. Immunohistochemical staining was done with a Dako Envision staining system (Dako). After endogenous peroxidase blocking with 0.03% H_2O_2 for 5 min, HIF-1α antigens were retrieved by pressure cooking in sodium citrate (770 W for 15 min, then 330 W for 15 min). Blocking of nonspecific protein binding was accomplished by incubation with 0.5% 2-nitro-5-thiobenzoate buffer for 30 min. The sections were then incubated or 60 min at room temperature with an anti–HIF-1α mouse monoclonal antibody (mouse IgG_{2b}, clone H1α67-sup, final concentration, 6 μg/mL; Novus Biologicals). Subsequently, the sections were incubated with an anti–mouse-conjugated peroxidase-labeled secondary antibody (Dako Envision, Dako) for 30 min on room temperature. Afterwards, the sections were incubated with biotinyl tyramide amplification reagent (Perkin-Elmer) at room temperature for 10 min. The sections were visualized with the application of diaminobenzidine substrate chromagen solution and hematoxylin counterstain.

The expression was evaluated by one anatomic pathologist (D.B. Seligson) in a blinded fashion to validate the diagnostic morphology of each array spot. The evaluation of expression involved site and degree of reactivity. Site of reactivity included evaluation of the relevant histologic subtype, as well as the subcellular localization (i.e., nucleus, cytoplasm). Degree of reactivity included evaluation for maximal staining intensity using a 0 to 3 scale (0, negative; 1, weak; 2, moderate; 3, strong), as well as the percentage of positive cells at each stated intensity. The overall score used for subsequent statistical analysis was the pooled mean from the three spots of the same tumor. The HIF-1α assay required tyramide amplification in order to appreciate the protein signals, and negative control IgG_{2b}-stained sections showed occasional, predominantly cytoplasmic staining (diffuse or coarsely granular), particularly in normal renal tubules as well as in stromal cells. These were considered background stainings. Sections stained with anti–HIF-1α also showed similar cytoplasmic background staining. Hence, only clearly specific nuclear localization of the HIF-1α–stained sections was included in the scoring.

Statistical analysis. The Kruskal-Wallis test was used for comparing expressions among pathologic variables. Correlation was assessed with

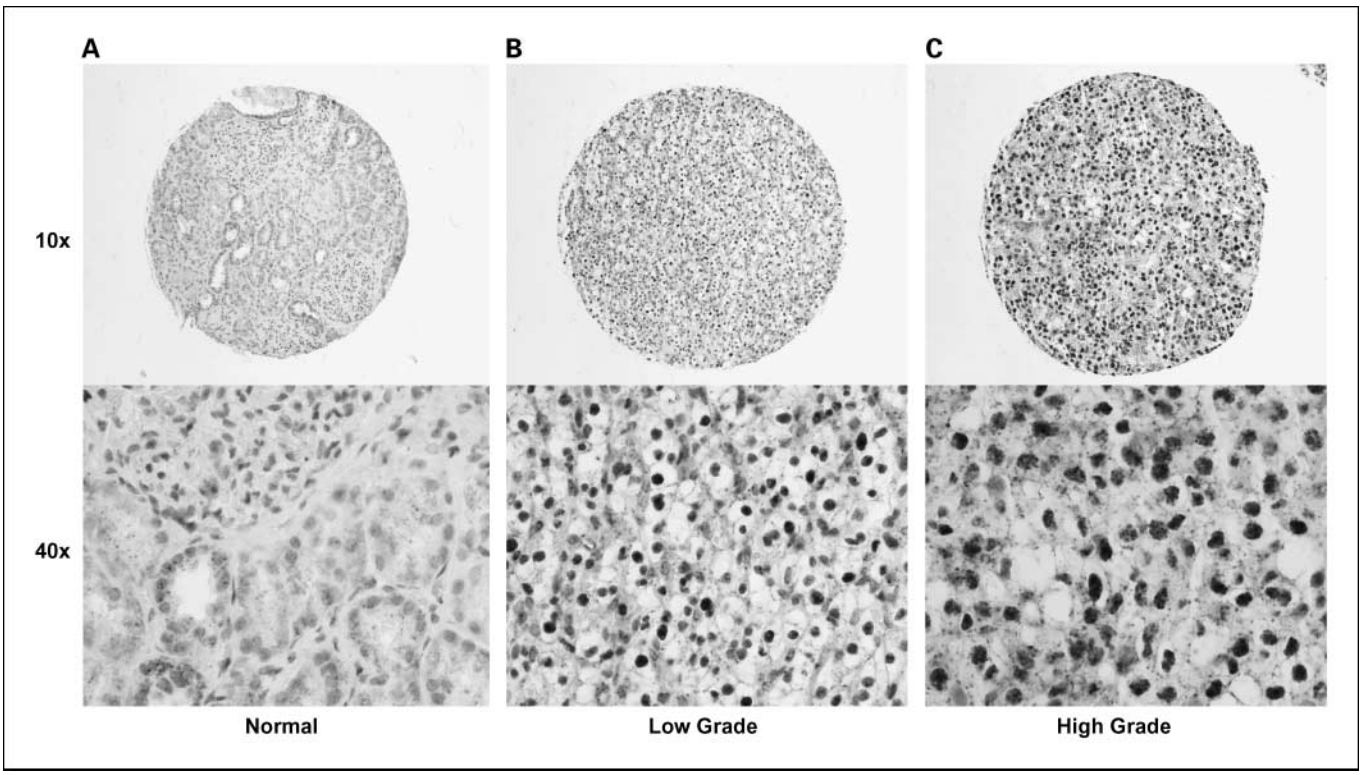

Fig. 1. Expression of HIF-1α by immunohistochemistry in renal tissues (*top,* magnification, ×10; *bottom,* magnification, ×40). *A,* morphologically normal cortical renal tissue demonstrating a lack of nuclear HIF-1α staining. Cytoplasmic background staining from the tyramide amplification system was seen in distal and proximal tubules and was disregarded in the scoring. *B,* low-grade clear cell RCC with well-defined scattered HIF-1α nuclear staining. *C,* high-grade clear cell RCC demonstrating abundant and heavy staining with frequent and strongly positive nuclear expression in tumor cells.

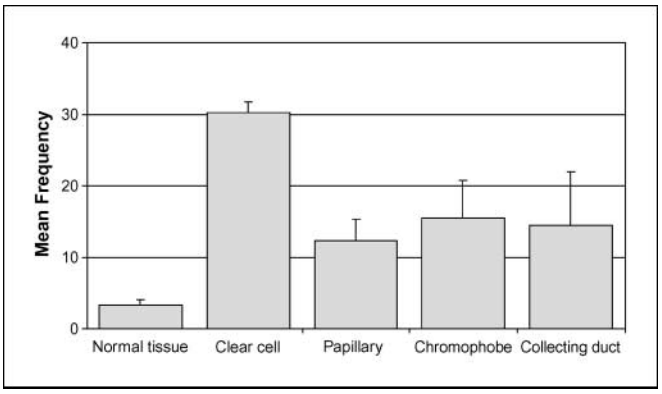

Fig. 2. HIF-1α expression frequency according to histologic subtype. Clear cell (mean expression ± SE, 30 ± 1; n = 308), papillary (12 ± 3, n = 37), chromophobe (15 ± 5, n = 8), and collecting duct RCC (15 ± 8, n = 4) had higher expression than normal renal tissue (3 ± 1).

the Pearson coefficient. The end point of this study was disease-specific survival time. Death from RCC was considered the event, whereas death from other reasons was censored at the date of death. The Kaplan-Meier method was used to generate the survival functions. To allow univariate survival analysis (log-rank test), we dichotomized the patient cohort according to a cutoff which was identified using recursive partitioning–based survival tree analysis. Univariate survival analysis was also done using univariate Cox regression analysis. Independent prognostic variables of survival were identified with a multivariate Cox regression analysis. To avoid overfitting of the regression model by using a dichotomized, categorical expression, we used a continuous marker expression. A significance level of 0.05 was used for all statistical tests. The statistical packages SPSS and R[4] were used for the analyses.

Results

HIF-1α overexpression in clear cell RCC. Normal renal tubules of both morphologically normal tissue and tumor tissue showed only rare scattered nuclear staining of HIF-1α. No staining of HIF-1α was observed in the glomeruli. Scattered positive nuclei, however, were seen in lymphoid clusters and rarely in endothelial cells. Tumors tended to have either predominantly negative or predominantly positive cells throughout each patient sample (Fig. 1).

HIF-1α staining intensities were generally increased in RCC compared with normal tissue (mean intensity, 0.4); clear cell had the highest mean intensity (1.3), followed by chromophobe (0.8), collecting duct (0.7), and papillary RCC (0.6). In addition to intensity, the greatest staining frequencies were also observed in the clear cell subtype (Fig. 2).

Because the highest HIF-1α expression was observed in clear cell RCC, subsequent analyses were only done for 308 patients with the clear cell variant of RCC. The characteristics of these patients are summarized in Table 1. No meaningful differences in HIF-1α expression were noted when the samples from clear cell patients were stratified according to T stage (P = 0.08), N stage (P = 0.46), M stage (P = 0.61), and Fuhrman grade (P = 0.93), but levels inversely correlated with tumor size (R = -0.14, P = 0.01).

HIF-1α expression correlates with upstream and downstream proteins. We have previously reported on the expression levels of p21, p27, p53, CAIX, CAXII, CXCR3, phosphorylated Akt (pAkt), PTEN, VEGF-A, VEGF-C, VEGF-D, VEGFR-1, VEGFR-2, and VEGFR-3 evaluated on the tumors of the same patients. Analysis of the relationship between HIF-1α expression and a panel of relevant kidney cancer tumor markers revealed a significant correlation with CAIX, CAXII, p21, p53, proteins of the mammalian target of rapamycin pathway (p27, pAkt), and proteins of the VEGF family (Table 2). HIF-1α was found to correlate with CAIX and CAXII only in localized (R = 0.23, P = 0.003 and R = 0.24, P = 0.003, respectively), but not in metastatic disease (R = 0.04, P = 0.63 and R = 0.12, P = 0.18, respectively). Conversely, a majority of the other tumor markers tested were significantly correlated in both localized and metastatic disease.

Survival. One hundred and forty-five (47%) of the subjects tested died from RCC after a median duration of 1.4 years (range, 0.1-11.6 years). Seventy-six patients died from other causes. The median follow-up of the patients alive was 6.8 years (range, 0.6-16.9 years).

Univariate Cox regression analysis showed no association between HIF-1α expression and survival in localized disease (P = 0.79), but predicted survival in metastatic RCC with a hazard ratio of 1.008. A 10% increase in HIF-1α expression was associated with an 8% increased risk of death from RCC (P = 0.04). Recursive partitioning-based survival tree analysis identified an ideal cutoff of 35% for further substratification of the metastatic patients. The 52 patients with high HIF-1α

Table 1. Patient and tumor characteristics

	No. (%)
Age (y)	
Median	61 (—)
Range	27-88 (—)
Gender	
Male	207 (67)
Female	101 (33)
ECOG PS	
0	114 (37)
1	181 (59)
2	12 (4)
3	1 (1)
Tumor size (cm)	
Median	6.7 (—)
Range	0.8-18.0 (—)
T stage	
T_1	117 (38)
T_2	36 (12)
T_3	141 (46)
T_4	14 (5)
N stage	
N_0	272 (88)
N_1	17 (6)
N_2	19 (6)
M stage	
M_0	172 (56)
M_1	136 (44)
Localized (N_0M_0)	167 (54)
Metastatic (N + M_0, M_1)	141 (46)
Fuhrman grade	
G_1	41 (13)
G_2	152 (49)
G_3	106 (34)
G_4	9 (3)

[4] http://cran.r-project.org

Table 2. Correlation analyses of HIF-1α with upstream and downstream proteins

Marker	All patients		Localized		Metastatic	
	R	*P*	*R*	*P*	*R*	*P*
p53	0.32	<0.001	0.37	<0.001	0.29	0.001
p21	0.32	<0.001	0.27	0.001	0.40	<0.001
CAIX	0.15	0.011	0.23	0.003	0.04	0.636
CAXII	0.18	0.002	0.24	0.003	0.11	0.179
p27 (cytoplasmic)	-0.05	0.362	-0.04	0.638	-0.06	0.447
p27 (nuclear)	0.50	<0.001	0.48	<0.001	0.53	<0.001
pAkt (cytoplasmic)	0.36	<0.001	0.42	<0.001	0.31	<0.001
pAkt (nuclear)	0.43	<0.001	0.43	<0.001	0.43	<0.001
PTEN	-0.01	0.976	-0.06	0.511	0.06	0.531
VEGF-A (epithelial)	-0.06	0.270	-0.11	0.170	-0.01	0.936
VEGF-A (endothelial)	0.09	0.101	0.07	0.377	0.16	0.066
VEGF-C (epithelial)	-0.21	<0.001	-0.20	0.011	-0.20	0.015
VEGF-C (endothelial)	-0.11	0.046	-0.17	0.034	-0.02	0.789
VEGF-D (epithelial)	0.08	0.161	0.08	0.329	0.09	0.268
VEGF-D (endothelial)	-0.05	0.415	-0.01	0.910	-0.07	0.427
VEGFR-1 (epithelial)	-0.04	0.444	-0.07	0.375	-0.01	0.952
VEGFR-1 (endothelial)	0.12	0.043	0.12	0.126	0.14	0.101
VEGFR-2 (epithelial)	-0.27	<0.001	-0.32	<0.001	-0.21	0.013
VEGFR-2 (endothelial)	0.15	0.011	0.17	0.031	0.13	0.123
VEGFR-3 (epithelial)	0.12	0.038	0.00	0.973	0.27	0.001
VEGFR-3 (endothelial)	0.02	0.762	0.08	0.343	-0.04	0.612
CXCR3	0.16	0.006	0.19	0.019	0.13	0.119

NOTE: The protein expression of the VEGF family is further subdivided into tumor epithelium (epithelial) and tumor-associated endothelium (endothelial).

expression (>35%) had significantly worse survival than the 89 patients with low expression (≤35%); median survival, 13.5 ± 2.6 versus 24.4 ± 5.1 months (P = 0.005; Fig. 3). The 1-, 2-, and 5-year survival rates were 51 ± 7%, 34 ± 7%, and 16 ± 5% for patients with high expression, and 69 ± 5%, 50 ± 5%, and 27 ± 5% for patients with low expression. There was no association between HIF-1α expression and response to immunotherapy (P = 0.19).

A multivariate Cox regression analysis was done to identify independent prognostic factors for the patients with metastatic clear cell RCC. The variables tested included ECOG PS, T stage, concomitant lymph node metastases, Fuhrman grade, number of metastatic sites and HIF-1α expression. To avoid overfitting of the model, HIF-1α expression was entered as a continuous variable. Only ECOG PS (hazard ratio, 1.45; 95% confidence interval, 1.06-2.00; P = 0.02) and HIF-1α expression (hazard ratio, 1.008; 95% confidence interval, 1.001-1.016; P = 0.03) were independent prognostic factors. Because it has been previously shown that CAIX plays a paramount role in predicting prognosis and response to immunotherapy in metastatic clear cell RCC (20, 21), we included continuous CAIX expression in a second Cox regression analysis. In this analysis, ECOG PS, HIF-1α, and CAIX expression were all found to be independent prognostic factors for patients with metastatic clear cell RCC. Both CAIX and HIF-1α were retained the strongest predictors of survival (Table 3). Paradoxically, high CAIX but low HIF-1α correlated with improved survival.

Discussion

The main findings of this study are that in metastatic clear cell RCC, (*a*) nuclear HIF-1α expression in the primary tumor is an independent prognostic factor, (*b*) levels of HIF-1α and CAIX expression are not correlated with each other, implying that each is independently regulated in metastatic disease, and (*c*) both markers used together improve prognostic modeling.

HIF-1α expression is affected by oxygen concentration, *VHL* status, and the activity of interrelated molecular pathways such as the mammalian target of rapamycin pathway (7, 8, 10).

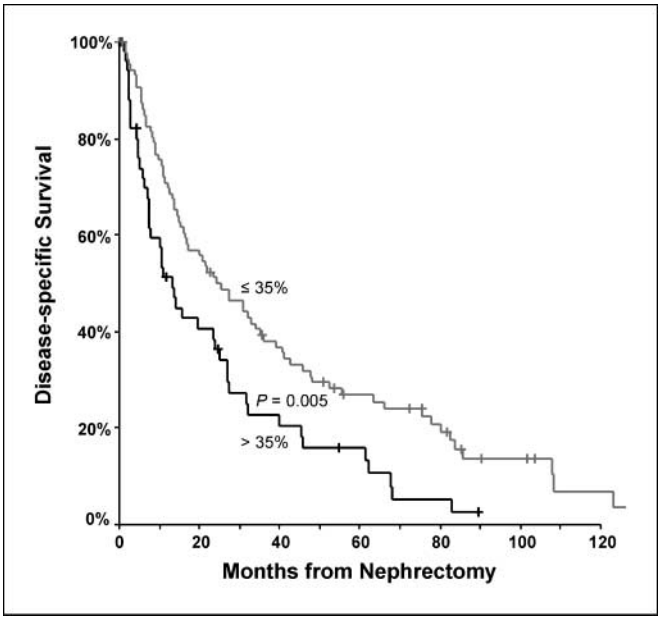

Fig. 3. Kaplan-Meier survival estimates for patients with metastatic clear cell RCC, substratified in high (>35%) and low (≤35%) HIF-1α expression.

Because HIF-1α is genetically linked to *VHL* inactivation (8), which occurs in >50% of sporadic clear cell RCCs, we limited our analysis to patients with this subtype. Dysfunction of the VHL protein (through mutation or hypermethylation of the *VHL* gene) and/or hypoxia lead to reduced degradation of HIF-1α through the 26S proteasome and thereby lead to intracellular stabilization and accumulation of HIF-1α (22, 23). HIF-1α then induces the transcription of several factors including VEGF, PDGF, CXCR, IGF-1, and CAIX (5).

The prognostic role of HIF-1α in RCC was recently evaluated by Lidgren et al. (13) who investigated HIF-1α expression of 66 clear cell, 20 papillary, and 6 chromophobe RCCs using Western blot analysis. Mirroring the present immunohistochemistry results, they showed that clear cell RCC had the highest expression of HIF-1α and that tumoral expression was significantly higher than in nonmalignant renal cortex tissue. No significant associations were observed between HIF-1α expression and tumor-node-metastasis stage, grade, tumor size, vein invasion, or DNA ploidy. Survival analysis, interestingly, showed that high HIF-1α expression was a favorable prognostic factor, even in multivariate analysis. Expanding on their findings, Lidgren et al. (14) conducted a immunohistochemical tissue microarray–based study on 176 patients with clear cell RCC. Unlike the Western blot–driven study, the survival difference between high and low expressors in this study did not reach statistical significance, although patients with higher expression tended to have a more favorable prognosis. In contrast with the studies of Lidgren et al., the authors of the present study observed high HIF-1α expression as an adverse prognostic factor, a finding which is in accordance with the results for other tumor entities (24, 25). This contradictory finding may be explained by the methodology of HIF-1α analysis used by Lidgren et al. (14), in which cytoplasmic HIF-1α staining, but not nuclear staining, was analyzed. This implies that the subcellular compartment (nucleus or cytoplasm) is important for the effectiveness of HIF-1α as it has been shown for other proteins such as p21 (26), and indeed, HIF-1α is a transcription factor that is active when translocated to the nucleus. Higher HIF-1α expression in the cytoplasm might indicate that HIF-1α has been translocated to the cytoplasm, does not transcribe DNA and, therefore, leads to less aggressive tumors and better prognosis. Because high HIF-1α expression was associated with poor survival, targeting HIF-1α may be a promising therapeutic approach (27). In fact, antisense HIF-1α has been shown to inhibit progression and metastasis, and to enhance the chemosensitivity of pancreatic cancer *in vivo* (28). Because HIF-1α was not correlated with CAIX, combined administration of antisense HIF-1α and the CAIX antibody G250 could produce a synergistic effect.

We did a comprehensive correlative analysis of nuclear HIF-1α expression in the primary tumor with its assumed upstream and downstream targets. We showed significant correlations between HIF-1α and proteins of the VEGF family, which corroborates the concept that HIF-1α accumulation leads to enhanced transcription of the respective DNA segments (5, 29–31). VEGF-A is the strongest proangiogenic factor and exerts its role via interaction with VEGFR-1 and VEGFR-2, whereas VEGF-C and VEGF-D are mainly involved in the formation of lymph vessels by interacting with VEGFR-2 and VEGFR-3 (31–33). It seems, however, that not all VEGF proteins are similarly regulated through HIF-1α accumulation.

Table 3. Multivariate Cox regression analysis

Covariate	Hazard ratio (95% confidence interval)	*P*
ECOG PS	1.430 (1.032-1.981)	0.031
T stage	1.249 (0.985-1.582)	0.066
N stage	1.159 (0.717-1.873)	0.548
Fuhrman grade	1.161 (0.860-1.567)	0.331
Number of metastatic sites	1.310 (0.985-1.744)	0.064
HIF-1α	1.011 (1.003-1.019)	0.006
CAIX	0.987 (0.981-0.993)	<0.001

NOTE: Expression of HIF-1α and CAIX were the strongest prognostic factors among patients with metastatic clear cell RCC.

For example, the expression levels of VEGF-C and VEGFR-2 within the tumor epithelium and HIF-1α were inversely correlated, implying that VEGF-C is negatively regulated through HIF-1α in RCC, consistent with the concept that VEGF-C is involved to a greater extent with lymphangiogenesis as opposed to angiogenesis. Correlation analysis also revealed that HIF-1α regulates chemokine receptors. Most research efforts have been focused on CXCR4, which is involved in cancer progression through its proangiogenic and immunomodulating properties (34, 35). Our data indicates that CXCR3, which has angiogenesis-inhibitory and immune enhancing effects (36), may also be regulated through HIF-1α. Additionally, it has been recently shown that HIF-1α is involved in apoptosis and cell cycle regulation by controlling p53 (12). It is accepted that hypoxia induces apoptosis by increasing the stability of p53 (37, 38), which itself induces apoptosis by acting as a transcription factor for cell cycle regulators such as p21 or mdm2 (11). It is worth noting that HIF-1α can only interact with wild-type p53 but not with the tumor-derived mutated p53 (39), reflecting different circumstances in apoptosis regulation through HIF-1α in benign and malignant tissue. Taken together, our correlation results corroborate the concept that HIF-1α regulates the cell cycle and apoptosis by modulating p21 and p53. Finally, correlation analysis between HIF-1α and pAkt and p27 further supports the theory that HIF-1α crosstalks with proteins of the mammalian target of rapamycin pathway (10, 40, 41).

Carbonic anhydrases are transmembrane enzymes that play a crucial role in the regulation of the pH value by catalyzing the reversible reaction of carbonic acid to carbon dioxide and water (42). Both hypoxia and *VHL* inactivation lead to increased cellular levels of HIF-1α, and subsequently, to an increase of carbonic anhydrases, especially of CAIX (43, 44). It has been shown that CAIX is an indicator for survival and response to systemic therapy among patients with metastatic clear cell RCC, with higher levels being associated with improved survival (20) and enhanced response to interleukin 2–based immunotherapy (21). Our investigation indicates that HIF-1α, CAIX, and CAXII are differentially regulated in localized and metastatic clear cell RCC. Expressions of these proteins were only significantly correlated in localized RCC. We hypothesize that carbonic anhydrases and HIF-1α may be regulated independently in metastatic RCC. In fact, Ihnatko et al. (45) recently described that CAIX expression in human glioblastoma cells was increased via a hypoxia-independent mechanism. Similar or other mechanisms can be assumed in RCC (42). It is

therefore possible that CAIX expression in metastatic RCC reflects *VHL* status, whereas HIF-1α expression might be more complex and be regulated by additional mechanisms and molecular pathways.

In conclusion, our data on HIF-1α expression in a large number of primary RCC cases shows that nuclear HIF-1α is an important indicator of prognosis in patients with metastatic clear cell RCC, with high HIF-1α expression predicting poor survival. Because they are not associated with each other, HIF-1α and CAIX may represent ideal complementary markers for predicting prognosis and may allow improved patient selection for systemic therapies. Integration of these markers in established prognostic models will result in more accurate survival prediction and will guide patient selection for systemic therapies. Finally, it is evident that CAIX and HIF-1α are differentially regulated in metastatic RCC.

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