Survival in Patients with Clear Cell Renal Cell Carcinoma Is Predicted by HIF-1α Expression

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Abstract. *Aim: To investigate hypoxia inducible factor-1α's (HIF-1α) immunohistochemical expression in clear cell renal cell carcinoma (ccRCC) treated with radical nephrectomy. Patients and Methods: One hundred and forty-eight patients were considered from those who underwent radical nephrectomy between 1983 and 1993. Archived materials were retrieved from the Institute of Pathological Anatomy for immunostaining. The features considered on the histological specimens were tumor stage, grade, as well as cellular and vascular HIF-1α expression. All considered parameters were correlated with time to recurrence (TTR) and overall survival (OS). Results: TTR was significantly longer in patients with low cellular HIF-1α expression; patients' survival was higher in those with low HIF-1α expression. Regarding vascular HIF-1α expression, the differences were not statistically significant when considering TTR and OS. On univariate analysis, age, clinical stage and HIF-1α cellular expression showed a significant association with OS. Conclusion: Cellular HIF-1α is an important indicator of prognosis in patients with ccRCC; high HIF-1α expression predicts poor survival.*

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Key Words: Hypoxia inducible factor, clear cell renal cell carcinoma, tumor stage, tumor grade, overall survival.

Clear cell renal cell carcinoma (ccRCC) is the most predominant type of sporadic kidney cancer with more than 25% of patients presenting with advanced disease (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 basic sciences have led to an increased understanding over the molecular pathways underlying the various RCC subtypes (4, 5). Hypoxia is one of the fundamentally important characteristics of solid cancer; it triggers a cascade of molecular events including angiogenesis and involves cell-cycle control proteins, which are closely associated with tumor growth, metastasis and poor prognosis (6-8).

In 1995, Wang *et al.* (9) isolated the hypoxia-inducible factor 1α (HIF-1α), which plays a central role in RCC tumorigenesis. In a physiological microenvironment, HIF-1α is rapidly degraded through the ubiquitin-proteasome pathway (10); however, under hypoxic conditions, HIF-1α is stabilized and accumulates due to the inactivation or absence of the von Hippel-Lindau tumor suppressor gene (*VHL*) and *via* activation of other independent mechanisms, such as the mammalian target of rapamycin pathway (mTOR) (11, 12). HIF-1a regulates angiogenesis, tumor growth, progression and metastatic spread by acting as a transcription factor for crucial proteins, such as vascular endothelial growth factor (VEGF) (12-16).

Over-expression of HIF-1α has been reported in many types of cancers, including lung, prostate, breast, colon and rectum carcinoma, and also in regional or distant metastases, implying that it may play a vital role in tumor progression (17-22).

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.

We investigated HIF-1α's immunohistochemical expression in a large series of ccRCC treated with radical nephrectomy by evaluating the correlation between HIF-1α expression on endothelial and tumor RCC cells and the outcome of patients at long-term follow-up.

Patients and Methods

The procedure for this research project conforms with the provisions of the Declaration of Helsinki. Our Institutional Review Board approved the study's design.

Patients. A total of 148 patients with ccRCC were randomly considered from those who underwent surgery at our Institute of Urology between 1983 and 1993. All patients underwent preoperative evaluation, including history and physical examination, urinalysis, blood count and serum chemistry. Preoperative imaging consisted of ultrasound in 100% of cases, chest and abdominal computerized tomography (CT) in 83% of cases (or chest and abdominal magnetic resonance -MR- in 17%). All patients were treated with radical nephrectomy with complete removal of the surrounding tissues, including the fat, adrenal gland, regional lymph nodes and Gerota's fascia. Follow-up examination at 3-months intervals in year 1 after surgery included serum creatinine determination, chest and abdominal CT or MR; thereafter, general examination, urinalysis, serum creatinine determination, chest and abdominal CT or MR were performed at 6-month intervals, while bone scan was performed when clinically required. At year 6 after surgery and thereafter, serum creatinine determination, chest X-ray, abdominal ultrasound and general examination were performed at 6-month intervals; at year 10 after surgery, all exams were performed yearly.

Archived material containing histological sections from the 150 patients were retrieved from the Institute of Pathological Anatomy and used for immunostaining. The features considered when evaluating the patients were tumor stage and grade, as well as cellular and vascular HIF-1α expression on the histological specimens. All considered parameters were correlated with time to recurrence (TTR) and overall survival (OS).

Tumor grade was based on the Fuhrman scheme (23) and tumor staging was based on the UICC classification (24). Disease recurrence was defined as evidence of measurable disease on imaging, including CT, MR imaging, bone scan or ultrasound, and cytological/histological evaluation of suspected lesions.

Immunohistochemistry. Immunohistochemistry was performed on conventional 5-μm-thick histological paraffin-embedded tissue sections on poly-L-lysine-coated glass slides. After heat-drying, sections were de-paraffinized in xylene and sequentially rehydrated in gradients of ethanol. To better unmask antigenic sites, sections were treated with TUF solution (Histo-line Laboratories, Milan, Italy) at 90˚C for 10 min and incubated overnight at 4˚C with the anti-HIF-1α monoclonal antibody (sc-10790, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The reaction was revealed using a secondary antibody and the streptoavidin-biotin-peroxidase technique (Dako-LSAB peroxidase kit; Dako-cytomation, Carpinteria, CA, USA). After incubation with 3.3-diaminobenzidine (0.05 diaminobenzidine in 0.05 M Tris buffer, pH 7.6 and 0.01% hydrogen peroxide), sections were counterstained with Mayer's Hematoxylin, coverslipped with Paramount and observed using a light microscope. Positive controls were represented by paraffin-embedded sections that had previously been shown to react with primary antibodies from gastric carcinomas. For negative controls primary antibodies were replaced with non-immune sera.

HIF-1α expression was semiquantitatively assessed independently by two different operators in the cytoplasm and nuclei of tumoral cells and in the cytoplasm of endothelial cells of the vessels branching within the tumoral cells, according to a 4-point arbitrary scale of 0 to 4: 0, no positive cells; 1+, less than 10% positive cells; 2+, 10% to 25% positive cells; 3+, 25% to 50% positive cells; and 4+, more than 50% positive cells.

The rate of 25% HIF-1α staining, *i.e.* the median cut-off, was arbitrarily chosen for further statistical correlations.

Statistical analysis. Differences in levels of HIF1α expression between age, gender and pathologic characteristics of the disease (TNM and Fuhrman grade) were tested with the Mann-Whitney *U*-test.

Survival analysis was conducted *via* the Kaplan-Meier product-limit method and the Mantel-Haenszel log-rank test was used to compare OS and TTR. A univariate Cox regression model was further fitted to the data to compare the weight of different levels of the variables in predicting probability of an event (death or recurrence). Significance levels were set at a 0.05 level for all tests. All *p*-values were two-sided. Statistical analysis was conducted with the R Statistical Software, version 3.0.1 (The R Company, Vienna, Austria).

Results

Patients' characteristics. In Table I the patients' characteristics are given; 96 of them were males and 52 females with a mean age of 61.03±7.6 years (range=38-85). A total of 69 tumors were in the right kidney and 79 in the left kidney; mean diameter was 6.3±3.2 cm (range=2.3-17). The patients' pathological stage and grade are also shown in Table I. The median follow-up was 217.43 months (range=5-327) and the median survival was 125.86 months (range=5-327 months).

Eighty-one patients had no evidence of metastasis, while sixty-seven patients (45.2%) developed distant metastases in the follow-up: four pT1 patients (8.3% of the pT1 patients) developed multiple lung and bone metastases between 10 and 51 months after surgery and died between 18 and 61 months thereafter; three pT2 patients (10%) developed disseminated metastases between 10 and 14 months after surgery and died between 10 and 18 months after surgery; fifty-three pT3 patients (78.4%) had metastases after a mean time of 26±23.1 months and died at 36.4±28.8 months (bone and liver metastasis in four cases, lung and bone metastases in 7 patients, liver metastasis in 16 patients, lung metastasis in 24 patients); the 7 pT4 patients (100% of the pT4) showed disseminated metastasis, on average, 2 months after surgery and died between 4 and 7 months.

In Figure 1, HIF-1α expression in tumoral cells and vessels is shown.

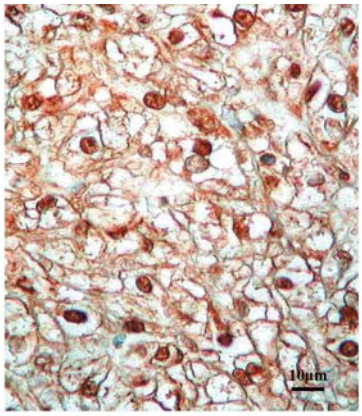

Figure 1. *Immunohistochemical expression of HIF-1α in the tumoral cells of clear cell RCC (immunoperoxidase).*

No significant differences of HIF-1α expression were found by considering patients' age and sex. The expression of cellular and endothelial HIF-1α were not correlated with grade (p=0.69 for cellular HIF-1α and p=0.31 for endothelial HIF-1α, respectively), T stage (p=0.14 and 0.73, respectively), absence or presence of nodal metastases (p=0.39 and p=0.27, respectively), absence or presence of distant metastases (p=0.47 and p=0.38, respectively).

Survival analysis. The overall patients' survival rate was 88.29% in pT1 tumors with a mean survival time of 232 months (range=195-279), 85.32% in pT2 tumors (mean survival time 211 months; range=138-327), 25.31 % in pT3 patients (mean survival time 88 months;, range, 48-131), the pT4 patient died 3-6 months after surgery; the differences are statistically significant (p=0.0003).

TTR was 144 months (range=11-327 months) for pT1 tumors, 154 months (range, 3-274 months) for pT2 tumors, 51 months (range=0-222) for pT3 tumors and 1 month (range=0-2 months) for pT4.

By comparing different tumor stages, a significantly higher OS and TTR was found for pT1-T2 tumors against higher stages (p<0.001) with a survival above the median in the T1-T2 group (95% confidence interval (CI)=165-NR) *vs*. 71 months (95% CI=39.4-127) in the T3-T4 group.

Furthermore, a significant difference in survival was found for lower Fuhrman grades (G1-G2) compared to higher (G3-4) against (p<0.001), irrespective of tumor stage, with an OS of 165 months (95%CI=132-NR) for grades 1-2 *vs*. 48 months (95%CI=28-115) for grades 3-4.

More than half (52.4%) of the patients expressed cellular and vascular HIF-1α at low-level (less than 25%), while the remaining showed high HIF-1α expression. No statistically significant correlation was found considering T stage *vs*. low and high HIF-1α expression (Mann-Whitney *U*-test p=0.47 for cellular HIF-1α and p=0.22 for vascular HIF-1α) and grading *vs*. low and high HIF-1α expression (p=0.27 for cellular HIF-1α and 0.94 for vascular HIF-1α).

Table I. *Patients' characteristics*.

Patients	148 (%)
Gender	
Male	96 (65)
Female	52 (35)
Age, years	61.03
Range	38-85
TNM Status	
T1	48 (32)
T2	30 (20)
T3	63 (42)
T4	7 (4)
N0	93 (63)
N1	34 (23)
N2	21 (14)
Grade	
1	52 (35)
2	62 (41)
3	26 (17)
4	10 (6)
Metastatic site	
Liver	21 (14)
Lung	42 (28)
Lymph-nodes	3 (2)
Pancreas	3 (2)
Brain	6 (4)
Bone	27 (18)
Other	6 (4)
Median OS (months)	125.86
Range	(5-327)

OS, Overall survival; TNM, tumor nodes metastases.

TTR was significantly longer in patients with low cellular HIF-1α expression compared to those with high expression (p=0.028, NR *vs*. 34 months, 9 – NR) (Figure 2); similarly, patients' survival was significantly higher in those with low HIF-1α expression (p=0.03, NR *vs*. 63 months, 19 – NR) (Figure 3). Regarding vascular HIF-1α expression, the differences were not statistically significant when considering TTR (p=0.278) (Figure 4) and OS (p=0.463) (Figure 5).

On univariate analysis, age, clinical stage and HIF-1α cellular expression showed significant association with OS at a significance level of p<0.2.

Discussion

The HIF-α subunits have emerged in recent years as potential therapeutic targets in ccRCC playing a central role in the development of ccRCC; several lines of evidence demonstrate that HIF-2α is the primary oncogenic driver in ccRCC (11, 25, 26).

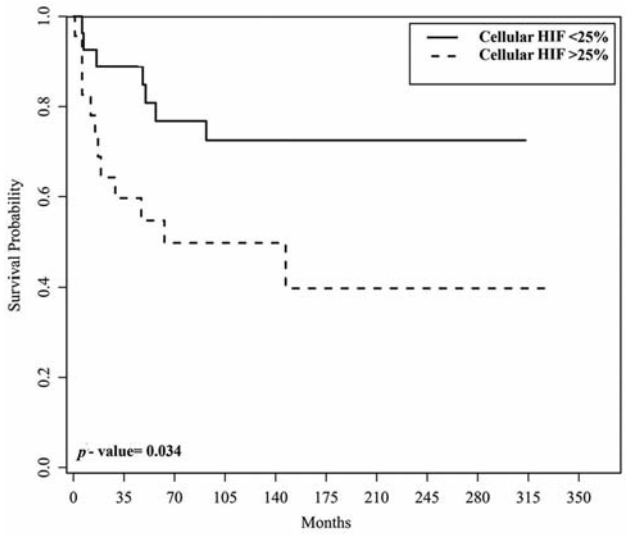

Figure 2. *Correlation between HIF-1α cellular expression and overall survival.*

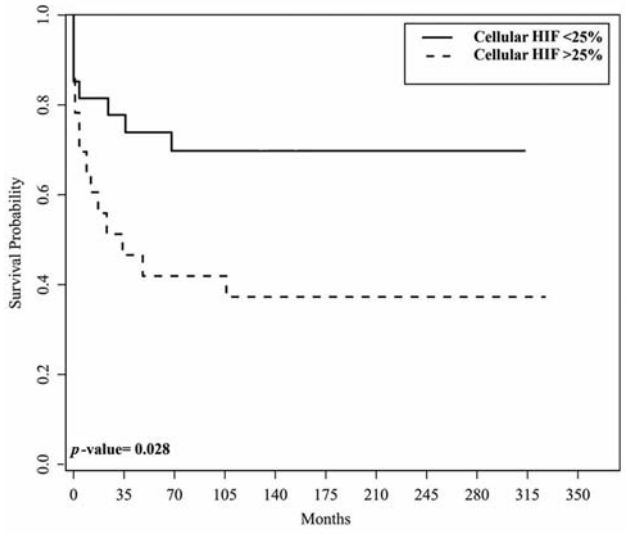

Figure 3. *Correlation between HIF-1α cellular expression and time to recurrence.*

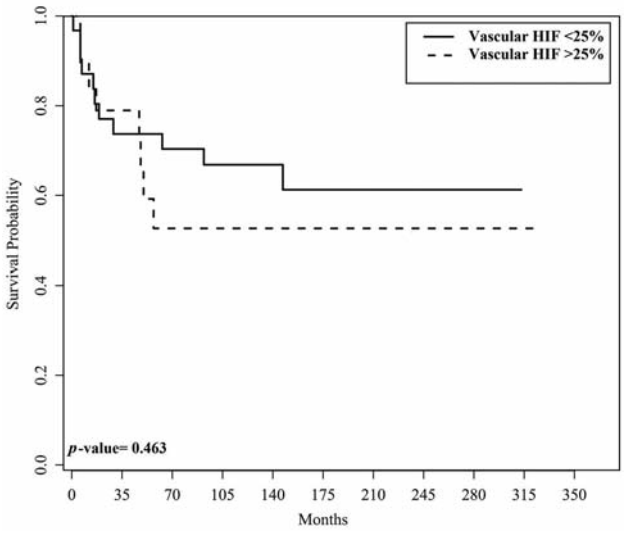

Figure 4. *Correlation between HIF-1α vascular expression and overall survival.*

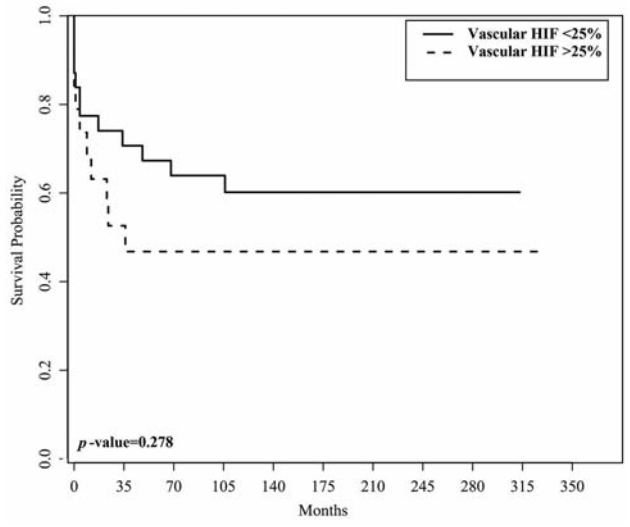

Figure 5. *Correlation between HIF-1α vascular expression and time to recurrence.*

The prognostic role of HIF-1α in RCC was evaluated by Lidgren *et al.* (27) who did not observe any significant associations between HIF-1α expression and tumor-node-metastasis stage, grade, tumor size, vein invasion; survival analysis showed that high HIF-1α expression was a favorable prognostic factor. Expanding on their findings (28), the same authors conducted an immunohistochemical–based study on patients with ccRCC where the survival difference between high and low expression did not reach statistical significance, although patients with higher expression tended to have a more favorable prognosis. These observations were not confirmed by others (12) who found that high HIF-1α expression as an adverse prognostic factor, a finding which is in accordance with the results for other tumor entities. Moreover, the presence of different *HIF* genotypes has been associated with the prognosis of RCC patients (29).

Staging of RCC is important for determining prognosis and selecting high-risk patients for systemic therapies as they become available. The TNM staging system has undergone systematic revision due to rapidly emerging data from longer patient follow-ups; the identification of various histological factors has led groups at many Centers to develop more comprehensive staging systems that integrate these factors and include patients with metastatic and local disease. Furthermore, the discovery of molecular tumor markers is expected to revolutionize RCC staging and lead to the development of new therapies based on molecular targeting (30). Adjuvant therapies are being investigated to increase cure rates and, ideally, these investigations should focus on patients with the worst prognosis, *i.e.* those with worst histological and molecular prognostic factors. In this regard, the results from the S-TRAC study (NCT00375674) on the use of sunitinib *vs.* placebo as adjuvant therapy in patients with high risk RCC and from the phase III study (PROTECT, NCT01235962) of pazopanib *vs.* placebo in patients with moderate and high risk RCC, are dramatically awaited.

Prognostic stratification should be considered as a major step forward for the management of ccRCC patients; many potential prognostic and predictive molecular biomarkers have now been identified in RCC, although none has yet entered into clinical practice as all require prospective validation in appropriately-designed randomized studies (31). Undoubtedly, such an approach could improve prognosis in patients without metastases at diagnosis and, thus, enable us to identify high-risk patients more likely to benefit from adjuvant therapy (30).

Because high HIF-1α expression was associated with poor survival, targeting HIF-1α may be a promising therapeutic approach (32, 33). In fact, antisense HIF-1α has been shown to inhibit progression and metastasis and to enhance the chemosensitivity of pancreatic cancer *in vivo* (34).

Our analysis showed that higher cellular HIF-1α expression is associated with higher malignant potential; therefore, these observations add important information to the current grading and staging system and suggest the possibility of a further substratification.

Our observations, expansion of a previously published study (35), were made in an untreated patient population and confirm that the HIF-1α expression level may be used in identifying, from a pathologic and prognostic point of view, different groups of ccRCC patients.

In addition to a potential role for prognosis, our findings have very important implications for therapeutic applications of HIF-1α inhibitors in RCC and it could be possible that they may have significant therapeutic implications in the selection of patients who will benefit from adjuvant anti-angiogenic therapies. Application of our results might also provide a means for determining which tumors will be best-treated with adjuvant anti-angiogenic therapies after conservative or radical surgery, independently of tumor stage (36-38).

Prospective studies are required to confirm the prognostic role of HIF-1α, as well as the predictive value of expression patterns in patients treated with HIF-1α pathway-targeting agents. Future clinical trials incorporating these agents in patients with RCC should stratify patients based on HIF-1α expression, aiming to develop an assay to improve patient selection for anti-angiogenic agents.

In conclusion, our data on HIF-1α expression in a large number of primary RCC cases show that cellular HIF-1α is an important indicator of prognosis in patients with ccRCC, with high HIF-1α expression predicting poor survival. Integration of this marker in established prognostic models will result in a more accurate survival prediction and will guide patient selection for systemic therapies.

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Received September 1, 2014
Revised October 1, 2014
Accepted October 8, 2014