The Expression of Hypoxia-Inducible Factor 1α Is a Favorable Independent Prognostic Factor in Renal Cell Carcinoma

Anders Lidgren,1 Ylva Hedberg,2 Kjell Grankvist,3 Torgny Rasmuson,4 Janos Vasko,2 and Börje Ljungberg1

Departments of 1Surgical and Perioperative Sciences, Urology and Andrology, 2Medical Biosciences, Pathology, 3Medical Biosciences, Clinical Chemistry, and 4Radiation Sciences, Oncology, Umeå University, Umeå, Sweden

ABSTRACT

Purpose: Renal cell carcinoma (RCC) is the most common malignancy of the kidney composed of specific tumor types. The sporadic conventional RCCs are, in contrast to the other RCC types, characterized by a high rate of von Hippel-Lindau (VHL) mutations and hypermethylation. The majority of these tumors lack functional VHL protein (pVHL) that leads to increased hypoxia-inducible factor 1α (HIF-1α) expression. The pVHL is the physiologic regulator of the activity of HIF-1α by targeting it to the proteasome for degradation under normoxia. Both pVHL and HIF-1α target other genes that are important for cancer survival and proliferation. Expression of HIF-1α has been linked to poor prognosis in different malignancies, although few studies have been done on the relation between HIF-1α and clinical variables in RCC.

Experimental Design: HIF-1α protein expression was analyzed in tumor tissue from 92 patients with RCC. HIF-1α was quantified by Western blot relative to a positive control.

Results: The HIF-1α protein was expressed as two bands which strongly correlated (r = 0.906, P < 0.001); therefore, they were added and the sum evaluated against clinicopathologic variables. There was no association between HIF-1α and gender, stage, grade, tumor size, or vein invasion. Conventional RCCs had significantly higher HIF-1α expression compared with papillary and chromophobe RCCs and kidney cortex. In conventional RCC, HIF-1α was an independent prognostic factor.

Conclusion: HIF-1α levels varied significantly between the different RCC types. In conventional RCC, HIF-1α was an independent prognostic factor. These data indicate that HIF-1α is involved in tumorogenesis and progression of RCC. Evaluation of other HIF target gene products and correlation to angiogenesis seems warranted.

INTRODUCTION

Renal cell carcinoma (RCC) is characterized by abundant neovascularization. Studies imply that there is a relationship between vascularity and clinical outcome in RCC. In a previous study, we showed a strong correlation between high serum protein vascular endothelial growth factor (VEGF) levels and local aggressive growth and poor outcome in patients with RCC (1). Conventional RCCs are more vascularized than papillary and chromophobe RCCs (2). In conventional RCC, mutations in the von Hippel-Lindau (VHL) suppressor gene are more common than in other RCC subtypes (3). The VHL gene product (pVHL) function is to bind to the hypoxia-inducible factor 1α (HIF-1α) in normoxic cells. Lack of the VHL protein leads to reduced degradation of HIF-1α, a state which is normally seen only in hypoxic cells. High levels of HIF-1α have been noted in conventional RCC (4) and the HIF-1α levels are thus mainly caused by genetic alterations of the VHL gene in addition to or despite stimulation through hypoxia. It has also been shown that VHL alterations remained as an independent prognostic factor for patients with stage I to III tumors after adjustment for sex, age, stage, grade, and symptomatic presentation (5).

HIF-1α induces transcription of several factors such as VEGF, platelet-derived growth factor, and erythropoietin. Overproduction of angiogenic factors due to HIF-1α up-regulation could explain the hypervascular nature of RCC and stimulate tumor development and growth. However, several other HIF-1α target genes relevant to cancer development and progression, such as vascularization factors, cell cycle regulators, and growth factors are also up-regulated (4–6).

Overexpression of HIF-1α has been detected in several human cancers (7). In cancer of the cervix and the breast, overexpression of HIF-1α was associated with an unfavorable prognosis (8, 9), whereas in lung cancer a favorable association was found (10). The aim of the study was to quantify HIF-1α in RCC and to correlate the relative expression to clinical and pathologic variables.

MATERIAL AND METHODS

Patients. Ninety-two patients with histopathologically verified RCC [66 conventional (clear cell), 20 papillary, and 6 chromophobe RCC] were included. Nonmalignant kidney cortex tissue samples from 15 corresponding patients and five other patients (2 with conventional RCC and 3 oncocytomas) were also analyzed. All patients were nephrectomized at the Department of Urology, Umeå University Hospital between 1987 and 1998. None of the patient was treated with any immunotherapy or hormonal therapy before nephrectomy. There were 53 men and 39 women, with a mean age of 66.0 years.
(range, 25-87 years). Informed consent was obtained from all patients.

Staging procedures included physical examination, chest radiography, ultrasonography, and computerized tomography of the abdomen. Tumor staging was done according to the tumor-node-metastasis (TNM) stage classification system 1997 (11). Tumor type classification was done according to Skinner et al. (12). RCC type was defined according to the Heidelberg consensus conference (13). The distribution of tumor stage and grade in conventional (clear cell) and papillary RCCs are illustrated in Table 1. All patients were followed with clinical and radiological examinations. Thirty-five of 66 patients with conventional RCC had died of the disease, 13 diseased of other causes, and 18 were alive at the end of the follow-up. Patients alive had a median follow-up time of 98 months (range, 67-142 months).

Protein Extraction and Quantification. All tumor samples were collected immediately after the nephrectomy, snap frozen in liquid nitrogen, and stored at −80°C. Sections of the fresh frozen tissues were fractionated and homogenized in two different extraction buffers, containing urea or Tris. The urea buffer containing 7 mol/L urea, 10% glycerol, 10 mmol/L Tris-HCl (pH 6.8), 1% SDS, Complete Mini protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany) was slightly modified from a protocol published by Wiesener et al. (14). The procedure for the Tris extraction has been described earlier (15). Protein expression levels were quantified by chemiluminescence (RPN 2135, Enhanced Chemiluminescence Advance, Amersham). The membranes were probed with anti-actin (1:3,000, Chemicon International, Temecula, CA) and anti-HIF-1α (1:250, Transduction Laboratories, Lexington, KY) monoclonal primary antibodies. A second incubation was done according to recommendations from the manufactures using Enhanced Chemiluminescence Advance (Amersham) and Fluor-S Multi Imager (Bio-Rad, Richmond, CA).

Western Blot Analysis. Electrophoresis with protein extract from each sample was done on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Hybond-N, Amersham). The membranes were probed with anti-actin (1:3,000, Chemicon International, Temecula, CA) and anti-HIF-1α (1:250, Transduction Laboratories, Lexington, KY) monoclonal primary antibodies. A second incubation was done with an anti-mouse antibody (NA 931 Amersham; 1:1,000 for h actin). The proteins were detected according to recommendations from the manufactures using Enhanced Chemiluminescence Advance (Amersham) and Fluor-S Multi Imager (Bio-Rad) and quantified by Quantity One (Bio-Rad). To ensure that equal amounts of protein were loaded, the membranes were stained with Ponceau red and β-actin was detected.

The protein expression of HIF-1α was quantified using HeLa CoCl2 Cell lysate, (Transduction Laboratories) as a positive control (PC). Thus, 5, 10, and 15 μg PC were loaded on each gel to create the standard curve. The relative HIF-1α concentration in the samples were thereafter calculated according to the linear regression standard curve based on HIF-1α expression of the PC.

Statistical Analysis. Spearman’s test of correlation, Fisher’s exact test, and the Mann-Whitney U test were used for statistical analyses. The log-rank test and Kaplan-Meier method were used for survival analyses. Multivariate analysis was done according to Cox proportional hazard model. The significance level was set to 0.05 and all tests were two sided. Statistical analyses were done using SPSS statistical software, version 11.0 (Statistical Package for Social Sciences, SPSS, Inc., Chicago, IL).

RESULTS

Protein Extraction and Mobility Shift. Protein extractions were done using two buffers containing urea or Tris. In both buffers, HIF-1α was detected as two bands, however weak in Tris buffer extraction as shown in Fig. 1. Using Tris extraction buffer, the HIF-1α signal was weak; thus, additional experiments were done to increase the signal. A secondary antibody with increased number of horseradish peroxidase conjugations as well as different dilutions of the antibodies was evaluated. It was problematic to increase the specific signal and still have a low background (data not shown). In contrast, using urea extraction buffer, the magnitude of the signal became evidently stronger in comparison to Tris extraction. Both HIF-1α bands were consistently shifted towards a higher molecular weight in the urea extracts compared with the Tris extracts (Fig. 1).

HIF-1α Expression. In the RCC tissue samples, HIF-1α was detected by Western blot as two distinct bands at ~116 kDa (Fig. 2A). In contrast, in the PC sample, only a single band was detected. The larger HIF-1α band in the RCC samples was defined as HIF-1αUpper and the band with a slightly lower molecular weight was defined as HIF-1αLower. To enable quantification, 5, 10, and 15 μg PC protein were loaded on each gel (Fig. 2B). The PC values obtained were used for quantification of the tissue samples by construction of a linear regression curve (Fig. 2C). The lower HIF-1α band was detected as two bands, however weak in the RCC samples.
regression standard curve, the average $r$ value for the PCs on all gels was 0.97. Analysis of 1, 5, 10, 20, 50, 100, and 200 μg PC showed a linear expression of HIF-1α thus enabling extrapolation within the protein range. The HIF-1α$I_{Upper}$ and HIF-1α$I_{Lower}$ protein expression relative values were added together and defined as HIF-1α$Total$.

**Protein Expression of HIF-1α in RCC Types and Nonmalignant Kidney Cortex Tissue.** The relative concentration of HIF-1α$I_{Upper}$, HIF-1α$I_{Lower}$, and HIF-1α$Total$ varied between the different RCC types and nonmalignant kidney cortex (Fig. 3A). The conventional RCCs had significantly higher expression of all three HIF-1α variables (HIF-1α$I_{Upper}$, HIF-1α$I_{Lower}$, and HIF-1α$Total$), compared with the other RCC types and with kidney cortex (Fig. 3A). Papillary RCC also expressed significantly higher relative values of all HIF variables compared with chromophobe RCC. When comparing papillary RCC and kidney cortex tissue, significant differences were observed only for HIF-1α$I_{Upper}$ and HIF-1α$Total$. There was no difference in HIF-1α expression between chromophobe RCC and kidney cortex (Fig. 3A). All three HIF-1α variables remained significantly higher in the 12 conventional RCCs compared with their corresponding nonmalignant kidney cortex tissues (Fig. 3B). There was a significant correlation ($r = 0.906, P < 0.001$) between HIF-1α$I_{Upper}$ and HIF-1α$I_{Lower}$ for all RCCs and kidney cortex tissues as well (Fig. 3C). Therefore, these relative values were added together and HIF-1α$Total$ was used in the following clinical statistical analysis.

**HIF-1α$Total$ and Clinicopathologic Variables.** The relative protein content of HIF-1α$Total$ in relation to tumor TNM stage and nuclear grade in conventional and papillary RCCs are shown in Table 1. When using mean, median, or upper quartile (22.9 and 12.2 relative concentration in chromophobe and papillary RCCs, respectively) HIF-1α$Total$ as cutoff values,

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**Fig. 1** Top, illustrations of HIF-1α expression at 116 kDa detected by Western blot in a sample extracted in urea (left) and in Tris buffer (right) in mini protean II (Bio-Rad). Protein samples (60 μg) were loaded in duplicate from tumor T9021. There was a mobility shift and difference in band intensity as shown using the two different extraction buffers. Bottom, β-actin (at 45 kDa) used as a loading control.

**Fig. 2** A, representative Western blot (Protean II xi, Bio-Rad) with 100 μg tumor (T) and nonmalignant kidney cortex samples (N). The PC with 15, 10, and 5 μg, respectively, consisted of CoCl$_2$-treated HeLa cells. β-Actin was used as loading control. B, illustration of a standard curve plot. The three concentrations of the PC sample were plotted (rings) to yield a linear standard curve. The tissue samples (■) were then quantified using the regression line ($r = 0.99$). In total, the average $r$ for the positive controls of all gels was 0.97 ($n = 14$; range, 0.90-0.99).
no correlation between HIF-1α\textsubscript{Total} expression and tumor stage, nuclear grade, gender, tumor size, vein invasion, or DNA ploidy was noted neither in conventional (n = 66) nor in papillary RCCs (n = 20; data not shown). No statistical analysis was done in chromophobe RCCs due to the low number of patients. The mean relative concentration of HIF-1α\textsubscript{Total} expression value of kidney cortex F2SD (4.4 ± 3.6) was defined as the cutoff of normal value (corresponding to 11.6 μg PC protein). Based on this cutoff value, two groups of tumors were defined as high HIF-1α\textsubscript{Total} and low HIF-1α\textsubscript{Total}. There was no correlation between low and high HIF-1α\textsubscript{Total} expression and tumor stage and grade in conventional (n = 66) and papillary RCCs (n = 20), respectively (data not shown). There was neither any correlation between pure clear cell and granular cells and low and high HIF-1α\textsubscript{Total} expression in the conventional RCCS nor between pure papillary and those with mixed cells in the papillary RCCs (data not shown).

HIF-1α and Survival of the Patients. In patients with conventional RCCs, those 44 with high HIF-1α\textsubscript{Total} tumor survived significantly longer than the 22 patients with low HIF-1α\textsubscript{Total} (P = 0.024, Fig. 4). In patients with papillary RCC (n = 20), no such statistical survival difference could be obtained (P = 0.224). In a multivariate analysis of conventional RCCs only, HIF-1α and tumor stage remained as independent prognostic factors as shown in Table 2.
RCC is known for its unpredictable clinical behavior. Patients with metastasized RCC have a median survival of ~8 months, whereas those with tumors confined to the kidney have 90% to 95% of 5-year survival rate (16). Clinical stage is the strongest survival predictor in RCC, but other histologic and biomolecular factors such as cytogenetic, apoptotic, proliferation, angiogenic, and classic tumor markers provide prognostic information (12, 17). Concerning angiogenesis, no angiogenic factor has been shown as an independent prognostic marker in RCC. We herein report that HIF-1α is an independent favorable prognostic factor for conventional RCC.

In the present study, two distinctly separated bands of HIF-1α were detected and quantified. Our results confirm that in RCC and its corresponding nonmalignant kidney cortex, two distinct HIF-1α bands can be detected with immunoblotting as previously found (18). These doublets are thought to represent different post-translational modifications (18, 19). Hydroxylations on HIF-1α initiate its degradation (20, 21), whereas phosphorylations seem to enhance its transcriptional activity (22, 23). We found that the two HIF-1α bands (HIF-1αUpper and HIF-1αLower) significantly correlated and was uniform between the different RCC types. Due to this strong correlation between the two bands, they were added (HIF-1αTotal), and evaluated as a single value.

Inactivation of the VHL gene by mutation or methylation occurs in up to 60% in sporadic conventional RCC (2, 4, 24). Tumor cells with impaired VHL expression have increased concentrations of HIF-1α and HIF target gene products under hypoxic as well as aerobic conditions (25, 26). Several other consequences of pVHL loss have been described, including effects on fibronectin assembly, apoptosis, and cell cycle exit (27–29). However, with the exception of loss of VHL function, oncogenic events upstream of HIF only rarely activate the HIF pathway (26). In addition, tumor suppression by pVHL could be overridden by a HIF variant that escaped pVHL control showing that HIF is a critical downstream target of pVHL (30). Hence, activation of the HIF target genes can promote tumorogenesis in vivo. In RCC, activation of the HIF system thus makes a quantitatively significant contribution to the downstream changes in gene expression associated with the VHL gene (31).

VHL gene alterations were strongly associated with more favorable cancer-specific survival for patients with stage I to III conventional RCC but not for stage IV tumors (5). The reason for a better prognosis for patients with an altered VHL gene is unclear. VHL alteration is the trigger of carcinogenesis and the expression of the conventional phenotype but not of the metastatic activity or other malignant phenotype, affecting the prognosis of the patients (6). Loss of pVHL causes accumulation of HIF-1α irrespective of the oxygen concentration and the deregulated activation of HIF target genes “turning on the angiogenic switch” in RCC (32, 33). Accumulation of HIF-1α caused by mutated VHL in tumor cells may result in VEGF overexpression. Most likely, this explains the increased vascularity of RCC. In the present study, we found that conventional RCC had significantly higher expression of HIF-1αTotal compared with the other RCC types as well as kidney cortex. Also, the papillary RCC had significantly higher HIF-1α expression than both chromophobe RCC and kidney cortex tissue. In conventional RCC, HIF-1α immunoreactivity was observed in cells throughout the tumor, consistent with HIF activation being caused by loss of VHL tumor suppressor function rather than microenvironmental hypoxia (31, 34). Several previous studies showed overexpression of VEGF in RCCs at the protein and mRNA levels and correlations with microvessel density (2, 35, 36). Other findings indicate that VHL gene alterations and HIF-1α protein expression correlate with a significant increase in VEGF production by RCC which in turn is associated with a more aggressive tumor phenotype (33). Schraml et al. (24) showed that regulation of angiogenesis and proliferation is not directly influenced by VHL, as they found no association between VHL alterations and tumor grade, stage, microvessel density, or tumor cells proliferation in RCC (24). Our study confirms these results. We found no association between HIF-1α levels and tumor stage, grade, and size, vein invasion, and DNA ploidy. Several reports indicate that larger tumors have an inadequate blood supply (37, 38). Thus, hypoxia causes up-regulation of VEGF expression (39). Tumor size and the presence of necrosis were found to be an important prognostic factor in papillary RCC (40). Considerable variations in VEGF expression and microvessel density could thus be expected within the same tumor (38, 39) and between RCC types (25).

The significant difference in HIF-1α expression between the different RCC types confirm findings in a previous study where we showed that conventional RCC had significantly higher VEGF121 mRNA levels than papillary RCCs (35).
In another study, we found that VEGF protein expression assessed by immunohistochemistry was present in most RCC cells (36). Furthermore, the correlation between VEGF expression and tumor stage and prognosis indicated the importance of VEGF for tumor growth in RCC (36).

The increased HIF-1α levels in RCC are independent of its position to vascular structures (4). The effects of renal artery clamping on the angiogenic cascade might influence the obtained result in the present study. The surgical procedure with ligature of the blood supply is necessary during nephrectomy, although its effects on the excised tissue and kidney cortex remain unclear. Wiesener et al. (4) also studied some of these inevitable effects on the excised tissue and kidney cortex but remain unclear. They showed that HIF-1α levels remained constant up to 60 minutes after nephrectomy indicating that preoperative quantitative changes of HIF-1α likely influenced our results. In the majority of their tumors, HIF-1α immunostaining was most intense in regions related to areas of necrosis. In contrast, in conventional RCC HIF-1α immunoreactivity was observed in cells throughout the tumor, consistent with HIF activation being caused by loss of VHL tumor suppressor function rather than microenvironmental hypoxia (31).

In the present study, HIF-1α was identified as an independent favorable factor in conventional RCC, although no association to tumor stage was found. Previous work from our group showed that high protein levels of the HIF-1α target gene VEGF correlated to adverse survival (1). However, in that study, no association between protein VEGF expression in serum and the different RCC types was found suggesting that VEGF also might be regulated by other factors than HIF. It is therefore necessary to evaluate other HIF-1α target gene products, their correlation to HIF-1α expression and their importance for patient survival.

In conclusion, HIF-1α differed significantly between the different RCC types. In conventional RCC, high expression of HIF-1α was an independent prognostic factor for favorable prognosis.

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