PROGNOSTIC ROLE OF TUMOR NECROSIS, MICROVESSEL DENSITY, VASCULAR ENDOTHELIAL GROWTH FACTOR AND HYPOXIA INDUCIBLE FACTOR-1α IN PATIENTS WITH CLEAR CELL RENAL CARCINOMA AFTER RADICAL NEPHRECTOMY IN A LONG TERM FOLLOW-UP

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Received August 9, 2007 – Accepted January 15, 2008

Angiogenesis is a critical step in the growth, invasive progression and metastatic spread of solid tumors. We investigated the importance of tumor necrosis, and microvessel density (MVD), vascular endothelial growth factor (VEGF) and hypoxia inducible factor 1α (HIF-1α) immunohistochemical expression in a large series of clear cell renal carcinomas treated with radical nephrectomy and assessed the prognostic value of their expression in terms of patient survival at long-term followup. Fifty patients with clear cell RCC were examined. The features considered when evaluating the patients were age, tumor size and grade, intratumoral vascular and renal capsula invasion, histological necrosis, and MVD, vascular and tumoral cell VEGF, and vascular, tumoral cytoplasmic and nuclear HIF-1α expression on the histologic specimens. All considered parameters were correlated with patient specific survival. Mean age was 62.06 ± 6.8 years. Median follow-up was 191.66 months; median survival was 120.86 months. Twenty-one patients developed metastases in the follow-up. Tumor necrosis, microvascular invasion and renal capsula infiltration are more likely to occur in high stage and grade RCC; cytoplasmic HIF-1α is highly expressed in high grade RCC. Survival is dependent upon tumor stage and grade, the presence of intratumoral vascular invasion and capsular infiltration, and tumor necrosis; MVD also resulted as being an important prognostic factor. VEGF and HIF-1α correlate with prognosis in high stage tumors where VEGF is the most important independent prognostic factor for cancer specific death. The histological and immunohistochemical parameters considered in our study can influence disease recurrence and survival in RCC.

Renal cell carcinoma (RCC) is the most common malignant tumor of the kidney; at diagnosis overt metastatic disease can be found in more than 30% of patients (1). RCC is characterized by abundant neovascularization, therefore several studies have been addressed to point out the relationship between

Key words: clear renal cell carcinoma, tumor necrosis, microvessel density, vascular endothelial growth factor, hypoxia inducible factor

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0394-6320 (2008)

vascularity and clinical outcome.

Rapidly growing tumors often develop spontaneous necrosis which in most cases represents an essential component for histological grading (2) and an important predictor of survival (3-4). Histologic necrosis is defined as any degree of microscopic tumor necrosis exclusive of degenerative changes such as hyalinization, hemorrhage, or fibrosis (3).

It has been observed that microvessel density (MVD) correlates with advanced pathological findings and poor clinical outcome in renal cancer and metastases are more likely to occur in patients with highly vascularized tumors, suggesting that tumor vascularization could be related to tumor outcome (5). MVD was highlighted by anti-CD34 antibody, the antigen present on immature haematopoietic cells (6), and it correlates well with vascular endothelial growth factor (VEGF) (7).

VEGF has been shown to be up-regulated in tumors and it correlates with tumor stage and progression (8-10).

Important insights have been gained through the study of hypoxia inducible factor (HIF) (11); the presence of hypoxic regions in solid tumors is associated with a more malignant tumor phenotype and worse prognosis; to obtain a blood supply and protect against cellular damage and death, oxygen deprived tumor cells alter their gene expression; therefore HIF-1α is activated to promote the transcription of several genes, including VEGF (12-13).

We investigated the role of tumor stage, grade, and renal capsula infiltration, histologic necrosis and CD34 (MVD), VEGF and HIF-1α immunohistochemical expression and in a series of conventional sporadic $pT_{1-4}N_0M_0$ clear cell renal carcinoma treated with radical nephrectomy. We assessed the prognostic value of the studied variables expression in terms of cancer specific survival at long-term follow-up (maximum 22 years).

MATERIALS AND METHODS

Patients

A total of 50 patients with clear cell RCC without sarcomatoid features were considered; all patients were treated with radical nephrectomy at our Institute of Urology between 1983 and 1984. Pre-operative imaging consisted of ultrasound in 100% of cases, and chest and abdominal computerized tomography (CT) in 93% of cases (or chest and abdominal magnetic resonance - MR- in 7%). For the purpose of this study only patients without lymph node involvement or distant metastases at diagnosis were included. Follow-up examinations at 3 month intervals in year 1 after surgery included chest and abdominal CT or MR; thereafter chest and abdominal CT or MR were carried out at 6-month intervals; bone scan was carried out when clinically required. At year 6 after surgery and thereafter chest X-rays, abdominal ultrasound and general examinations were carried out at 6-month intervals; at year 10 after surgery they were carried out yearly.

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.

Histologic features

Archived materials containing histological sections from the 50 patients were retrieved from the Institute of Pathological Anatomy and were used for the study. Tumours grade was based on the Fuhrman scheme. Fuhrman grade is on a scale of I-IV, where grade I carries the best prognosis and grade IV the worst (14); tumor staging was based on UICC classification (15).

The features considered in this study were patient age, tumor size and grade, microvascular invasion and renal capsula infiltration, histologic necrosis, MVD, vascular and tumoral cell cytoplasmic VEGF, and vascular, cytoplasmic and nuclear HIF-1α expression. All considered parameters were correlated with patient specific survival.

Extent of necrosis was graded using the following scale: 0 = no necrosis, 1 = <25% of necrosis, 2 = 25-50% of necrosis, 3 = 51-75% of necrosis, 4 = 76-100% of necrosis (2); the rate of 50%, i.e. the median cutoff, was arbitrarily chosen for further statistical correlations.

Immunohistochemistry

Immunohistochemistry was carried out on conventional 5 μm thick histological paraffin-embedded tissue sections on poly-L-lysine-coated glass slides. After heat-drying, the sections were deparaffinized in xylene and sequentially rehydratated in gradients of ethanol. To better unmask antigenic sites, the sections were treated with TUF solution (Histo-line Laboratories, Milano, Italy) at 90°C for 10 min and incubated overnight at 4°C with the following antibodies: anti-CD34 (347660, dil. 1:20, BD Biosciences, Becton Dickinson, USA), anti-VEGF-165 (sc-7269, dil. 1:200, Santa Cruz Biotechnology, Santa Cruz, CA) and anti-HIF-1α (sc-10790, dil 1:100 Santa

Table I. *Pathological stage and grade of RCC carcinoma in our patients.*

		GRADE				
		G1	G2	G3	G4	Total
STAGE	pT1	2	7	9	1	19
	pT2	2	1	3	1	7
	pT3	3	7	5	8	23
	pT4			1		1
Total		7	15	18	10	50

Table II. *Multivariate analysis of death-specific predictive factors.*

Factor	p value	Hazard ratio	95.0% CI	
			Lower	Upper
AGE	0.629	0.984	0.922	1.050
GRADE	0.392	1.293	0.718	2.329
STAGE	**0.003**	**2.669**	**1.396**	**5.103**
NECROSIS	0.579	0.767	0.301	1.957
CD34 (MVD)	0.349	0.995	0.986	1.005
e-VEGF	0.870	1.109	0.322	3.820
t-VEGF	0.437	1.662	0.461	5.989
e-HIF1	0.333	1.805	0.546	5.967
t-HIF1	0.121	2.851	0.759	10.713
nt-HIF1	0.532	0.698	0.226	2.157

e-VEGF = endothelial VEGF-1α
t-VEGF = tumoral VEGF-1α
e-HIF-1α = endothelial HIF-1α
t-HIF-1α = tumoral HIF-1α
nt-HIF-1α = nuclear tumoral HIF-1α

Cruz Biotechnology, Santa Cruz, CA). The reaction was revealed using the secondary antibody and streptoavidin-biotin-peroxidase technique (Dako-LSAB peroxidase kit, Dako-cytomation, Carpinteria, CA). 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, previously shown to react with primary antibodies, from gastric carcinomas. For negative controls primary antibodies were replaced with non-immune sera.

Microvessel density was assessed independently by two different operators, counting individual microvessels on 10 fields at 400x magnification, i.e. a 40x objective

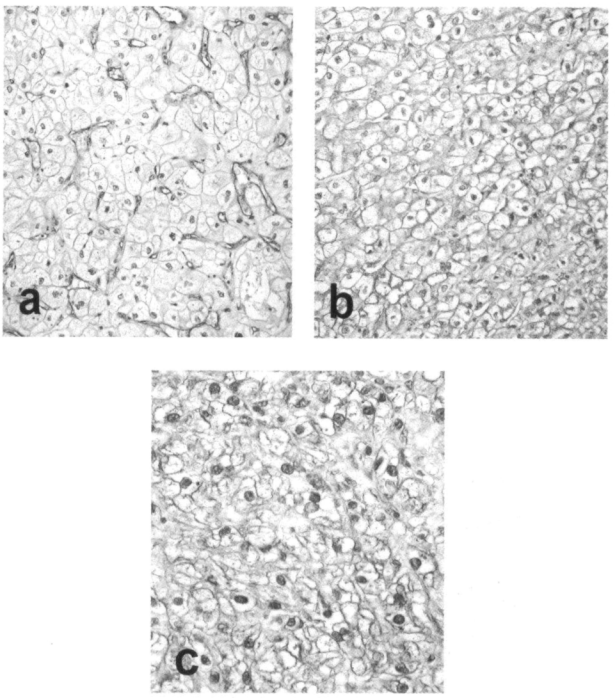

Fig. 1. *Immunohistochemical staining of RCC analyzed by immunoperoxidase technique as described in Materials and Methods section:*
a) *CD34 positive microvessels (x200 original magnification);*
b) *VEGF expression (x200 original magnification);*
c) *HIF-1α expression (x250 original magnification).*

lens and 10x ocular lens with 0.22 mm^2 per field, in a highly vascular tumor area (hot spot), excluding areas with prominent hyalinization and necrosis. Microvessels were defined as any CD34 positive endothelial cell or endothelial cell cluster with or without a viable lumen. In tumors showing a dense microvasculature network each branch was interpreted as a single vessel. For each tumor, the mean number of microvessels counted per 400x magnification on 10 fields was considered MVD. Values are expressed as the number of vessels per mm^2.

VEGF staining was semiquantitatively assessed independently by two different operators in the cytoplasm of tumoral cells according to a 4 point arbitrary scale of 0 to 4, that is 0: no positive tumor cells, 1+ less than 10% positive tumor cells, 2+ 10% to 25% positive tumor cells, 3+ 25% to 50% positive tumor cells and 4+ more than 50% positive tumor cells.

HIF-1α expression was semi-quantitatively assessed independently by two different operators in the cytoplasms and nuclei of tumoral cells according to a 4 point arbitrary scale of 0 to 4, that is: 0 no positive tumor cells, 1+ less

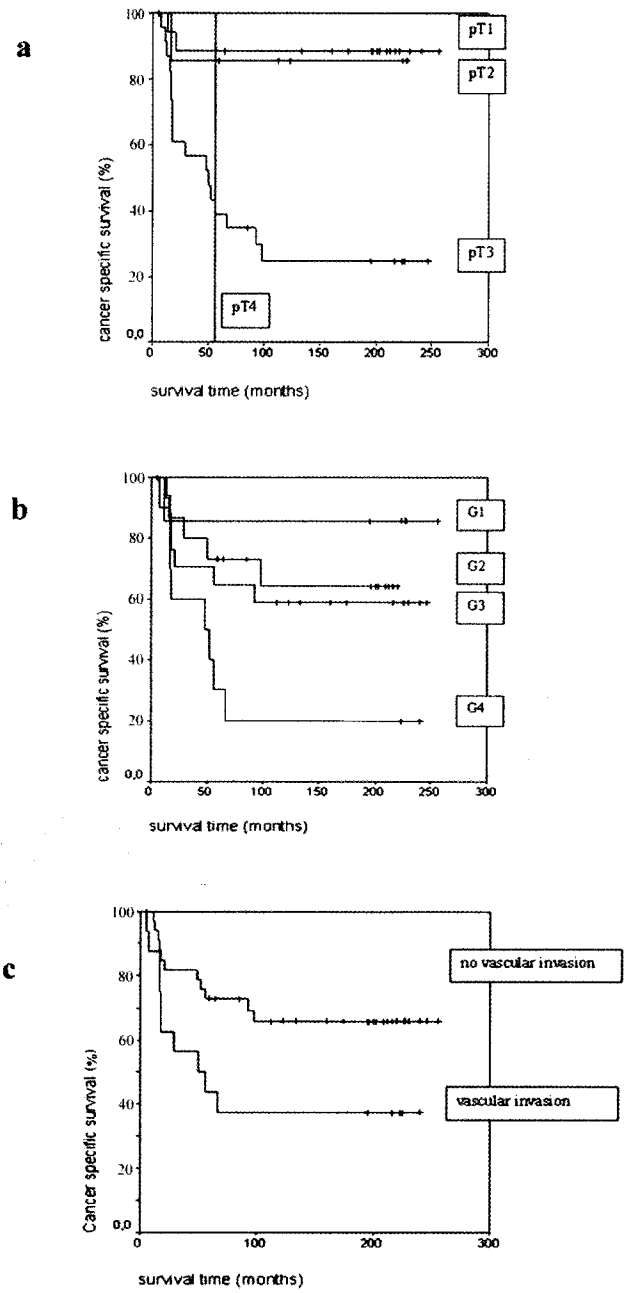

Fig. 2. ***a)*** *Kaplan-Meier survival curve for tumor stage (p=0.0003, χ^2 18.96, log rank 18.99);*
b) *Kaplan-Meier survival curve for tumor grade (p=0.310, log rank= 8.88);*
c) *Kaplan-Meier survival curve for microvascular invasion in ouer series of RCC (p=0.0682, log rank= 3.33).*

than 10% positive tumor cells, 2+ 10% to 25% positive tumor cells, 3+ 25% to 50% positive tumor cells and 4+ more than 50% positive tumor cells.

The rate of 25% VEGF and HIF-1α staining, i.e. the

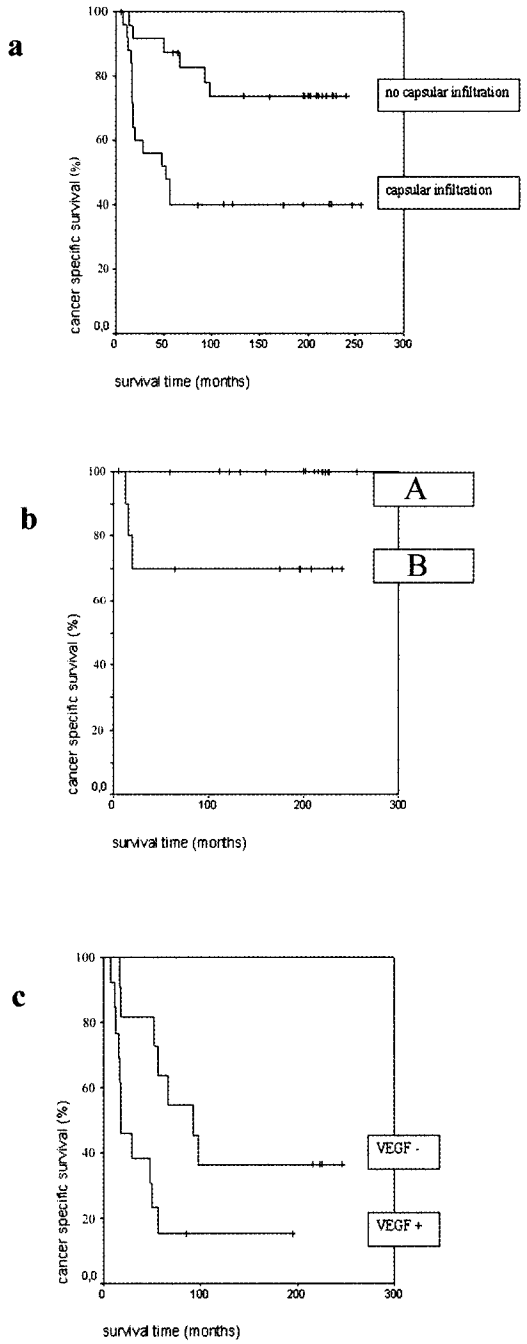

Fig. 3. ***a)*** *Kaplan-Meier survival curve for renal capsula infiltration in our series of RCC (p=0.0065, log rank= 7.42);* ***b)*** *Kaplan-Meier survival curve for microvessel density in our series of RCC (p=0.0020, log rank= 9.52), measured by the number of CD34 positive vessels*
A: < CD34+ 150 vessels/mm²; B: > CD34+ 150 vessels/ mm²
c) *Kaplan-Meier survival curve for vascular VEGF expression in pT3-4 renal tumors (p=0.027, log rank= 4.84).*

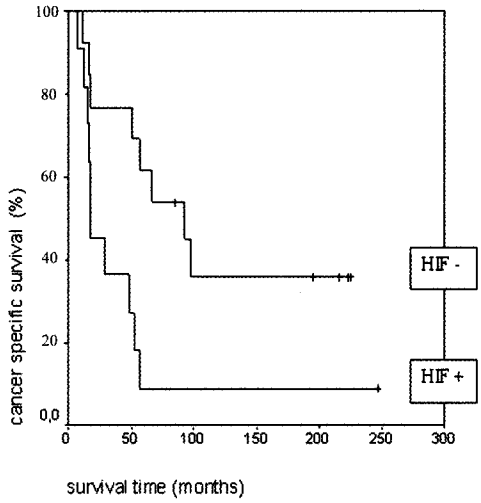

Fig. 4. *Kaplan-Meier survival curve for tumoral cells cytoplasmic HIF-1α expression in pT3-4 renal tumors (p=0.0016, log rank=5.73).*

median cutoff, was arbitrarily chosen for further statistical correlations.

Two different operators also independently evaluated semi-quantitatively the differences of VEGF and HIF-1α expression in the cytoplasms of endothelial cells of the vessels branching within the tumoral cells, according to a 2 point arbitrary scale, that is: 1-low staining intensity, 2- high staining intensity.

Statistical analysis

Statistical analysis was performed using the Kolmogorov-Smirnov normality test for all the considered parameters. The Mann-Whitney U and Kruskal-Wallis tests were used to compare non-parametric data. The Fischer and $\chi 2$ tests were used to compare nominal data. Kaplan-Meier curves were designed to compare survival parameters. The influence of each parameter on survival was assessed using Cox proportional hazard models.

RESULTS

Patients

Of the 50 patients, 32 were male and 18 female. Mean age was 62.06 ± 6.8 years (range 38 to 83). A total of 23 tumors were in the right kidney and 27 in the left kidney. Mean diameter was 6.9 ± 2.9 cm (range 2.5 to 17). Pathological stage and grade are shown in Table I. Median follow-up was 191.66 months (range 5 to 264); median survival was 120.86 months (range 5 to 264 months).

Twenty-nine patients had no evidence of metastasis while twenty-one patients (42%) developed distant metastases in the follow-up: two pT1 patients developed multiple lung and bone metastases 12 and 47 months after surgery and died 21 and 57 months respectively thereafter; a pT2 patient developed disseminated metastases 12 months after surgery and died at month 16; seventeen pT3 patients had metastases after a mean time of 25 ± 21.5 months and died at 36.4 ± 28.8 months (overall the patients had respectively multiple bone and liver metastases in one case, multiple lung and bone metastases in 3 patients, multiple liver metastases in 5 patients, multiple pulmonary metastases in 8 patients); the pT4 patient showed disseminated metastases 2 months after surgery and died at month 5. None of the patients had local relapse. All the patients received adjuvant systemic therapy with Interferon at the time of metastases discovery and thereafter.

Histo-pathological features

The histologic and macroscopic tumor observation showed:

a) a homogeneous distribution of the Furhman grade among the different pathologic stages (p= 0.300);
b) a significant increase of microvascular invasion and renal capsula infiltration in pT3 and pT4 patients compared to pT1 and pT2 patients (p = 0.009 and 0.002, respectively) and in G3-G4 tumors compared to G1-G2 tumors (p= 0.004 and 0.072, respectively). Tumors with microvascular invasion had a significantly larger diameter than patients without it (8.1 ± 2.9 vs 6.4 ± 2.8 cm, p= 0.066); similarly, tumors showing renal capsula infiltration had a significantly greater diameter (8.18 ± 3.3 cm vs. 5.74 ± 1.8 cm, p= 0.002);
c) tumor necrosis was correlated with tumor stage (p= 0.022) and grade (p= 0.004).

Immunohistochemistry

The mean of microvessel density (MVD) observed by CD34 expression (Fig. 1a) was 141.5 ± 64.35 (min 23.63, max 292.7), scored as reported in the Materials and Methods section, and did not correlate with tumor stage and grade, nor with the presence of microvascular invasion and renal capsula infiltration.

The endothelial and tumoral cells cytoplasmic expression of VEGF (Fig. 1b) did not correlate with tumor stage and grade, nor with microvascular invasion and renal capsula infiltration and with tumor necrosis; a significant correlation between the endothelial and tumoral cells cytoplasmic expression of VEGF was observed (Rho = 0.410, p = 0.003).

The expression of endothelial and tumoral cells cytoplasmic and nuclear HIF-1α (Fig. 1c) did not change with stage, or with the presence of microvascular invasion and renal capsula infiltration, nor with tumor necrosis; cytoplasmic HIF-1α had a significantly higher expression in high grade RCC (17/28 G3-4 vs. 6/22 G1-2).

Endothelial VEGF expression was significantly correlated with CD34 expression (Rho= 0.345, p= 0.019), and a strong correlation was observed between endothelial VEGF and endothelial HIF-1α expression (Rho= 0.590, p= 0.0001).

Prognosis

Overall patient survival was 89.47% in pT1 tumors with a mean survival time of 229 months (range 195-264), 85.71% in pT2 tumors (mean survival time 197 months, range 142-252), 26.09 % in pT3 patients (mean survival time 90 months, range 51-128), the pT4 patient died 5 months after surgery; the differences are statistically significant (p=0.0003, χ^2 18.96, log rank: 18.99) (Fig. 2a).

Fuhrman grade resulted to be statistically significant for prognosis (p= 0.0310, log rank: 8.88), survival being 85.71% in G1 tumors (n= 7 patients), 66.67% in G2 (n= 15), 61.11% in G3 (n= 18) and 20% in G4 (n= 10) (Fig. 2b).

Microvascular invasion resulted as being important for survival, as shown in Fig. 2c; survival was 37.50% and 67.65%, respectively in patients without and with vascular invasion (p = 0.0682, log rank: 3.33). Similarly (Fig. 3a), patients showing renal capsula infiltration had a significantly worse prognosis than those without it (40% vs. 76%, p = 0.0065, log rank: 7.42). Moreover, tumor necrosis was important for survival; in fact it was lower for those patients showing a necrosis rate higher than 50% (p=0.0128; log rank 8.72) (data not shown).

CD34, scored as reported in the Materials and Methods section, resulted as being an important prognostic factor; in fact, in patients showing less

than 150 vessels/mm^2 (p = 0.0020 log rank = 9.52) we observed a higher survival (Fig. 3b).

Considering all 50 patients, we found that endothelial VEGF expression did not correlate with survival; in fact the latter was 50% in patients showing respectively low expression compared to 60% in those with high expression (p = 0.5626, log rank: 0.34); similarly the tumor cells cytoplasmic VEGF expression did not correlate with survival, which was 50% in patients with low expression and 73% in those with high expression (p = 0.2023, log rank: 3.20).

Considering all 50 patients, vascular, tumoral cell cytoplasmic and nuclear HIF-1α expression did not correlate with prognosis (p = 0.3562, log rank: 2.06; p = 0.1435, log rank: 3.88; p = 0.801, log rank: 0.06, respectively).

The multivariate analysis of death-specific predictive factors showed that only pathologic stage was an independent prognostic factor (n= 50, H.R. 4.8, p= 0.001) (Table II).

Considering at this point the 26 patients with organ-confined cancer (pT1-2), stage and grade did not seem to influence prognosis; MVD, VEGF and HIF-1α are not important for prognosis; an indirect correlation was observed between vascular VEGF and nuclear HIF-1α (Rho = - 0.553, p = 0.003) (data not shown).

Considering finally the 24 patients with locally advanced disease (pT3-4), stage and grade correlated with prognosis, vascular VEGF expression influenced survival, which was lower in patients showing high expression (15.4%) compared to those with low expression (36.4%) (p = 0.027, long rank: 4.84) (Fig. 3c); tumoral cell cytoplasmic HIF-1α expression inversely correlated with survival (p = 0.016, log rank: 5.73) (Fig. 4). Vascular and tumoral cell VEGF expression showed a significant correlation between them (Rho = 0.514, p =.010). The tumoral cell HIF-1α expression was inversely correlated with CD34 expression (Rho= - 0.556, p= 0.009).

Vascular VEGF was therefore the most important independent prognostic factor for cancer specific death in pT3-4 cancers (data not shown).

DISCUSSION

Surgery is the most efficacious therapy for patients with non-metastatic, localized renal cell carcinoma. Many patients with high stage and grade RCC are at risk for progression and death and several factors are paramount to the successful treatment of these patients (16).

To date, no widely accepted kidney cancer biomarkers that would stratify patients into high-risk groups have been identified; promising markers examined in RCC involve the mechanisms associated with the hypoxia-inducible pathway (13, 17-18).

CD34 expression (MVD) on tumor-associated vessels is augmented as compared to vasculature in the normal tissue (19-20). Increased MVD not only supply the tumor with more oxygen and nutrients, but also provide a ready portal for metastatic spread, as angiogenic vessels tend to be leaky (21).

As tumors progress to more advanced stages of malignancy, levels of VEGF continue to increase and hypoxia develops (9, 13, 22).

HIF is a critical factor in the cellular response to hypoxia. Highly aggressive tumors rapidly outgrow blood supply, leaving the cells starved of oxygen; tumors cells adapt to hypoxia by increasing their synthesis of HIF, which in turn binds to and activates several genes (13, 23).

Several authors have found that HIF-1α is highly expressed in renal cell carcinoma tissues in close correlation with VEGF and the expression levels are significantly higher in metastatic RCC, involving the regulation of a functional VHL gene of sporadic RCC (24-25).

The aim of the present study is to study the importance of tumor stage, grade and renal capsula infiltration on patient survival; also to investigate the role of histologic necrosis, MVD, VEGF and HIF-1α immunohistochemical expression on cancer specific survival.

At the long term follow-up of patients included in our study, we made the following observations:

1. tumor necrosis, microvascular invasion and renal capsula infiltration are more likely to occur in high stage and grade RCC;
2. MVD, VEGF and HIF-1α expression are not related to tumor stage and grade, nor to the presence of microvascular invasion and renal capsula infiltration;
3. survival is dependent upon tumor stage and grade, presence of intratumoral vascular invasion and capsular infiltration, and tumor

necrosis;
4. MVD resulted as being an important prognostic factor in all stages of RCC;
5. only in high stage tumors (i.e. pT3 – pT4) VEGF and HIF-1α correlate with prognosis, and VEGF is the most important independent prognostic factor for cancer specific death.

From our study, a significant correlation between endothelial VEGF and CD34 expression has been shown, underlining a key role of VEGF in angiogenesis of RCC; a correlation between tumoral VEGF and vascular VEGF expression was also observed, confirming that VEGF of tumor renal cells could induce angiogenesis, monitoring VEGF expression in intratumoral endothelial cells; our data also confirm the regulation induced by HIF-1α on VEGF (26), as suggested by the strong correlation between endothelial VEGF and endothelial HIF-1α expression. Therefore, our study provides important pathological evidence that HIF-1α expression correlates with a significant increase in VEGF and CD34 production in RCC specimes. Up-regulated VEGF expression within tumor tissue can be regarded as an indicator of hypoxia, which is commonly found in solid tumors of various origin. Selection of hypoxia may render tumor cells resistant to hypoxia inducible apoptosis; these cells with a reduced apoptotic potential may also explain the resistance of many solid tumors to chemotherapeutics (13). The cellular response to hypoxia defined by death, adaptation and/or survival results from, in part overlapping, signaling components as a reaction towards decreased oxygen tension; in general, hypoxia induces cell death via pro-apoptotic mechanisms and/or secondary to stressors; adaptation results from anti-apoptotic and proliferative mechamisms, which might represent a cellular response to hypoxia itself and/or the secondary stress factors. Chemoresistance is caused by a balance of cell death and adaptive mechanisms, allowing selection of cell clones which adapt to unfavourable living conditions in part by genetic and epigenetic mechanisms (13).

The histologic and immunohistochemical parameters considered in our study can influence disease recurrence and survival in RCC; these markers will enhance our ability to predict an individual tumor's behavior and to stratify patients into risk categories: with the assistance of these prognostic factors clinicians can easily identify those patients who may benefit from adjuvant therapy (27-28). Moreover, the advancements of molecular biology and genetics will help clinicians to incorporate further prognostic models.

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