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ORIGINAL ARTICLE

Prognostic significance of vascular endothelial growth factor expression in clear cell renal cell carcinoma

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Abstract

The purpose of this investigation was to analyze and correlate the immunohistochemical pattern of vascular endothelial growth factor (VEGF) expression with the average of microvessel density (MVD) and other clinicopathologic parameters in clear cell renal cell carcinoma (CCRCC) in order to determine its prognostic significance. Surgical specimens of 93 CCRCC were immunohistochemically analyzed for VEGF expression, MVD with anti-CD31, and Ki 67 proliferative index. VEGF expression was recorded as the percentage of positive tumor cells ($<75\%$ and $>75\%$) and as diffuse or perimembranous VEGF expression according to cytoplasmic distribution. Sixty-three (68%) RCC had $<75\%$ and 30 had (32%) $>75\%$ of VEGF expression. A diffuse cytoplasmic pattern of VEGF expression was found in 61(66%) RCC and a perimembranous one in 32 (34%) RCC. Statistical analysis showed that tumors with $>75\%$ of VEGF expression were characterized by lower MVD value ($p = 0.034$), higher nuclear grade ($p = 0.018$), and higher Ki 67 proliferation index ($p = 0.023$). Moreover, a higher nuclear grade of tumor cells was characterized by diffuse cytoplasmic VEGF distribution ($p = 0.005$).

This tumor model did not confirm the postulated simple relationship between VEGF overexpression and angiogenesis through high microvessel count. However, the study results indicated that overexpression of VEGF was a worse histologic prognostic parameter in CCRCC.

Keywords: Carcinoma; Renal cell; CD31; VEGF; Survival rate

Introduction

Renal cell carcinoma (RCC) has been characterized by a constant increase in its incidence and mortality over the last 60 years [11,15]. Although the pathologic stage has been considered as the most powerful prognostic marker in patients with RCC, many investigations have been performed to discover a new predictive marker for this tumor [24]. Since Folkman [9], in 1971, proposed that tumor growth is dependent on angiogenesis, many studies have examined the prognostic significance of microvessel density (MVD) in different tumor entities

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[27,30,36]. The majority of these studies have revealed that more vascular tumors are associated with an increased risk of metastasis and a less favorable prognosis. However, the mechanism by which the tumor induces neovascularization remains obscure.

Angiogenesis is controlled by angiogenic factors that provide the regulation of extracellular matrix remodeling, endothelial cell proliferation, capillary differentiation, and anastomosis necessary to establish blood supply. Angiogenic stimuli are released by tumor cells, stromal cells, and inflammatory cells recruited to the tumor site [33]. Among several identified peptides with angiogenic properties, the vascular endothelial growth factor (VEGF) is thought to play a major role in tumor angiogenesis [8]. Increased expression and prognostic relevance of VEGF have been described in various epithelial [1,2] and mesenchymal neoplasms [3,4,5,13], melanomas [25], and malignant gliomas [26]. Furthermore, comparable associations were observed with other tumors overexpressing factors stimulating angiogenesis, i.e., hypoxia inducible factors (HIFs), basic fibroblast growth factor (bFGF), and thymidine phosphorylase (TP), and these observations have shown that both tumor growth and tumor spread are dependent on high angiogenesis [33].

The aim of this study was to analyze the immunohistochemical pattern of VEGF expression and to investigate angiogenesis by average MVD in clear cell renal cell carcinoma (CCRCC) in order to demonstrate the correlation or differences in tumor stage, nuclear grade, proliferative activity and patient survival time, and, finally, to find the possible relationship between MVD and VEGF expression.

Materials and methods

Clinicopathologic data

In this study, we reviewed clinical and pathologic findings of 142 CCRCC patients treated by radical nephrectomy at University Department of Urology, Rijeka University Hospital Center, Rijeka, Croatia, between 1989 and 1994. Ninety-three archival nephrectomy specimens with RCC (formalin-fixed and paraffin-embedded material), stored at the Department of Pathology, Rijeka University School of Medicine, Croatia, were selected for immunohistochemistry. Patients with all available clinical data were included. There were 62 (67%) male and 31 (33%) female patients, mean age 58 (range 30–82) years.

Subtyping of tumors was done according to the WHO classification [6] of renal cell neoplasms, and staging according to the TNM classification [29]. The mean tumor size was 6.4 (range 1.9–15) cm. There were 77 (80%) tumors confined within the kidney (pT1 and pT2) and 16 (20%) tumors with progression (pT3 and pT4). Ten patients had lung metastases, eight presented with tumor spread to regional lymph nodes, and one patient had liver metastasis. Nuclear grade was assessed using the four-tiered system of Fuhrman et al., combining nuclear grades 1 and 2 into one group, leaving nuclear grades 3 and 4 as another group [10]. Follow-up information was obtained from patient medical records and the files of the Croatian Cancer Registry. Follow-up was available for 93 patients with a 5-year survival rate of 62%; 22% of patients died from CCRCC within 2 years.

Immunohistochemistry

Immunohistology analysis was done on paraffin-embedded sections to determine MVD, VEGF expression, and Ki 67 proliferative index. Epitope retrieval was achieved by immersing slides in 10 mM citrate buffer (pH 6.0) in a microwave oven in four 5-min cycles. Indirect immunoperoxidase staining was performed using the DakoCytomation LSAB2 HRP system on an automatic immunostainer (DakoCytomation, TechMate^{TM} Horizon, Glostrup, Denmark) according to the manufacturer's protocol.

Microvessels were visualized by anti-CD31 (clone JC70A, dilution 1:50, Dakocytomation, Glostrup, Denmark). The quality of staining was judged using blood vessels in adjacent benign renal parenchyma as internal control. For microvessel quantitation, the slides were examined at medium power magnification ($\times 200$) to identify the areas of the highest number of vessels within the tumor. In each tumor, three areas (hot spots) considered to have the highest vascularization were selected. The highest power magnification ($\times 400$) field in each of these three areas was counted. The highest counts of these three areas were recorded. Vessels with thick muscular walls and vessels in sclerotic areas were excluded from the count. Single endothelial cells or clusters of endothelial cells with or without the lumen were considered to be individual vessels. In the neovascular hot spots, MVD was calculated by test counting using the computer-aided image analysis system (Issa 3.1 software, Vams, Zagreb, Croatia). By this procedure, tumor areas with the true highest MVD within the tumor could be selected. Microvessels were counted as the number of marked vascular structures per scanned field of counting with a size of 0.076049 mm^2 [14].

Anti-VEGF polyclonal antibody (C-1:sc-7269, dilution 1:750, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used to determine VEGF expression. Smooth muscle cells in vascular walls served as an internal positive control. The results of the immunohistochemically obtained expression rates in CCRCC were analyzed semiquantitatively. Four groups were formed

according to the percentage of positive carcinoma cells with cytoplasmic staining: 1, <25%; 2, 26–50%; 3, 51–75%; and 4, 76–100%. VEGF cytoplasmic expression was described as diffuse or perimembranous, and the VEGF histologic pattern as homogeneous (regular distribution and intensity of VEGF staining in tumors) or heterogeneous (irregular VEGF staining) [12].

Proliferative activity in tumors was assessed by detecting Ki 67 protein using a monoclonal antibody (clone MIB-1, dilution 1:50, Dakocytomation, Glostrup, Denmark). Immunoreaction for Ki 67 antigen was also quantified by image analysis using Issa 3.1 software (Vams, Zagreb, Croatia) and assessed by scoring 500 tumor cells at $\times 400$ magnification in the region with the highest proliferative activity.

Statistical analysis

Statistical analysis was performed using Statistica 6.1 software (StatSoft, Inc., Tulsa, OK, USA). Pearson's χ^2-test was used to assess the significance of correlation between categorical data. The mean values of continuous data such as MVD, Ki 67 proliferation index, and tumor size were compared using the Mann–Whitney *U*-test. Survival probabilities were estimated by the univariate Kaplan–Meier method, and survival curves were compared using the log-rank test. The correlation of immunohistochemical staining for VEGF with patient survival was evaluated using the Kaplan–Meier method, and differences between groups were tested by the log-rank test. Statistical differences with a p value less than 0.05 were considered significant.

Results

Immunohistochemical assessment of VEGF expression, microvessel density, and Ki 67 proliferation index

In normal renal tissue, VEGF expression was limited to the cytoplasm of tubular epithelium, smooth muscle cells and macrophages in the interstitial tissue, and mesangial cells in the glomerule (Fig. 1A, B). In CCRCC, VEGF was expressed in the cytoplasm of tumor cells, endothelial cells, and stromal fibroblasts. VEGF staining in tumor was predominantly heterogeneous, with strong immunoreactivity more often observed at the edge than in the center of tumors (Fig. 1C, D). A heterogeneous histologic pattern was observed in 67 (72%), and a homogeneous pattern with regular staining intensity and distribution of VEGF-positive cells in 26 (28%) CCRCC cases. A diffuse cytoplasmic pattern of VEGF expression was found in 61 (66%) (Fig. 2D), and a perimembranous one in 32 (34%) renal carcinomas (Fig. 2C). The frequency distribution of immunohistochemical staining patterns for VEGF is shown in Table 1.

Microvessels were heterogeneously distributed, always more abundant at the periphery of the tumor. The mean number of microvessels *per* tumor standard area was 37.78 ($\pm$20.11), and the mean value of Ki 67 proliferation index in CCRCC 7.29% ($\pm$7.03).

VEGF expression in relation to clinicopathologic parameters

VEGF expression was correlated with immunohistochemically determined MVD and Ki 67 proliferative index in order to establish the influence on angiogenesis and tumor proliferation, and with nuclear grade, tumor size, pathologic stage, and 5-year patient survival in order to establish the prognostic significance of VEGF expression.

Statistical analysis using the four graded system of VEGF expression showed a significant association only with tumor size, while correlation with other mentioned parameters did not turn out to be statistically significant (data not shown). When using the cut-off of 75% (namely, grouping first four categories together), VEGF expression >75% was associated with lower MVD ($p = 0.034$) and higher Ki 67 proliferative index ($p = 0.023$) at the statistically significant level (Table 2). Furthermore, those tumors were characterized by higher nuclear grade ($p = 0.018$) and larger tumor size ($p = 0.009$), but were not statistically significantly associated with the pathologic stage ($p = 0.233$) (Table 3).

Besides the statistics, including the percentage of VEGF staining tumor cells, further analysis was based on VEGF cytoplasimc distribution, which was compared with the same parameters (Tables 2 and 3). A diffuse cytoplasmic staining pattern was found in tumors with higher nuclear grade ($p = 0.005$) and proliferative index ($p = 0.004$), but it was not statistically significantly associated with MVD ($p = 0.152$). There was a nearly significant difference between diffuse and perimembranous cytoplasmic expression of VEGF according to pT stage ($p = 0.056$) but not according to tumor size ($p = 0.371$).

The 5-year survival rate was 71.67% in VEGF expression <75% and 64.29% in VEGF expression >75%, the difference being not statistically significant ($p = 0.651$) (Table 3). A similar result was observed according to cytoplasmic distribution, since the 5-year survival rate was 70.97% and 67.86% in the perimembranous and diffuse VEGF expression group, respectively ($p = 0.687$).

Discussion

The growing awareness of the central role of angiogenesis in the progression of tumors can be used

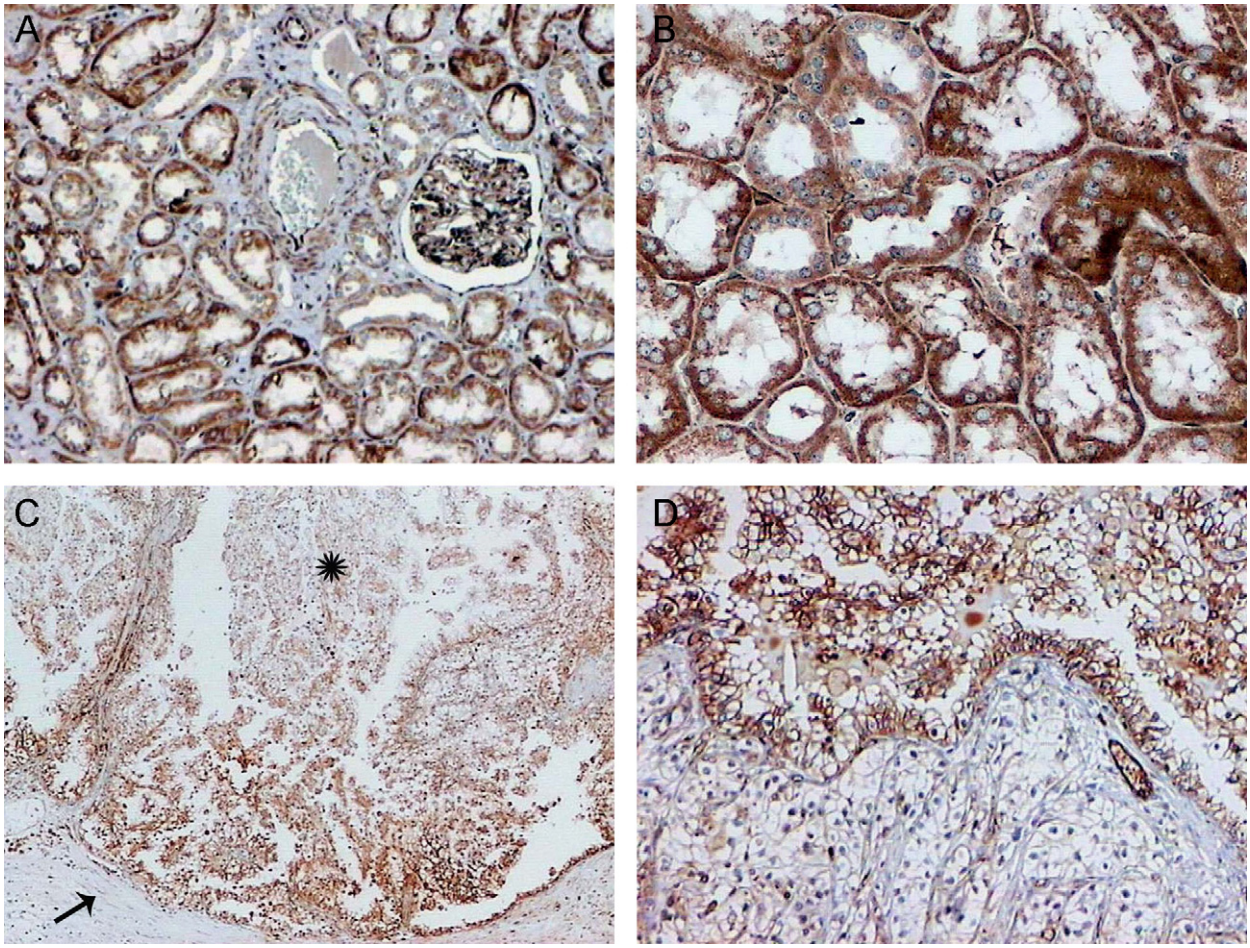

Fig. 1. Immunohistochemical staining for vascular endothelial growth factor (VEGF) in normal renal tissue showing VEGF expression in smooth muscle cells, macrophages in interstitial tissue, mesangial cells in glomerules and in tubular epithelium cytoplasm (A, magnification $\times 100$; and B, magnification $\times 200$). In clear cell renal cell carcinoma (CCRCC), VEGF staining is predominantly heterogeneous, with strong immunoreactivity at the periphery near the pseudocapsule (arrow) and weak or negative staining in the central part of the tumor (asterisk) (C, magnification $\times 40$). Higher magnification of the same tumor sample showing positive staining of VEGF in the upper part of the field (D, magnification $\times 100$).

in the development of antiangiogenic therapy, which specifically targets at suppressing tumor growth and metastasis. As RCC does not respond to any current treatment, there is the need for further identification of tumor characteristics, and tumor angiogenesis might serve as a potential drug target in RCC [7,11,31,32,35].

The results of this study show that normal renal parenchyma and RCC constitutively express VEGF, an angiogenic cytokine with a more homogeneous expression in normal renal parenchyma and heterogeneous overexpression in CCRCC. In some immunohistochemistry analyses, expression of VEGF was not observed in the normal kidney [23], whereas others detected VEGF in the cytosol of normal renal tubular cells [21]. Serum levels of VEGF measured by ELISA showed a highly significant difference between healthy and RCC groups, confirming that it could provide some very useful information for patient screening [4,17,35]. In this study, attention was paid especially to the pattern of VEGF expression according to pathologic and clinical parameters relevant for the disease prognosis. Higher nuclear grade and tumor size, known as independent prognostic parameters, significantly correlated with extensive VEGF expression, i.e., $>75\%$ positive tumor cells. Moreover, higher nuclear grade, higher proliferative rate, and higher pT stage were found in CCRCC with diffuse and intensive cytoplasmic VEGF distribution in tumor cells. These results are in agreement with previous observations that VEGF overexpression is related to tumor progression, having a prognostic significance in RCC [19,20,22,37,38,41]. However, in our study, the prognostic significance of VEGF overexpression was emphasized not only through the percentage of positive tumor cells but also through characterization of the cytoplasmic staining pattern, similar to the report by Yildiz et al. [38].

An interesting finding is the inverse correlation between VEGF expression and microvessel count, also

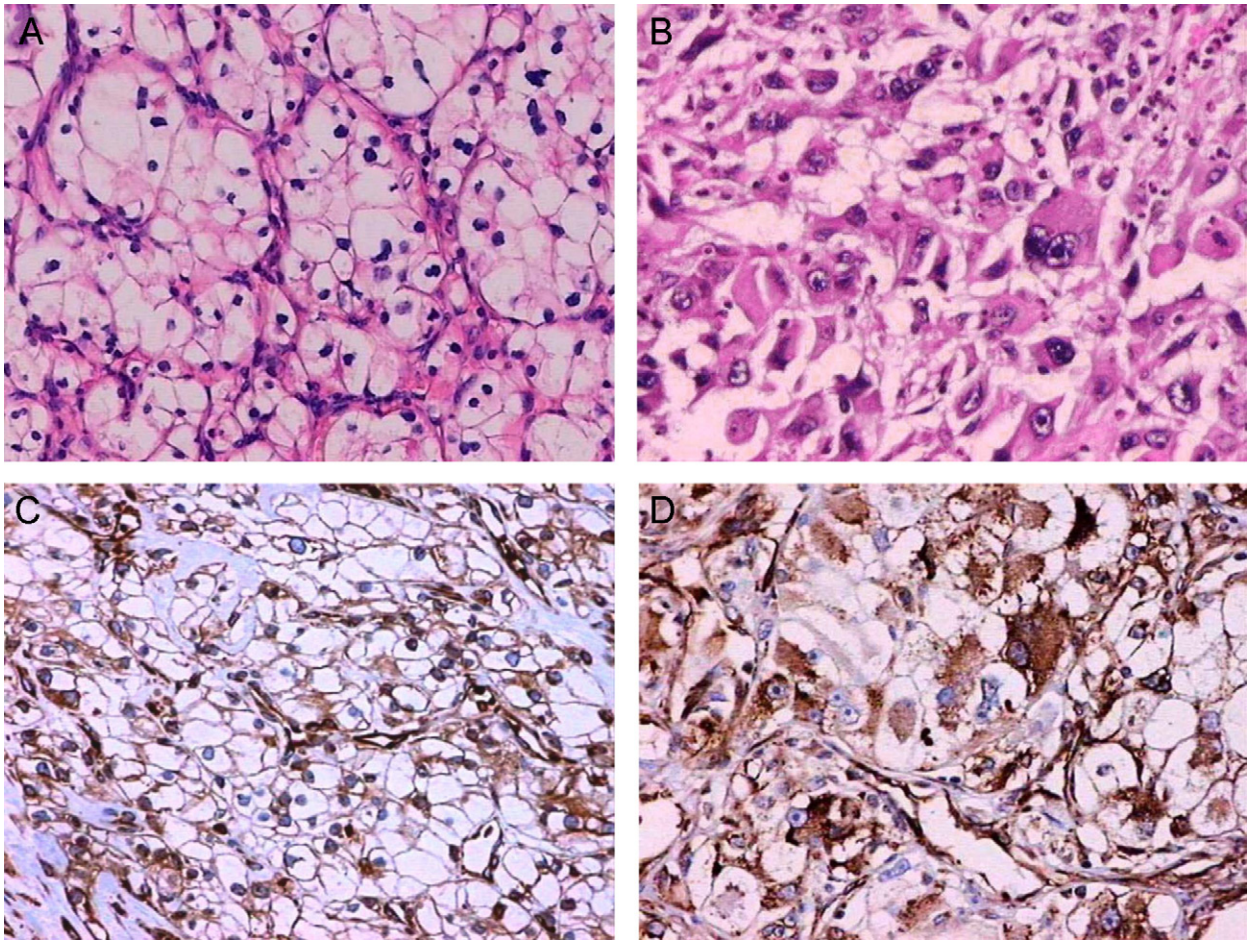

Fig. 2. Comparison between low (A, C) and high (B, D) nuclear grade of clear cell renal cell carcinoma. H&E staining (A, B). Perimembranous (C) and diffuse cytoplasmic immunohistochemical staining of vascular endothelial growth factor (D). Magnification × 200.

Table 1. Frequency distribution of immunohistochemical staining of CCRCC[a] for VEGF[b]

VEGF		*n* (%)
Percentage of positive carcinoma cells staining	<25	25 (27)
	26–50	22 (24)
	51–75	18 (19)
	76–100	28 (30)
Cytoplasmic distribution	Perimembranous	32 (34)
	Diffuse	61 (66)
Staining pattern	Heterogeneous	67 (72)
	Homogeneous	26 (28)

[a]Vascular endothelial growth factor.
[b]Clear cell renal cell carcinoma.

reported by some authors [14,16,18]; however, most studies found opposite results [32,39]. The low degree of vascularity might be explained by increased permeability of the vessel wall and changes in the tumor vascular bed, with the development of large-diameter vascular channels as frequently observed in large tumors [40]. Thus, VEGF alone as a potent inducer of microvascular hyperpermeability and endothelial growth factor could be associated with tumor aggressiveness, increased metastatic potential, and poor prognosis in quite the same way as the increased MVD. The inverse association between tumor grade and MVD could also be explained by the fact that transformed tumor cells are hypoxia-tolerant with reduced oxygen requirements, without the need for neovascularization [28,34]. Some authors also consider MVD as a differentiation parameter. Namely, RCC imitates tubule formation, which is closely associated with blood vessels. High MVD may be considered to reflect normal tissue organization of the renal tubular system. This is supported by the observation that microvascular endothelial cells influence tubulogenesis mediated by VEGF [14]. If MVD is considered a differentiation marker of RCC, the small microvessel count in high-grade tumors becomes understandable. Contradictory results regarding angiogenesis in RCC are very often explained by diverse methodologies and criteria of MVD evaluation used in these studies. We assume that

Table 2. Expression and cytoplasmic distribution of VEGF[a] according to NG[b], MVD[c], and Ki67 proliferative index in CCRCC[d]

VEGF	MVD (mean ± SD)	*p*-value	Ki67 index (%) (mean ± SD)	*p*-value
Expression				
<75	40.8 ± 2.561	0.034	5.5 ± 0.801	0.023
>75	31.2 ± 3.391		10.5 ± 1.708	
Cytoplasmic distribution				
Perimembranous	42.3 ± 3.772	0.152	3.9 ± 0.8	0.004
Diffuse	35.8 ± 2.511		8.709 ± 1.037	

[a]Vascular endothelial growth factor.
[b]Fuhrman nuclear grade.
[c]Microvessel density.
[d]Clear cell renal cell carcinoma.

Table 3. Expression and cytoplasmic distribution of VEGF[a] according to clinicopathologic parameters and 5-year survival rate in patients with CRCC[b]

VEGF	TS[c] (cm) (mean ± SD)	*p*-value	NG[d] (*n*)		*p*-value	PT[e] (*n*)		*p*-value	Survival rate (%)	*p*-value
			1	2		1	2			
Expression										
<75	6.367 ± 0.355	0.009	46	17	0.018	54	9	0.233	71.67	0.651
>75	8.172 ± 0.625		14	16		23	7		64.29	
Cytoplasmic distribution										
	Perimembranous								6.684 ± 0.586	0.371
27	5	0.005	30	2	0.056	70.97	0.687			
Diffuse	7.059 ± 0.387		33	28		47		14		67.86

[a]Vascular endothelial growth factor.
[b]Clear cell renal cell carcinoma.
[c]Tumor size.
[d]Nuclear grade.
[e]Pathologic stage.

the problem also lies in the heterogeneity of architectural properties of the selected tumors.

In conclusion, in spite of the inverse correlation between VEGF expression and MVD, which can be found in CCRCC, overexpression of this angiogenic factor is a worse histologic prognostic factor, since it is associated with higher nuclear grade, higher proliferation, and larger tumors. Antiangiogenic therapy in this tumor would be useful not only for blocking the angiogenesis but probably also for suppressing the autocrine mitogen activity of VEGF.

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