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Expression of vascular endothelial growth factor in renal cell carcinomas

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Abstract Vascular endothelial growth factor (VEGF) is an angiogenic factor that may be involved in tumor growth and metastasis. Only a few data concerning the role of VEGF in renal cell carcinomas (RCCs) are available, and no studies have yet evaluated its prognostic value. The aim of the present study was to assess VEGF expression in a large series of renal tumors with a long follow-up, correlated with the usual histoprognostic factors and survival. VEGF immunostaining was performed on formalin-fixed, paraffin-embedded archival tissue from 74 renal carcinomas (62 conventional renal cell and 12 papillary carcinomas). Positivity of immunostaining was semi-quantitatively scored by two pathologists. Angiogenesis was evaluated by immunostaining with anti-CD34 antibodies on serial sections. Cytoplasmic VEGF expression was detected in tumor cells in 35% (26/74) of RCCs, including 18 out of the 62 (29%) conventional RCCs and 8 out of the 12 (67%) papillary carcinomas (P=0.02). In the group of conventional RCCs, VEGF expression was positively correlated with both nuclear grade (P=0.05) and size of the tumor (P=0.05). Furthermore, a significant correlation was observed between VEGF expression and microvascular count (P=0.04). Finally, cumulative survival rate was significantly lower in the group of patients with conventional RCCs expressing VEGF (log rank test, P=0.01). In the Cox model, VEGF expression was a significant independent predictor of outcome, as well as stage and nuclear grade. This study suggests that VEGF is involved in angiogenesis in conventional RCCs and appears to be a potential prognostic factor in these tumors.

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Introduction

Vascular endothelial growth factor (VEGF) is expressed by many different cell types during various normal and pathologic angiogenesis-related processes, such as embryogenesis, wound healing, ischemic heart disease, tumor growth and metastasis [4, 8, 17]. In contrast to other angiogenic growth factors, such as bFGF and TGF-β, the activity of VEGF is tightly restricted to vascular endothelial cells. VEGF stimulates endothelial cell proliferation in vitro and has also an angiogenic activity in vivo [6, 16].

Clinical and experimental studies have shown that angiogenesis is a prerequisite for both tumor growth and metastasis [17, 27]. Microvessel density (MVD) is correlated with advanced pathological findings and poor clinical outcome in various cancers, including breast, prostate, and bladder carcinomas, and melanomas [2, 12, 14, 26]. Furthermore, it has been shown that metastases are more likely to occur in patients with highly vascularized tumors, suggesting that tumor vascularization could be related to clinical outcome [1]. Since recent data showed that MVD correlated with VEGF expression, this growth factor may play a role in the vascular biology of some tumors as a mediator of angiogenesis [19]. Indeed, VEGF mRNA up-regulation has been demonstrated in several primary human cancers, including central nervous system neoplasms, hepatocellular carcinomas and renal cancers [5, 21, 24, 25]. From these data, it can be hypothesized that VEGF may have prognostic value.

Renal cell cancers (RCCs) are classified in various types according to genetic and morphologic data [15]. Most common are the conventional renal cell carcinomas (mostly composed of clear cells), the papillary and the chromophobe renal cell carcinomas. Conventional renal cell carcinomas are characterized by rich neovascularization and display a fine vascular network around tumor

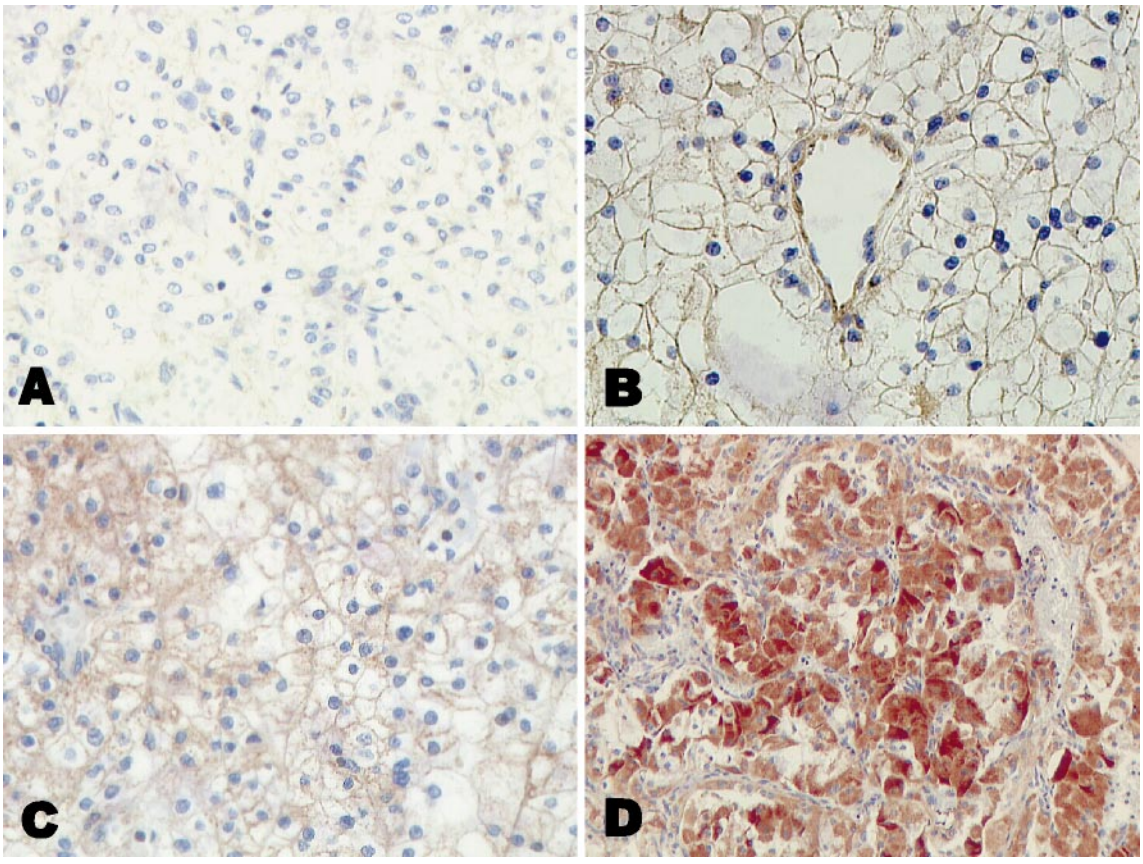

Fig. 1A–D Patterns of VEGF immunostaining in renal conventional renal cell carcinomas. **A** No staining of tumor cells (grade 0). ×40 **B** Membranous without cytoplasmic immunostaining of tumor cells (grade +). Note the labeling of endothelial cells. ×20 **C** Diffuse membrane with cytoplasmic staining in some tumor cells (grade ++). ×40 **D** Diffuse and strong cytoplasmic staining in most tumor cells (grade +++). ×40

cells. Whereas several studies showed that VEGF expression was increased in renal cancers, its relationship with tumor vascularization and its potential prognostic value are unknown [5, 20, 25].

The aim of the study was to investigate VEGF expression in a large series of conventional RCCs with a long follow-up and to assess its relationship with other clinicopathological features including microvessel density. Finally, the prognostic value of VEGF expression was studied in terms of patient survival.

Materials and methods

Tissue samples

Sixty-two conventional renal cell carcinomas were retrospectively selected from the files of the department of pathology of our institution on the basis of available paraffin blocks and adequate follow-up. All cases were reviewed by two pathologists in independent readings. Discordances were resolved by a common reading. For each case, the following histopathological criteria were evaluated: size of the tumor, histological type, pathological stage according to the recently revised TNM system [7], Fuhrman nuclear grade [9], vascular invasion, and presence of necrosis and hemorrhagic areas. The mean age of patients was 60 years, and the mean size of their tumors was 6.6±4.3 cm. Staging was pT1 in 39 cases, pT2 in 7 cases, pT3a in 7 cases and pT3b in 9 cases. Grading according to Fuhrman was 1 in 1 case, 2 in 22 cases, 3 in 30 cases, and 4 in 9 cases.

A group of 12 papillary carcinomas was also studied. The mean age was 60 years and the mean tumor size was 6.3±2.7 cm. Staging was pT1 in 6 cases, pT2 in 3 cases, pT3a in 2 cases and pT3b in 1 case.

Disease-free interval and survival time were obtained for each patient from the attending physicians. Disease-free interval was defined as no evidence of recurrence and/or metastasis at the time of the last follow-up.

Immunohistochemistry

For each case, two representative samples of the RCC and one sample of adjacent normal tissue were selected. The immunohistochemical procedure was performed on paraffin-embedded 5-µm sections using an automated immunostainer (Techmate 500, Dako, Carpinteria, Calif.) with the avidin-biotin-peroxidase method. Rabbit polyclonal antisera to VEGF reacting with VEGF isoforms 121, 165, 189 and 206 (Biogenex, San Ramon, Calif.), and CD34 (Dako) were used at a 1:100 dilution with microwave pretreatment. As negative controls, we used normal sheep, phosphate-buffered saline or irrelevant antibodies instead of primary antibodies.

Immunostaining was independently evaluated by two pathologists, and discordances were resolved in a common reading. For VEGF, staining was semi-quantitatively assessed according to a four-grade scale (0 absence of faint membranous staining of rare tumor cells (<20%), + membrane staining of most tumor cells, ++ diffuse membrane staining and cytoplasmic staining of groups of

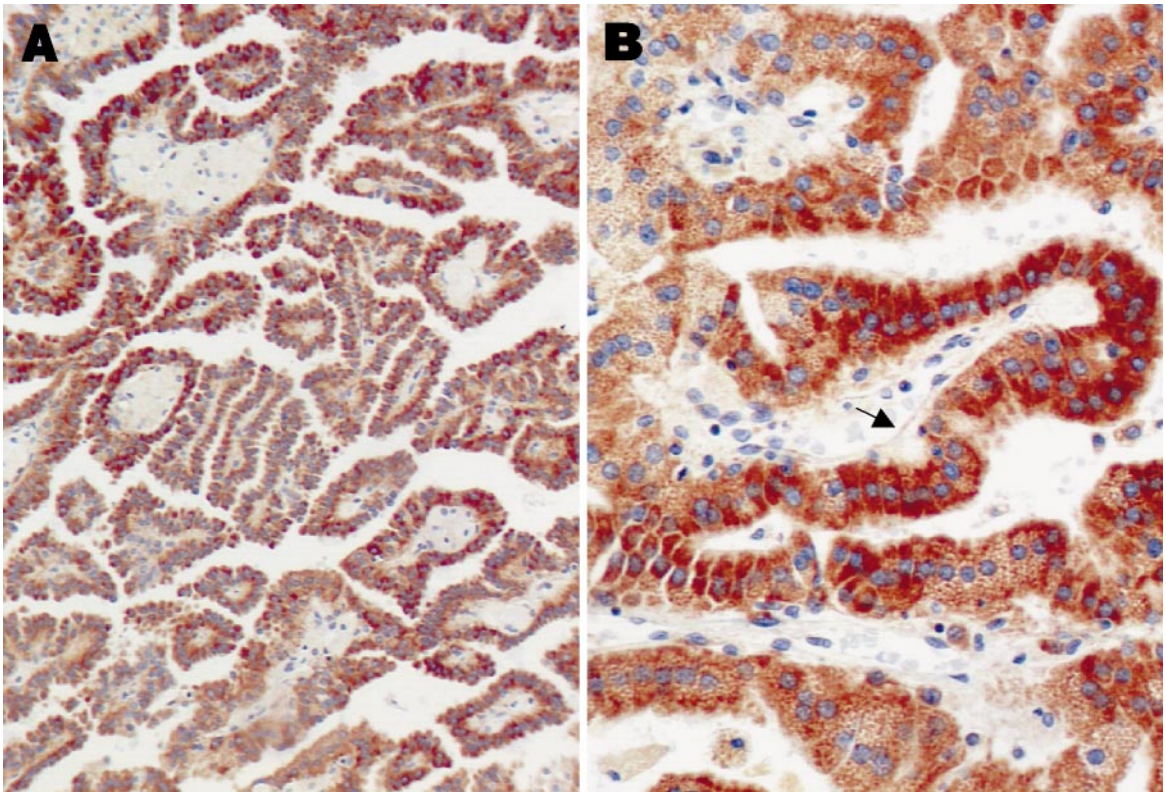

Fig. 2A, B Patterns of VEGF immunostaining in renal papillary carcinomas. **A** All tumor cells display a strong cytoplasmic immunostaining. ×20 **B** At higher magnification, there is no or faint staining of microvessel in the tumoral stroma (*arrow*). ×40

tumor cells (<50%), and +++ significant cytoplasmic staining in most tumor cells, often associated with membranous reinforcement (Fig. 1).

To estimate intratumoral microvessel density (MVD), CD34 immunostaining was performed on the same blocks. The 5 most highly vascularized fields, assessed by CD34 immunostained microvessels (hot spots) at ×200 magnification, were selected and digitalized using the Transpath system (Leica). Microvessels were counted on each digitalized image using a grid, and MVD was determined as the mean value of stained microvessels per HPF.

Statistics

Contingency tables and Fisher's exact tests were used to analyze the relationship between VEGF immunostaining and other pathological features. Each semi-quantitative variable was dichotomized for application to this analysis. Statistical endpoints were overall survival and disease-free period, which were measured from the date of surgery. Life tables were estimated by Kaplan-Meier statistics, and survival curves were compared using the log-rank test. Surviving patients were censored at the time of their last clinical control. A case was censored if death resulted from unrelated disease. Cox multiple regression analysis was performed to evaluate the independent predictive value. All factors that were potentially prognostic at a significant level according to previous single factor analysis were entered in the model. The same evaluation was performed using overall survival and disease-free interval as endpoints. $P<0.05$ was considered statistically significant.

Results

Immunohistochemistry of VEGF

VEGF immunostaining was successfully performed in all cases. In normal kidneys, mild staining of glomerular epithelial cells and proximal epithelial cells was observed. In carcinomas, staining was observed mainly in tumor cells and also in intratumoral microvessels. VEGF staining was graded 0 in 18 cases (24%), + in 30 cases (41%), ++ in 16 cases (22%), and +++ in 10 cases (13%) (Fig. 1). For the rest of the study, results were dichotomized in negative VEGF (0 and +, absent or only membranous staining, 48 cases) versus positive VEGF (++ and +++, membranous and cytoplasmic staining, 26 cases). Among the 62 conventional RCCs, 18 (29%) expressed VEGF cytoplasmic staining. Among the 12 papillary carcinomas, 8 (67%) display cytoplasmic VEGF. In this group, endothelial cells of tumoral microvessels did not show significant immunostaining (Fig. 2). Distribution differed significantly according to the histological type (Fisher's exact test=6.2, $P=0.02$).

According to these results, further analysis was separately performed in the group of conventional renal cell carcinomas and in the group of papillary carcinomas. In the group of conventional renal cell carcinomas, there was a significant association of VEGF expression with size ($P=0.05$) and grade ($P=0.05$). No correlation was observed with age, sex, stage, or presence of necrotic or hemorrhagic areas.

In the group of papillary carcinomas, VEGF expression was inversely correlated with stage ($P=0.005$) but

Table 1 Correlations between vascular endothelial growth factor (*VEGF*) expression and pathological data in conventional renal cell carcinomas (*MVD* microvessel density)

	VEGF negativity (grade 0 or +)	VEGF positivity (grade ++ or +++)	Fisher's exact test	*P*-value	MVD
Conventional renal cell carcinomas					
Size <4 cm	3	19			26±9
Size >4 cm	15	25	4	0.05	22±3
Grade 1 or 2	3	20			24±9
Grade 3 or 4	15	24	4.5	0.05	21±9
Papillary carcinomas					
Stage pT1 or pT2	8	1	8	0.005	9±7
Stage pT3 or pT4	0	3			4±1

not with age, sex, grade or presence of necrotic or hemorrhagic areas. Detailed data are reported in Table 1.

Microvessel quantification

Microvessels were stained by CD34 both in normal and tumoral tissue. No immunostaining was observed in tumor cells. MVD mean value, obtained in the 5 most highly vascularized tumor areas, was 22.3±9.3 in the group of 62 conventional RCCs, and 6.5±5 in the group of papillary carcinomas. This difference was significant (*P*=0.01). In the group of conventional renal cell carcinomas, tumors displaying VEGF had a higher MVD than those without VEGF expression (24.9±12.4 versus 15.4±7.6, *P*=0.04). In this group, MVD was not correlated either with grade or with size of the tumor (Table 1).

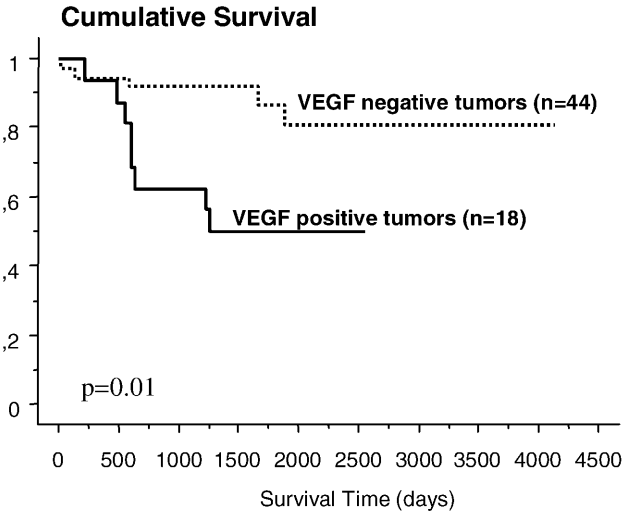

Fig. 3 Kaplan-Meier plots of estimated probability of survival according to cytoplasmic VEGF immunostaining (negative or positive)

Patient survival

The median follow-up of the 62 patients with conventional renal cell carcinomas was 53 months. Eighteen patients died during the follow-up period; 13 of these deaths were related to the disease and 5 to causes other than tumor relapse. Five patients still alive at their last follow-up had tumor recurrence (local relapse or metastases). The cumulative survival at 1, 3 and 5 years was 93%, 82% and 75%, respectively. The disease-free cumulative survival at 1, 3 and 5 years was 86%, 73% and 63%, respectively.

In the group of papillary carcinomas, the mean follow-up was 67 months. Three patients died during follow-up. Two deaths were unrelated to the renal carcinoma. The third was observed 3 months after surgery and the renal tumor did not show any VEGF immunostaining.

Univariate analysis

In conventional renal cell carcinomas, VEGF positivity was associated both with patient survival and with disease-free survival. Patients with RCC displaying VEGF had shorter survival than those with VEGF-negative tumors (log-rank test: *P*=0.01) (Fig. 3). The 5-year cumulative survival in the group of VEGF-negative tumors was 81% versus 50% in the group with VEGF-positive tumors. Patient survival was also significantly correlated with age (*P*=0.02), size (*P*=0.01), stage (*P*=0.001), nuclear grading (*P*=0.05), and MVD (*P*=0.01). The same associations were observed for disease-free survival.

Table 2 Pathological factors independently associated with increased cumulative survival in the group of conventional renal cell carcinomas (*RR* relative risk)

	β	*P*-value	RR
VEGF expression	1.06	0.04	2.9
Stage	1.19	0.01	3.28
Grade	0.69	0.05	2.01

Multivariate analysis

Cox regression analysis, including variables significantly associated with survival in univariate analysis of conventional renal cell carcinomas, showed that stage, VEGF expression and nuclear grade emerged as independent prognostic factors. Detailed data are reported in Table 2.

The same results were obtained when overall or disease-free survival was considered.

Discussion

Angiogenesis, a process occurring in both normal physiological and pathological conditions including cancer, is determined by the balance between angiogenesis inducers and inhibitors [3, 13]. In malignant tumors, angiogenesis mainly results from an increase in the secretion of inducers such as VEGF. Conventional renal cell carcinomas are highly vascularized tumors, and increased levels of VEGF mRNA have been found in most hypervascular tumors [5, 25]. Furthermore, enhanced secretion of VEGF has been correlated with inactivation of the von Hippel-Lindau tumor suppressor gene, which is commonly observed in sporadic and familial conventional renal cell carcinomas [11, 23]. Although the physiological relevance of VEGF overexpression in tumoral angiogenesis is well accepted, its potential prognostic value is subject to debate [25]. Therefore, we investigated neovascularization and VEGF expression in a large series of conventional renal cell carcinomas in relation to survival.

In this study, VEGF immunostaining was initially semi-quantitatively assessed on a four-grade scale, and then divided into two groups according to presence of cytoplasmic immunostaining. According to the four-grade scale, 44 out of the 62 (71%) conventional renal cell caarcinomas exhibited VEGF immunostaining. These data confirm that, as previously described, VEGF is commonly expressed by tumor cells in these tumors. In most conventional renal cell carcinomas, we also observed a significant labeling of neocapillaries, whereas blood vessels in the normal adjacent kidney were unstained. Previous studies have shown that intratumoral blood vessels express mRNA encoding the VEGF receptors. Taken together, these results suggest that VEGF released by tumor cells binds to VEGF receptors on endothelial cells of tumoral stroma [5].

In this study, VEGF expression was positively correlated with the size of the tumor in the conventional renal cell carcinoma group. This result supports the hypothesis that VEGF is associated with tumor growth and progression. Since tumor growth requires the development of neovascularization in the tumoral stroma, VEGF appears to be a key factor in angiogenesis. In support of our data, in two previous studies up-regulation of VEGF was observed in most renal carcinomas studied [20, 25]. Interestingly, these two studies failed to demonstrate any correlation between VEGF expression, assessed either by Western or Northern blot, and the usual clinicopathological prognostic factors such as grade and stage. These results appear to be discordant with our findings, but in the present study, when we compared the group of conventional renal cell carcinomas without any VEGF expression (grade 0) versus those graded +, ++ or +++, we still did not observe any correlation with pathological data or follow-up (data not shown). By contrast, taking as significant only cases showing cytoplasmic immunostaining (++ or +++), we found that these cases were of a significantly higher grade or stage and had a worse prognosis than those without cytoplasmic VEGF immunostaining. Furthermore, in multivariate analysis, cytoplasmic VEGF expression emerged as an independent prognostic factor. This suggests that, although most conventional renal cell carcinomas express VEGF, only a high level of expression has prognostic significance.

Although a correlation was observed between MVD and VEGF expression level, we did not find, as in a previous study, any independent prognostic value for MVD in the conventional renal cell caarcinomas [10, 18].

In this study, VEGF expression was also investigated in papillary carcinomas. Our results show that there are striking differences in terms of VEGF expression between the group of conventional renal cell carcinomas and the group of papillary carcinomas. In the group of papillary cell carcinomas, 68% displayed significant labeling of VEGF. This rate is considerably higher than that observed in conventional renal cell carcinomas. Taking into account our previous results showing a relationship between VEGF expression and poor prognosis, this result might appear surprising, since papillary tumors are usually considered to be of better prognosis. However, this finding must be analyzed with respect to MVD in papillary tumors. Indeed, MVD is significantly lower in the group of papillary carcinomas than in the group of conventional renal cell carcinomas. No or only faint immunostaining of VEGF on microvessel walls was observed in papillary renal cell carcinomas. Overexpression of VEGF immunostaining in the absence of neovascularization suggest that, in papillary tumors, VEGF is not a mediator of neoangiogenesis, and that other factors, such as overexpression of anti-angiogenic factors, the absence of VEGF receptors or defect in VEGF activation, might counteract the angiogenic effect of VEGF. Further studies of VEGF receptor expression in blood vessels and the production of antiangiogenic molecules are required.

In conclusion, VEGF is an angiogenic growth factor involved in the biological behavior of conventional renal cell carcinomas, and our results suggest that it is an independent prognostic factor in survival. Therefore, VEGF might be a therapeutic target of choice in the management of conventional renal cell carcinomas.

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