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Original Article

Vascular Endothelial Growth Factor - Its Relation to Neovascularization and Their Significance as Prognostic Factors in Renal Cell Carcinoma

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Angiogenesis is a series of processes that include endothelial proliferation, migration and tube formation. Vascular endothelial growth factor (VEGF) is regarded as a potent mediator of angiogenesis, vascular permeability and tumor cell growth in renal cell carcinoma. This study was designed to evaluate the expression of VEGF and the microvessel count (MVC) and to determine their prediction efficacies for prognosis in renal cell carcinoma. The relationship between the expression of VEGF and MVC were evaluated immunohistochemically in 50 patients with renal cell carcinoma who received a radical nephrectomy at Wonju Christian Hospital between 1989 and 1997. Microvessels were identified by immunostaining endothelial cells for CD-31 antigen. The mean follow-up was 96 months (3 - 133 months). Overall 5-year survival rate was 71.5%. VEGF was expressed in the tumor cell cytoplasm. Of the 50 tumors, 23 (46%) were weak to strongly positive for VEGF but 27 (54%) were unreactive. The respective 5-year survival rates for patients with positive and negative expressions of VEGF were 70% and 73% ($p > 0.05$). The overall mean MVC was 13.4 in a 400x field. Mean MVCs were significantly higher in VEGF-positive tumors (17.6 $\pm$ 12.1) than in VEGF-negative tumors (9.9 $\pm$ 5.4), and the MVCs of the high vascular density group and the low vascular density groups were significantly different. The 5-year survival rates of patients with high vascular density and low vascular density were 59% and 86%. The median survival period for patients with MVCs higher than or equal to 10 vessels/field was 85 months, whereas for those with MVCs lower than 10 vessels/field the median survival time was 102 months. These results suggest that MVC may be a better prognostic factor in renal cell carcinoma than the expression of VEGF.

Key Words: Renal cell carcinoma, vascular endothelial growth factor, microvessel density

INTRODUCTION

In adults, 80 - 90% of all primary cancers originating from the kidneys are renal cell carcinomas, with distant metastasis discovered at the time of diagnosis in 20 - 50% of all cases. Even in low stage renal cell carcinomas, follow-up studies after radical nephrectomy reveal a 30 - 60% rate of metastatic cancer occurrence.[1] It was also noted that small carcinomas show metastasis, and differences in survival rate and the frequency of distant metastasis are apparent for carcinomas of the same stage. Therefore, since the natural progress of renal cell carcinoma is very hard to estimate, current studies, using molecular biology, are focused on estimating these characteristics during the early stages of cancer.[2-4]

The growth and spread of solid tumors are dependant on the establishment of adequate angiogenesis, which can occur by either sprouting or non-sprouting processes. Sprouting angiogenesis occurs via the branching of new capillaries from existing vessels, whereas non-sprouting angiogenesis results from the enlargement, splitting, and fusion of existing vessels produced by the proliferation of endothelial cells

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within the wall of a vessel.[5] The non-sprouting mechanism is not yet known, though VEGF, which plays a pivotal role in developmental, physiological, and pathological neovascularization, is a candidate effector. VEGF stimulates the proliferation and migration of endothelial cells, and the overexpression of VEGF in tumor cells is known to enhance tumor growth and metastasis in several animal models by stimulating vascularization.[6] The quantitation of microvessel count in the histologic specimens of various neoplasms, including those of the urinary tract, has provided an indication for the risk of metastasis, and many reports have demonstrated a close association between neovascularity and tumor biology or patient clinical outcome.

Renal cell carcinoma is characterized by abundant neovascularization,[7] and a study by Nicol et al.[8] showed that the expression of VEGF increased in renal cell carcinomas. They concluded that angiogenesis might be a prognostic factor in renal cell carcinoma. Thus, angiogenic factors are thought to be involved in the growth and metastasis of renal cell carcinomas, but conclusive studies have yet to be done. Therefore, by studying angiogenic factors, such as VEGF and microvessel density (MVC) in renal cell carcinoma, we hoped to identify the significance of VEGF and MVC as prognostic factors.

MATERIALS AND METHODS

Patients

Fifty of the patients that underwent radical nephrectomy after being diagnosed with renal cell carcinoma at Wonju Christian Hospital between 1989 and 1997, and who we were able to follow-up for at least 6 months, were included in this study. The average age of the patients was 57.7 years (38-76 years), the sex ratio was 1.9 male to 1 female (33:17), and the location of the renal cancer was on the right for 29 cases (58%), and on the left for 21 (42%). Clinical parameters and survival rates we obtained for all subjects and tumor staging was reevaluated by classifying T1 and T2 as smaller or larger than 7 cm; the standard used in the 1997 revised TNM system of the American Joint Committee on Cancer (AJCC). Stage I was the most frequent, found in 22 cases (44%), stage II in 7 cases (14%), stage III in 13 cases (26%), and stage IV in 8 cases (16%). The most frequent histological cell type was the clear cell type with 27 cases (54%)(Table 1). Using H&E staining, specific paraffin blocks representing the histological diagnosis of each patient were chosen and immunohistochemical staining performed.

Immunohistochemistry

Immunohistochemical staining using the anti-VEGF polyclonal antibody (A-20, 1:200, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), which recognizes the VEGF121, VEGF165, and VEGF189 was performed to observe the distribution of angiogenic factors in the neoplastic tissues.

Paraffin sections (4 μm) were made and transferred to gelatin coated slides. These were then deparaffinized and rehydrated through the following solutions: xylene 3 times for 3 minutes each, and serially through 100%, 95%, 80%, and 75% ethanol for 3 minutes each. The tissues were then put in an antigen exposing citric acid solution and boiled in a microwave oven for 10 minutes, incubated with 10% normal goat serum for 30 minutes (to avoid any non-specific reactions), and incubated for 24 h in PBS (4℃) con-

Table 1. Clinical and Histopathologic Characteristics of the Patients

Characteristics	No.
Age (mean) yr	38-76(58)
Sex (Male : Female)	33 : 17
Site (Right : Left)	29 : 21
Tumor stage*(N=50)	
I	22
II	7
III	13
IV	8
Histologic type	
Clear cell	27
Chromophobe cell	8
Mixed cell	8
Papillary cell	3
Collecting duct cell	2
Sarcomatoid	2

*according to the AJCC staging(1997).

taining the primary antibody, 0.3% Triton X-100, 0.5 mg/ml bovine serum albumin and 1.5% normal horse serum. After each incubation step, they were washed three times with PBS for a total of 15 minutes. Tissues were then treated with biotinylated goat anti-mouse IgG (1:200) for 30 minutes at room temperature, followed by treatment with avidin-peroxidase for 30 minutes. After treatment with 1% H_2O_2 and 3-amino-9-ethyl carbazole (AEC) containing phosphate buffered saline for 5 minutes (for colour development), sections were counterstained in hematoxylin for 30 seconds. Finally the prepared slides were coverslipped and observed under the microscope. Sections not treated with the primary antibody, or adjacent normal tissue were used as negative controls. The sections were observed under 100X, and 400X magnification by two pathologists to confirm VEGF expression. The intensity and distribution of the staining were both assigned scores from 0 to 3, which were summed, and if this sum was less than 3 the sample was considered negative and if greater than 4 it was considered positive (Fig. 1). Immunohistochemical staining was performed on the same block using anti-CD 31 monoclonal antibody (1:400, DAKO Inc., Carpinteria, CA, USA) to estimate intratumoral MVC. The MVCs of renal cell carcinoma tissues were calculated by first observing the complete tissue under a low power light microscope, to find an area of high vascular distribution, and then counting either the microvasculature or the endothelium stained by anti-CD31 monoclonal antibody at high magnification (Fig. 2). The average of the 5 highest density areas of were chosen from the section was calculated, but excluding large vessels of more than 6 RBCs. The presence of a vascular lumen was not necessary to identify a microvessel. Patients were subsequently divided two groups, the high vascular density group (MVC $\geq$ 10 vessels) and low vascular density group (MVC $<$ 10 vessels).

Statistical analysis

Statistical analyses were performed using the SPSS for windows 7.5 statistical program. Student t-test, the Chi-square test and the Kaplan-Meier

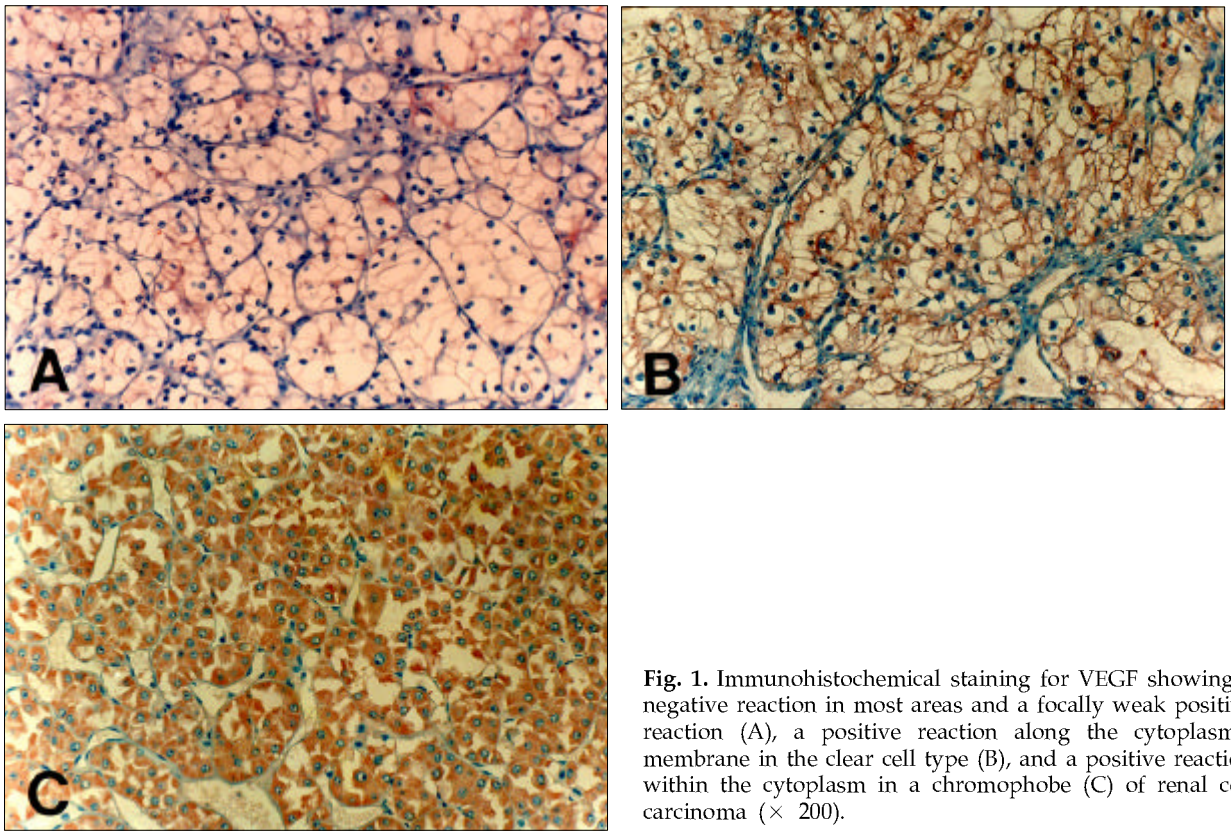

Fig. 1. Immunohistochemical staining for VEGF showing a negative reaction in most areas and a focally weak positive reaction (A), a positive reaction along the cytoplasmic membrane in the clear cell type (B), and a positive reaction within the cytoplasm in a chromophobe (C) of renal cell carcinoma (× 200).

survival curve were used to analyze differences between stages and differences between the two groups. Statistical significance was accepted at $p < 0.05$.

RESULTS

Mean follow-up was 96 months (3-133 months). During the period of follow-up 15 patients died of renal cell carcinoma, and the overall 5-year survival rate was 71.5%. Immunohistochemical staining for VEGF revealed a positive reaction along the cytoplasm in the clear cell type and within the cytoplasm in the chromophobe type (Fig. 2). Expression of VEGF according to tumor size showed 14 positive cases (28%) when the size was less than 7 cm, and 9 positive cases (18%) when size was greater than or equal to 7cm, with no statistical significance (Table 2). In addition, no difference in the expression of VEGF was seen when the cell types were divided into the clear cell and the non-clear cell types, and no statistical significance was observed versus nuclear grade and stage. However, the relationship between MVC and VEGF expression showed statistical significance. 16 cases (32%) were negative reaction of VEGF expression and 6 cases (12%) were positive reaction for the low vascular density group and 17 cases (34%) were positive reaction and 11 cases (22%) were negative reaction for the high vascular density group in the high power field (Table 2) ($p < 0.05$). When MVC was divided into low vascular density group and high vascular density group subsequent clinical and pathological relationships were found. For a tumor size < 7 cm, 16 cases (59.3%) were low density and 11 cases (40.7%) were high density, similarly, for a tumor size > 7 cm, 6 cases (26.1%) were low den-

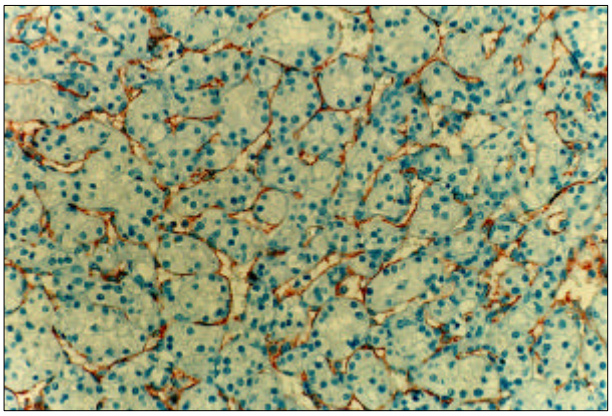

Fig. 2. Immunohistochemical staining for CD 31 in the clear cell type of renal cell carcinoma (× 200). The MVCs of renal cell carcinoma tissues were calculated by first observing the complete tissue under a low power light microscope, to find an area of high vascular distribution, and then counting either the microvasculature or the endothelium stained by anti-CD31 monoclonal antibody at high magnification.

Table 2. Relationship between Expression of Vascular Endothelial Growth Factor and Clinicopathologic Parameters

Parameter	Expression of VEGF		*P*-value
	Negative	Positive	
Size			$p > 0.05$
< 7 cm	13	14	
≥ 7 cm	14	9	
Cell type			$p > 0.05$
clear cell	17	10	
non-clear cell	10	13	
Nuclear grade*			$p > 0.05$
low (1, 2)	16	14	
high (3, 4)	11	9	
Stage			$p > 0.05$
low (I, II)	11	11	
high (III, IV)	16	12	
MVC			$p > 0.05$
Low	16	6	
High	11	17	

VEGF, vascular endothelial growth factor; MVC, microvessels count.
*Fuhrman grade.

sity and 17 cases (73.9%) were high density (Table 3) ($p < 0.05$). In addition, for the lower stage cancers (I, II), 16 cases (72.7%, 5.9 ± 2.2) were low density, and of the higher stage cancer (III, IV), 22 cases (78.5%, 17.0 ± 7.6) were high density, therefore showing statistical significance.

However, no statistical significance was found for cell type or nuclear grade (Table 3). The relationship between MVC and VEGF expression showed 23 cases (46%, 17.6 ± 12.1) to be VEGF positive, and 27 cases (54%, 9.9 ± 5.4) to be VEGF negative, showing that MVC increased as VEGF expression increased (Fig. 3) ($p < 0.05$). However, no statistical significance was found between the degree of expression and distribution. As shown in the Kaplan-Meier survival curve (Fig. 4), the 5-year survival rates of patients with positive and negative VEGF expressions were 70% and 73% ($p > 0.05$). Nevertheless, there was a statistical difference in the MVCs of the high vascular density group and the low vascular density group (Fig. 5) ($p < 0.05$). The 5-year survival rate for patients with high vascular density and low vascular density was 59% and 86%, and the median survival time for patients with MVC greater than or equal to 10 vessels/field was 85 months, whereas that of those with an MVC lower than 10 vessels/field was 102 months.

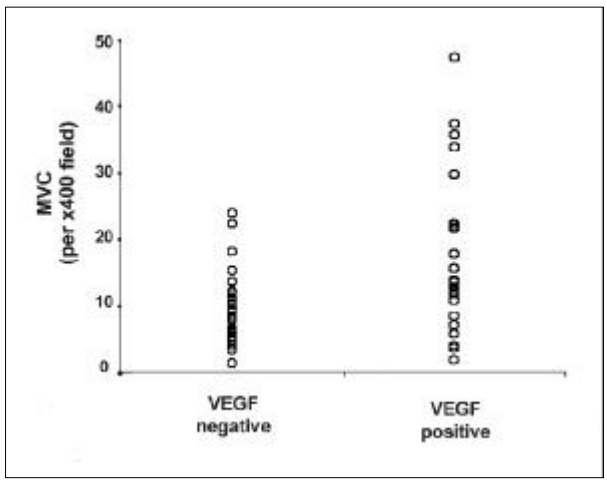

Fig. 3. Microvessel count in case of VEGF-positive and VEGF-negative renal cell carcinoma ($p < 0.05$).

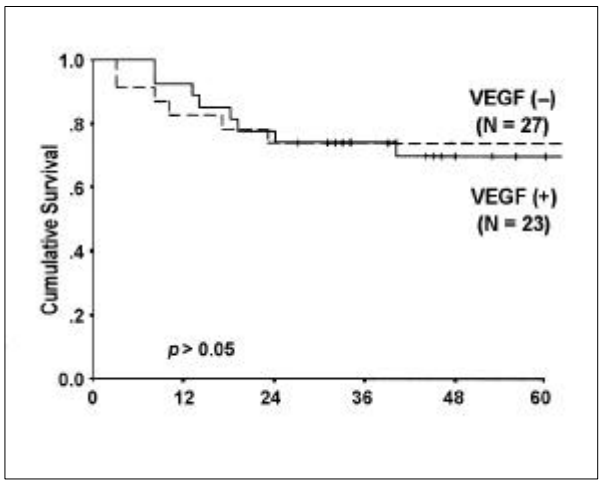

Fig. 4. Kaplan-Meier survival curve according to the expression of VEGF.

Table 3. Relationship between Mean Microvessel Count and Histopathologic Parameters

Parameter	MVC < 10/HPF		MVC ≥ 10/HPF		p-value
	No. of Pts. (%)	MVC (± SD)	No. of Pts. (%)	MVC (± SD)	
Size					$p < 0.05$
< 7 cm	16 (32)	5.9 (± 2.3)	11 (22)	22.7 (± 12.5)	
≥ 7 cm	6 (12)	6.9 (± 2.7)	17 (34)	16.9 (± 6.9)	
Cell type					$p > 0.05$
clear cell	11 (22)	6.4 (± 2.1)	16 (32)	19.8 (± 11.3)	
non-clear cell	11 (22)	6.0 (± 2.8)	12 (24)	18.3 (± 7.4)	
Nuclear grade*					$p > 0.05$
low (1,2)	16 (32)	6.1 (± 2.3)	14 (28)	21.2 (± 11.7)	
high (3,4)	6 (12)	6.5 (± 3.0)	14 (28)	17.1 (± 7.0)	
Stage					$p < 0.05$
low (I, II)	16 (32)	5.9 (± 2.3)	6 (12)	27.1 (± 12.9)	
high (III, IV)	6 (12)	6.9 (± 2.7)	22 (44)	17.0 (± 7.6)	

MVC, microvessels count.
*Fuhrman grade.

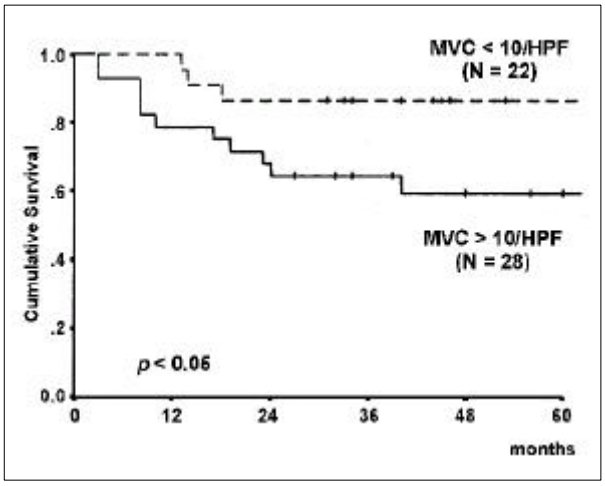

Fig. 5. Kaplan-Meier survival curve according to the microvessel count.

DISCUSSION

Neovascularization in renal cell carcinoma is frequently found during selective renal angiography. In addition, the removal of the primary site of renal cell carcinoma results in the occasional natural disappearance of the metastatic site, and also results in occasional recurrence and metastasis a few years after surgery. The tumor stage at the time of surgery remains the most important prognostic factor for renal cell carcinoma.[9] Therefore, many studies have focused on finding a better prognostic factor or a tumor marker of this carcinoma.

Neovascularization is a process in which many types of angiogenic factors react with the receptors of vascular endothelial cells to stimulate splitting and migration to form new vessels. Recent studies have found that angiogenesis involves the interactions of positive (VEGF, PDGF, bFGF, angiogenin, etc.) and negative regulators (thrombospodins, TIMP-1, TIMP-2, etc.), which are delicately controlled in time and space. They temporarily appear and disappear according to the development stage, i.e., differentiation in the embryo, or reproduction and wound repair in the adult. However, in the case of a malignant tumor, the formation of positive regulators is insufficient during growth, infiltration and progression, which leads to the continuous formation of vessels. This newly formed vasculature supplies nutrients and oxygen to the tumor cells and excretes waste, and through this mechanism the tumor cell can grow endlessly and can migrate to other organs.[10,11] Also, recent studies have shown that angiogenesis greatly influences the growth and migration of tumors, such as malignant melanoma, breast cancer, ovarian cancer, non-small cell lung cancer, and prostate cancer, and the MVC of the tumor has been found to be a significant prognostic factor in estimating long term survival.[12-16] Many authors have stated that angiogenesis in tumor cells is a significant prognostic factor predicting recurrence, and is also closely correlated with tumor growth rates, metastasis, and survival rates in patients with renal cell carcinoma who undergo radical nephrectomy. VEGF expression has also been proven to increase the occurrence of renal cell carcinoma.[17,18]

The VEGF gene encodes a disulfide-linked dimeric glycoprotein, moreover, four different molecular species with 121, 145, 165, 189, and 206 amino acids are determined by alternative splicing of VEGF mRNA. Tomisawa et al. reported that VEGF 121 + 165 + 189 expressions in human renal cell carcinoma were 70.2%,[19] and Takahashi et al. reported that two shorter forms, such as VEGF121 and VEGF165 were predominantly expressed.[7] Jacobsen and colleagues[20] showed that the expression of serum VEGF mRNA in renal cell carcinoma was higher than in patients with benign renal tumors, and VEGF mRNA expression was also associated with stage and differentiation, and particularly with renal vein invasion. Furthermore, Takahashi et al. reported that VEGF expression was evident in 26 of 27 patients (96%) with renal cell carcinoma, and that this was 3-13 times higher than in normal tissue.[7] However, Edgren and colleagues[21] reported that no correlation was observed between VEGF and the presence or absence of metastases, nor with patient survival. In our study, the expression of VEGF was found not to affect the survival rate or to be a statistically meaningful prognostic factor, but Sasaki[18] found a close correlation between VEGF expression and MVC. Therefore, although the expression of VEGF did not impact the survival rate, it was strongly correlated with MVC, and thus could be of some value as a prognostic factor. To confirm the possible increase of neovasculari-

zation, immunohistochemical staining for von Willebrand's factor (factor VIII), an endothelial antigen, or more recently immunohistochemical staining of surface antigens, such as CD-31, CD-34, are being used. MacLennan and Bostwick found that microvessel density in renal cell carcinoma was of no prognostic significance. We believe that this is because microvessel density is more than 3 times higher in renal cell carcinoma than in other malignant tumors, such as skin melanoma, invasive breast cancer, or non-small-cell lung cancer.[22] However, Yoshino and colleagues showed that the survival of patients with less than 30 microvessels per 200 x field was significantly better than that of patients with more than 30 microvessels per 200 x field in localized renal cell carcinoma.[19] Nativ and colleagues reported similar results.[23] Our study also show that a standard MVC of 10 at a magnification of 400X, shows that MVC impacts the survival rate. In addition, MVC was also found to correlate with clinical stage and VEGF expression.

In conclusion, angiogenesis of the neovasculature is a necessary factor in the local growth, metastasis, recurrence and malignant tendency of tumors. Therefore, many studies have attempted to identify angiogenic factors. However, all treatment options, except radical nephrectomy, for renal cell carcinoma are the subjects of much criticism, and the best treatment plan is reported to be early diagnosis and early radical nephrectomy. Even when a tumor is diagnosed early, many cases have shown micrometastasis, and distant metastasis at the time of diagnosis, and these situations tend to have a negative influence on prognosis. Thus, if an angiogenic factor could be applied as a prognostic factor, patients with poor prognoses could receive a more aggressive treatment plan to help improve survival.

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