Expression of Vascular Endothelial Growth Factor in Renal Cell Carcinoma Is Correlated With Cancer Advancement

Ching-Chiang Yang,^{1*} Kang-Chu Chu,² and Wen-Meng Yeh³

¹Department of Urology, China Medical College Hospital, China Medical College, Taichung, Taiwan

²Department of Pathology, China Medical College Hospital, China Medical College, Taichung, Taiwan

³Department of Urology, China Medical College Peikang Hospital, Peikang, Taiwan

Vascular endothelial growth factor (VEGF) functions as a regulator of neovascularization in malignant cells. VEGF as a mitogen is thought to alter renal cell carcinoma formation and tumor progression. We investigated the expression of the VEGF gene in order to evaluate its clinical significance in renal cell carcinoma. Tissue samples from 198 patients with renal cell carcinoma were examined with an immunohistochemical stain for the expression of the VEGF gene. The expression rate was compared to 34 normal renal cortical samples obtained from renal surgery from noncancer patients. There were significant differences between normal renal cortex

(0%) and cancer tissue (54.5%) in positive staining of VEGF protein ($P < 0.001$). With the progression of tumor grade, the positive rate of VEGF gene expression significantly increased. The expression rate of the VEGF gene in the advanced group, such as with lymph node involvement or vein invasion, was greater than that in the locally confined group ($P < 0.001$). The results revealed that expression of the VEGF gene is proportional to the formation and progression of renal cell carcinoma, which may allow VEGF to be used as a prognostic marker for renal cell carcinoma. *J. Clin. Lab. Anal.* 17:85–89, 2003. © 2003 Wiley-Liss, Inc.

Key words: renal cell carcinoma; vascular endothelial growth factor gene; VEGF

INTRODUCTION

Renal cell carcinoma (RCC) is a multifactorial, complex disease involving both environmental and genetic factors. RCC is characterized by a solid tumor arising from the proximal convoluted tubules of the kidney. The clinical behavior of RCC is unpredictable. Metastasis of RCC can spontaneously regress after the primary tumor is removed, or metastasize many years after a complete surgical treatment. Although clinical staging is an important prognostic factor, because of variable clinical outcomes it is not sufficient for predicting tumor advancement. Therefore, the combination of clinical tumor staging and other markers may help to predict the tumor behavior. RCC is also known to be highly vascular, and angiogenesis may play an important role in the tumor growth and metastasis. Neovascularization is an essential event for the growth of solid tumor and is regulated by a number of angiogenic factors (1,2). One such factor, vascular endothelial growth factor (VEGF), is an angiogenic factor that may be involved in tumor advancement and metastasis (3–5), and is believed to exert potential angiogenic activity (6).

The human VEGF gene is located at chromosome 6p12. Sato et al. (7) and Jacobsen et al. (8) measured serum levels of VEGF in controls and patients with RCC, and found significant differences between the two groups. They determined that serum levels of VEGF were significantly correlated with tumor volume. Their primary conclusions were that tumor tissue of RCC liberates VEGF into the systemic blood flow, and that serum VEGF is a possible marker for RCC. However, in an analysis of VEGF in 53 patients with RCC, Edgren et al. (9) found no correlation between VEGF serum levels and tumor stage, patient survival, or tumor metastasis. The serum level of VEGF as a tumor marker appears to be of limited value. In the present study, immunohistochemical staining was used to examine and compare VEGF

*Correspondence to: Ching-Chiang Yang, M.D., Department of Urology, China Medical College Hospital, China Medical College, 2 Yu-Der Road, Taichung 404, Taiwan. E-mail: drtom@www.cmch.org.tw

Received 15 August 2002; Accepted 9 October 2002

DOI 10.1002/jcla.10074

Published online in Wiley InterScience (www.interscience.wiley.com).

gene expression in RCC and normal renal cortex in order to elucidate the role played by VEGF in tumor formation and progression.

PATIENTS AND METHODS

Source of Specimens

Specimens were obtained from 198 diagnosed RCC patients (127 males and 71 females, 38–82 years old, average age 64.3 years) who had undergone radical nephrectomy in two medical centers between June 1987 and June 1995. As a control group, 34 specimens of normal renal cortex were obtained from trauma, nephrolithiasis, and congenital malformation patients who had undergone nephrectomies. Informed consent was obtained from all of the subjects. Pathological grading was performed according to Skinner et al. (10) as follows: grade 1 = 36; grade 2 = 54; grade 3 = 86; and grade 4 = 22. Positive lymph node involvement, including the perirenal hilum, para-aortic, and inferior vena cava, was found in 83 patients. Seventy-nine patients had either renal vein or inferior vena cava invasion.

Immunohistochemical Staining for VEGF

Sections (4 μ m) of formalin-fixed, paraffin-embedded tissues mounted on poly-d-lysine-coated slides were

deparaffinized in xylene and rehydrated through serial baths of alcohol and water. The slides were placed in 6 M urea and heated in the microwave oven at 700 W for 5 min. The hydrated sections were then treated in methanol containing 0.3% hydrogen peroxide for 30 min to eliminate endogenous peroxidase activity. They were then washed in phosphate-buffered saline (PBS). The primary antibody used in this study was a polyclonal antibody to VEGF (Santa Cruz, CA). The polyclonal antibody-treated slides were rinsed in PBS solution and incubated with a biotinylated secondary antibody (Signet, Dedham, MA). The slides were washed in PBS, and then incubated with an avidin-biotin-peroxidase complex (Signet, Dedham, MA) for 30 min. After the slides were washed with PBS, a chromogenic reaction was developed by incubating the slides with a freshly prepared solution of 3,3'-diaminobenzidine tetrahydrochloride (0.04%) and hydrogen peroxide (0.03%). A reddish-brown substrate in the cytoplasm indicated a positive reaction to VEGF (Fig. 1). For the negative controls, we used all reagents except the primary antibody.

The expressions of the VEGF proteins were analyzed in the entire section of microscopic fields. The mean fraction of these fields was calculated and used in further statistical analysis. Tumor cytoplasm were considered positive for VEGF when a clear

Fig. 1. Positive immunohistochemical staining in cytoplasm of VEGF can be seen in the reddish-brown particle (20 $\times$ 40 magnification).

TABLE 1. Expression rate of vascular endothelial growth factor in renal cell carcinoma cancer tissue by comparison with normal kidney tissue

Classification	Positive	Negative	Total	χ^2	P value
Normal control	0 (0%)	34 (100%)	34 (100%)		
Cancer	108 (54.5%)	90 (45.5%)	198 (100%)		<0.001 ^a
Pathological grading					
Grade I+II	30 (33.4%)	60 (66.6%)	90 (100%)		
Grade III+IV	78 (72.2%)	30 (27.8%)	108 (100%)	29.94	<0.001 ^b
Lymph node involvement					
Negative	35 (30.4%)	80 (69.6%)	115 (100%)		
Positive	73 (87.9%)	10 (12.1%)	83 (100%)	64.32	<0.001 ^b
Vein invasion					
Negative	45 (37.8%)	74 (62.2%)	119 (100%)		
Positive	63 (79.7%)	16 (20.3%)	79 (100%)	33.67	<0.001 ^b
3-Year survival					
Survival	9 (15.8%)	48 (84.2%)	57 (100%)		
Death	66 (82.5%)	14 (17.5%)	80 (100%)	59.78	<0.001 ^b

^aFisher's exact test.^bChi-square test.

staining signal was present. The cutoff limits of VEGF were set at 10%.

Statistical Processing

The chi-square test and Fisher's exact test were used to compare the differences between normal bladder epithelium and cancer tissue. The SPSS[®] (Statistical Package for the Social Sciences) system was used for the calculations. Results were considered statistically significant when the probability of findings occurring by chance was <5% ($P < 0.05$).

RESULTS

Expression of VEGF in RCC

Table 1 shows the expression rates of VEGF in normal renal cortex and cancer tissue. The positive expression rate of VEGF in RCC was 54.5%, while that of the normal renal cortex group was 0% ($P < 0.001$, Fisher's exact test). In pathological grading, the rate of positive expression of VEGF was 33.4% (grades 1 and 2) and 72.2% (grades 3 and 4). There were statistically significant differences between these classes ($P < 0.001$). There was a significant difference between positive and negative lymph node involvement in the expression of VEGF ($P < 0.001$, chi-square test). The expression of VEGF in the vein invasion group (79.7%) was significantly higher than in the negative vein invasion group (37.8%) ($P < 0.001$).

DISCUSSION

This study showed a positive correlation between VEGF expression and cancer progression. The VEGF gene was expressed in 0 of 12 normal renal cortical

samples (0%), while in the RCC group 108 out of 198 samples (54.5%) showed positive expression. It is clear that positive expression of VEGF is associated with RCC. Positive expression of VEGF protein appears to be related to a higher incidence of cancer formation and tumor progression. The current data indicate that expression of VEGF is associated with advanced tumors; therefore, the expression of VEGF may be a useful predictive marker for tumor advancement.

VEGF, a homodimeric glycoprotein of relative molecular mass 45,000, is the only mitogen that specifically acts on endothelial cells (11). It may be a major regulator of tumor angiogenesis in vivo. Folkman (12) noted the importance of VEGF and its receptor system in tumor growth, and suggested that intervention in this system may provide promising approaches to cancer therapy (12). Helmlinger et al. (13) proposed that autocrine endothelial VEGF contributes to the formation of blood vessels in a tumor and promotes its survival. VEGF is involved in angiogenesis in conventional RCCs and appears to be a potential prognostic marker (3). Our data are compatible with previous reports indicating that VEGF may be of clinical significance in the prognosis of RCC.

Tumor growth to the metastatic tumor focus needs additional factors to enhance neovascularization in favor of tumor growth on the vascular bed. Therefore, there is a relationship between vascularization and clinical outcome in RCC. Increased serum and urinary levels of VEGF have been associated with poor prognosis in RCC (7,8,14). However, previous studies demonstrated low levels of mRNA expression localized to glomerular visceral epithelial cells in the normal kidney (15). Hemmerlein et al. (16) found that the expression of VEGF in RCC is due to a highly

proliferative tumor that outgrows its vascular supply and develops chronic hypoxia, causing decreased proliferation. Therefore, the expression of VEGF indicates advanced tumor progression.

Krupski et al. (17) found that overexpression of VEGF is organ/site-specific, which suggests that tumor microenvironmental factors also determine the mode of tumor spread. VEGF mediates neo-angiogenesis during tumor progression, and is probably associated with a signaling system and other factors. Fibroblast growth factor (FGF) is also correlated with VEGF as a synergistic means of mediating neo-angiogenesis of prostate cancer (18). Cvetkovic et al. (19) found that an increased level of hypoxia is associated with increased expression of VEGF in human prostate cancer, which indicates that upstream signaling of VEGF must be activated. VEGF stimulates tumor growth via binding to fetal liver kinase (Flk-1) and is promoted by tumor cell autostimulation and neo-angiogenesis signals, such as hypoxia (20). Therefore, the VEGF gene may play a partial role in the progression of cancer. Androgen, interleukin-1, and tumor necrosis factor have been reported to regulate VEGF in the enhancement of neovascularization (21,22). Other studies have indicated that VEGF combines upstream signals and other growth factors to enhance tumor growth and progression (23).

Although the current data showed a higher expression rate of VEGF in advanced tumor and metastasis than in the localized and lower-grade tumor, a discrepancy still remains. Yano et al. (23) studied the relationship between VEGF expression and brain metastasis *in vitro*, and demonstrated that VEGF is necessary but not sufficient for the production of metastasis in RCC. Other tumors, such as colon cancer and lung adenocarcinoma without VEGF expression, can produce fast-growing in brain metastasis, which indicates that different tumor cells may have different responses to angiogenesis. Edgren et al. (9) reported no correlation between tumor stage and serum levels of VEGF in advanced RCC. However, in agreement with other reports (14, 16), our study showed a positive correlation. Further research as to whether anti-angiogenesis inhibits VEGF in advanced RCC may help explain this discrepancy.

VEGF plays both paracrine and autocrine roles in tumor progression, which means that it is both an angiogenic factor and a mediator of tumor cell motility (24). VEGF has been proposed to be a useful prognostic marker for RCC (3). Our data showed an increased expression of VEGF in RCC tumor cells compared to normal renal cortex. The expression was higher in advanced tumors than in lower-grade tumors. Based on results from a 3-year follow-up, VEGF expression is

associated with poor prognosis. Therefore, we conclude that expression of the VEGF gene is correlated with tumor formation and progression in RCC.

REFERENCES

1. Folkman J, Shing Y. Angiogenesis. *J Biol Chem* 1992;267:10931–10934.
2. Liotta LA, Steeg PS, Stetler-Stevenson WG. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 1991;64:327–336.
3. Dvorak HF, Brown LF, Detmar M, Dvorak AM. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 1995;146:1029–1039.
4. Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 1995;55:3964–3968.
5. Takahashi Y, Tucker SL, Kitadai Y, et al. Vessel counts and expression of vascular endothelial growth factor as prognostic factors in node-negative colon cancer. *Arch Surg* 1997;132:541–546.
6. Paradis V, Lagha NB, Zeimoura L, et al. Expression of vascular endothelial growth factor in renal cell carcinomas. *Virchows Arch* 2000;436:351–356.
7. Sato K, Tsuchiya N, Sasaki R, et al. Increased serum levels of vascular endothelial growth factor in patients with renal cell carcinoma. *Jpn J Cancer Res* 1999;90:874–879.
8. Jacobsen J, Rasmuson T, Grankvist K, Ljungberg B. Vascular endothelial growth factor as prognostic factor in renal cell carcinoma. *J Urol* 2000;163:343–347.
9. Edgren M, Lennernas B, Larsson A, Kalkner KM. Angiogenic factors: vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (b-FGF) are not necessarily elevated in patients with advanced renal cell carcinoma. *Anticancer Res* 2001;21:1423–1430.
10. Skinner DG, Colvin RB, Vermillion CD, Pfister RC, Leadbetter WF. Diagnosis and management of renal cell carcinoma. A clinical and pathologic study of 309 cases. *Cancer* 1971;28:1165–1177.
11. Ferrara N, Houck KA, Jakeman LB, Winer J, Leung DW. The vascular endothelial growth factor family of polypeptides. *J Cell Biochem* 1991;47:211–218.
12. Folkman J. Clinical applications of research on angiogenesis. *N Engl J Med* 1995;333:1757–1763.
13. Helmlinger G, Endo M, Ferrara N, Hlatky L, Jain RK. Formation of endothelial cell networks. *Nature* 2000;405:139–141.
14. Chang SG, Jeon SH, Lee SJ, Choi JM, Kim YW. Clinical significance of urinary vascular endothelial growth factor and microvessel density in patients with renal cell carcinoma. *Urology* 2001;58:904–908.
15. Brown LF, Berse B, Tognazzi K, et al. Vascular permeability factor mRNA and protein expression in human kidney. *Kidney Int* 1992;42:457–461.
16. Hemmerlein B, Kugler A, Ozisik R, Ringert RH, Radzun HJ, Thelen P. Vascular endothelial growth factor expression, angiogenesis, and necrosis in renal cell carcinomas. *Virchows Arch* 2001;439:645–652.
17. Krupski T, Harding MA, Hecce ME, et al. The role of vascular endothelial growth factor in the tissue specific *in vivo* growth of prostate cancer cells. *Growth Factors* 2001;18:287–302.
18. West AF, O'Donnell M, Charlton RG, et al. Correlation of vascular endothelial growth factor expression with fibroblast

- growth factor-8 expression and clinico-pathologic parameters in human prostate cancer. *Br J Cancer* 2001;85:576–583.
19. Cvetkovic D, Movsas B, Dicker AP, et al. Increased hypoxia correlates with increased expression of the angiogenesis marker vascular endothelial growth factor in human prostate cancer. *Urology* 2001;57:821–825.
 20. Kollermann J, Helpap B. Expression of vascular endothelial growth factor (VEGF) and VEGF receptor Flk-1 in benign, pre-malignant prostate tissue. *Am J Clin Pathol* 2001;116:115–121.
 21. Joseph IB, Nelson JB, Denmeade SR, et al. Androgen regulate vascular endothelial growth factor content in normal and malignant prostatic tissue. *Clin Cancer Res* 1997;3:2507–2511.
 22. Ferrer FA, Miller LJ, Andrawis RI, et al. Vascular endothelial growth factor (VEGF) expression in human prostate cancer: in situ and in vitro expression of VEGF by human prostate cancer cells. *J Urol* 1997;157:2329–2333.
 23. Yano S, Shinohara H, Roy S, et al. Expression of vascular endothelial growth factor is necessary but not sufficient for production and growth of brain metastasis. *Cancer Res* 2000;60:4959–4967.
 24. Soker S, Kaefer M, Johnson M, et al. Vascular endothelial growth factor-mediated autocrine stimulation of prostate tumor cells coincides with progression to a malignant phenotype. *Am J Pathol* 2001;159:651–659.