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Original Paper

Expression Pattern of Vascular Endothelial Growth Factor Isoform is Closely Correlated with Tumour Stage and Vascularisation in Renal Cell Carcinoma

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Vascular endothelial growth factor (VEGF) has five isoforms (VEGF206, 189, 165, 145 and 121). Increased VEGF expression in renal cell carcinoma (RCC) is associated with angiogenesis, but, it is not apparent which isoform is involved in this effect. We examined the isoform patterns of VEGF by reverse transcription–polymerase chain reaction (RT–PCR) in 47 RCCs. All showed increased VEGF expression as compared with extraneoplastic renal tissue. Four of the 47 RCCs showed VEGF121 alone, 10 showed VEGF121 + 165, and 33 showed the VEGF121 + 165 + 189 pattern. Patients with pathological stage pT3–4 RCC showed the VEGF121 + 165 + 189 isoform pattern at a significantly higher incidence (10/10, 100%) than those with pT0–2 (23/37, 62%) ($P < 0.022$). The VEGF121 + 165 + 189 isoform pattern was also significantly associated with high vessel counts and density ($P = 0.0002$, Mann–Whitney *U* test). These observations suggested that the VEGF189 mRNA isoform is closely associated with angiogenesis and results in the growth of RCC. © **1999 Elsevier Science Ltd. All rights reserved.**

Key words: vascular endothelial growth factor, mRNA isoform, angiogenesis, vascularisation, renal cell carcinoma
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INTRODUCTION

It is well known that renal cell carcinoma (RCC) accompanied by prominent vascularisation may lead to distant metastasis. There are many factors known to modulate angiogenesis, including basic fibroblast growth factor (b-FGF), transforming growth factor-β (TGF-β) and tumour necrosis factor-α (TNF-α). Vascular endothelial growth factor (VEGF) has been studied as an angiogenic factor [1, 2]. VEGF was discovered because of its ability to increase the permeability of the microvasculature to circulating macromolecules [3]. VEGF plays an important role in neovascularisation in various kinds of cancer, and overexpression of VEGF has been demonstrated in various genitourinary neoplasms including RCC as compared with normal tissue [4].

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Five different isoforms of VEGF transcripts have been identified [5]. The isoforms encoding polypeptides of 206, 189, 165, 145 and 121 amino acids are produced by alternative splicing from a single gene, and these isoforms possess different biological activities [6, 7]. The two larger isoforms (VEGF189 and VEGF206) are considered to be cell associated because of their stronger affinities for cell surface proteoglycans, whereas the other shorter isoforms (VEGF121 and VEGF165) are secreted in soluble form. VEGF165 and VEGF189 isoforms possess heparin binding activity, whilst VEGF121 is not a heparin binding protein [8]. VEGF165 has been assumed to be the predominant form in most tissues [9], but each of the five different isoforms possesses specific biological properties. The precise expression patterns of VEGF isoforms have not been determined *in vivo*. Extracellular and specific cleavage of the VEGF189 isoform is essential for its mitogenic effect on angiogenesis [10]. Colon

cancer showed a significantly higher frequency of VEGF189 expression than extraneoplastic tissue [11], whilst the VEGF mRNA isoform patterns were similar between tumour and non-tumour tissues in the liver and brain [12, 13]. We have demonstrated that cell associated isoform VEGF189 was essential for distant metastasis and was associated with poorer prognosis in colon cancer [11].

There have been some studies of the correlation between VEGF overexpression and vessel count in RCC [14–16]. The pathological significance of the isoform expression patterns of VEGF mRNA is not well understood in RCC. In this study, we analysed the correlation between the VEGF isoform pattern and pathological features in RCC.

MATERIALS AND METHODS

Subjects and tissue samples

The subjects were 47 patients (11 females and 36 males, mean age 55.4 years) with RCC who underwent surgical resection between July 1993 and September 1997 at Tokai University Hospital. All patients were evaluated by TNM score (UICC, 1978). Surgical specimens were rapidly frozen and stored at − 80°C until analysis. Total cellular RNA was prepared from frozen specimens [17].

Expression and isoform pattern of VEGF mRNA

We evaluated the isoforms of VEGF mRNA by reverse transcription–polymerase chain reaction (RT–PCR) under the conditions reported previously [12]. The following primers were used: V-S, 5-AAGCCATCCTGTGTGCCCCT-GATG-3; V-S4, 5-CGGATCAAACCTCACCAAGGCC-3; V-A, 5-GCGAATTCCTCCTGCCCGGCTCAC-3; V-A7, 5-CTTTCTCCGCTCTGAGCAAGGC-3 (Figure 1). Probes (378 bp) were prepared by PCR amplification with primers V-S and V-A. RT was performed at 42°C for 60 min (1 μg total cellular RNA; 100 pM random primers, Boehringer Mannheim, Mannheim, Germany; reverse transcriptase, GIBCO-BRL, Maryland, U.S.A.). VEGF cDNA fragments were amplified by 30 rounds of PCR consisting of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C with a Gene Amp PCR System 9600 (Perkin Elmer, California, U.S.A.) and Taq DNA polymerase (TOYOBO, Japan). Blots of products (Zeta-Probe, Bio-Rad, California, U.S.A.) were hybridised with photochemically labelled probes (ECL; Amersham, Buckinghamshire, U.K.), and exposed to Kodak AR film. The expression patterns of the isoforms were not affected by increasing the number of PCR cycles or the amount of template. We also confirmed the identity of the VEGF189–PCR fragment using an oligonucleotide probe (p189) specific for exon 6 (53 bases, 597–649) (Figure 1).

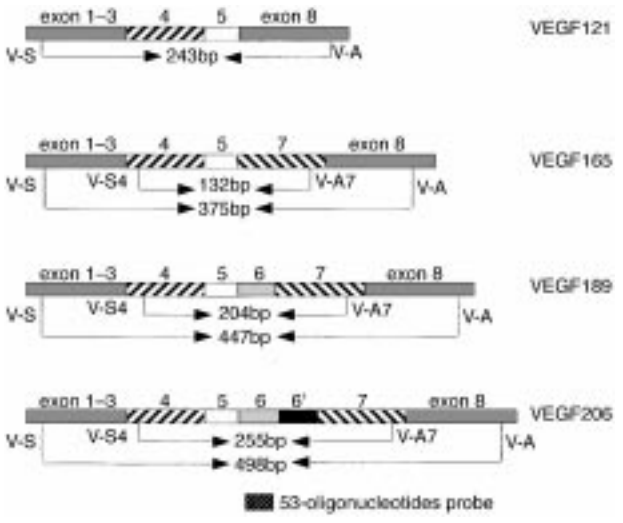

Figure 1. Primers for vascular endothelial growth factor (VEGF) isoforms: polymerase chain reaction (PCR) with V-S and V-A gave VEGF121 (243 bp), VEGF165 (375 bp), VEGF189 (447 bp) and VEGF206 (498 bp) fragments. PCR with V-S4 and V-A7 gave VEGF165, VEGF189 and VEGF206 fragments. VEGF189 specific oligoprobe: 53 oligonucleotides in exon 6 (597–649).

Northern blots of total cellular RNA (15 μg, GeneScreen Plus, New England Nuclear, Massachusetts, U.S.A.) were hybridised with ^{32}P-labelled VEGF cDNA probes (see above). The levels of VEGF gene expression were estimated by densitometry (Interactive Build Analysis System, Zeiss, Jena, Germany). Southern blots of cellular DNAs (13 μg) digested with EcoRI (for 20 h at 37°C, Boehringer Mannheim; Nytran, MSI, Massachusetts, U.S.A.) were hybridised with ^{32}P-labelled VEGF cDNA and exposed to Kodak RP film for 1 week at − 80°C.

Vascularisation in RCC

RCC specimens were fixed with 10% formalin, and embedded in paraffin according to routine procedures. Formalin-fixed, paraffin-embedded sections of the tumour tissue were examined immunohistochemically with mouse antihuman CD34 monoclonal antibody (NCL-end, Novo Castra, Newcastle Upon Tyne, U.K.). After blockage of endogenous peroxidase activity (methylalcohol, 3% H_2O_2) and non-specific binding (10% normal goat serum), the specimens were incubated with anti-CD34 antibody (1:20) at room temperature for 60 min. Sections were serially incubated with biotin-labelled antimouse IgG (Nichirei, Tokyo, Japan) and horseradish peroxidase-conjugated streptavidin (Nichirei). The reaction products were visualised with 3,3′-diaminobenzidine. Light microscopy was used to identify two regions within or immediately adjacent to the cancer containing the highest number of vessels. The microvessel counts and densities were evaluated at ×200 magnification (×20 objective and ×10 ocular, 0.739 mm^2 per field) by using a computerised image analyser (Interactive Build Analysis System, Zeiss).

Expression of VEGF receptor (flt-1, KDR)

VEGF receptor gene expression (flt-1, KDR) was estimated by RT–PCR with the following primers: flt1-S, 5-ATGAGC-AGTGTGAGCGGCTCCC-3 (2669–2690); flt1-A; 5-AA-GCTTTCGCTGCTGGTGACGC-3 (3125–3146); KDR-S, 5-CGTCATGGATCCAGATGAACTCCC-3 (2406–2429); KDR-A, 5-CTTGACGGAATCGTGCCCCTTTGG-3 (2813–2836); under conditions similar to those described above. Both specific probes for flt-1 (477 bp) and KDR (430 bp) were prepared by PCR amplification with primer sets flt1-S, A and KDR-S, A and their sequences were confirmed with a genetic analyser (ABI PRISM 310, Perkin Elmer).

Statistical analysis

Differences in survival between subgroups of patients were compared with the log-rank test, and survival curves were plotted according to the method of Kaplan and Meier. The

χ^2 test or Fisher's exact test was applied for comparisons between group frequencies. Differences in mean vessel counts and density among the groups were analysed by the Mann–Whitney *U* test.

RESULTS

VEGF mRNA isoform pattern

All 47 RCC specimens expressed VEGF121. The isoform patterns were classified into three groups: VEGF121; VEGF121 + VEGF165 (VEGF121 + 165); VEGF121 + VEGF165 + VEGF189 (VEGF121 + 165 + 189). Four of the 47 RCCs showed only VEGF121 expression. 10 patients showed VEGF121 + 165 expression, and 33 had the VEGF121 + 165 + 189 pattern. No RCC expressed VEGF206. These isoform patterns of VEGF mRNA were confirmed by RT–PCR with two sets of primers designed to amplify different portions of the VEGF cDNA (Figures 1, 2a,b). The VEGF189 fragments were specified by oligonucleotide hybridisation (Figures 1, 2c).

The corresponding extraneoplastic tissues of the RCC expressed the VEGF gene. Twenty-nine of the 47 extraneoplastic specimens showed VEGF121, whilst the other extraneoplastic specimens expressed VEGF121 + 165 + 189 isoforms. VEGF189 expression was found at a significantly higher incidence in RCC (33/47) than in extraneoplastic tissue (18/47) ($P = 0.002$, χ^2 test).

Pathological features and VEGF mRNA isoform pattern

Four of the 47 RCCs were papillary RCCs and the remaining 43 were common RCCs. VEGF189 expression (VEGF121 + 165 + 189) was significantly correlated with tumour size (pT3) ($P < 0.05$, Fisher's test, Table 1). VEGF189 expression was significantly correlated with stages 3 and 4 by pTNM classification of RCC ($P < 0.05$, χ^2 test, Table 1). 15 of the 17 (88%) patients with stage 3 or 4 disease showed VEGF189 expression, whilst 18 of the 30 (60%) patients with stage 1 or 2 disease expressed this isoform. No correlation was apparent between VEGF isoform expression pattern and venous involvement (pV), histological grade or histological type (Table 1). VEGF189 (VEGF121 + 165 + 189) expression was not correlated with poorer prognosis of RCC ($P > 0.05$, χ^2 test, Table 1).

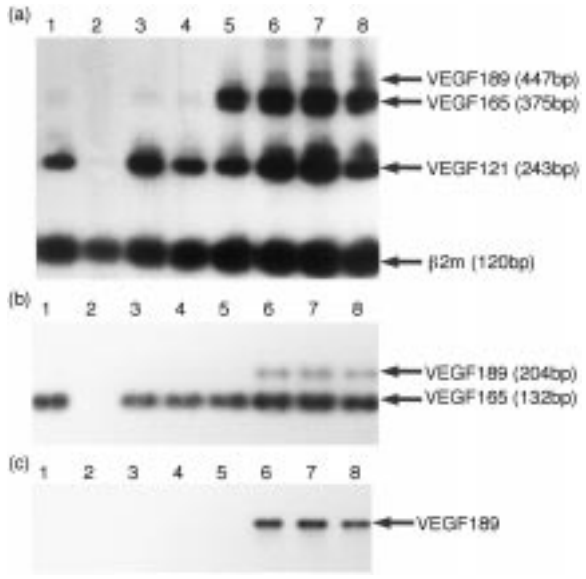

Figure 2. Vascular endothelial growth factor (VEGF) isoform in renal cell carcinoma (RCC). (a) Reverse transcription–polymerase chain reaction (RT–PCR) with primers V-S and V-A, showing VEGF121 (243 bp), VEGF165 (375 bp), and VEGF189 (447 bp). (b) RT–PCR with primers V-S4 and V-A7, showing VEGF165 (132 bp), and VEGF189 (204 bp) fragments. (c) RT–PCR products hybridised with VEGF189 specific oligoprobe.

VEGF gene expression levels

Northern blot analyses showed overexpression of VEGF in RCC tissues (data not shown). Levels of VEGF mRNA upregulation were varied. Extraneoplastic specimens showed faint expression of the VEGF gene compared with corresponding RCC. The VEGF isoform pattern was not correlated with the VEGF expression level. Southern blot analyses showed neither amplification nor rearrangement of the VEGF gene.

Vascularisation and VEGF isoform expression

The mean vessel count in RCCs expressing VEGF189 (VEGF121 + 165 + 189) was 135.5 ± 45.4 per $\times 200$ field (range 82–195), whilst that of RCCs expressing no

Table 1. *Patient characteristics and results of univariate analysis of the associations between vascular endothelial growth factor (VEGF) isoform pattern and patient or tumour characteristics*

Variable	VEGF189 negative *n* (%)	VEGF189 positive *n* (%)	*P* value
Total	14 (30)	33 (70)	
Histology grade			0.372
Grade 1	4 (9)	14 (30)	
Grade 2	10 (21)	19 (40)	
Histological type			1
Papillary	1 (2)	3 (6)	
Common	13 (28)	30 (64)	
v-factor			0.263
= V0	9 (19)	27 (57)	
≤ V1 +	5 (11)	6 (13)	
ly-factor			0.657
= ly0	13 (28)	27 (57)	
≤ ly1 +	1 (2)	6 (13)	
T-staging			0.022*
T0, T1, T2	14 (30)	23 (49)	
T3, T4	0 (0)	10 (21)	
N-stage			0.653
N0	13 (28)	28 (60)	
N1, N2, N3	1 (2)	5 (11)	
M-stage			0.136
M0	13 (28)	23 (49)	
M1	1 (2)	10 (21)	
Tumour stage			0.042†
Stage 1, 2	12 (26)	18 (38)	
Stage 3, 4	2 (2)	15 (32)	
flt-1 expression			0.023*
Yes	10 (21)	32 (68)	
No	4 (9)	1 (2)	
KDR expression			1
Yes	14 (30)	33 (70)	
No	0 (0)	0 (0)	

VEGF189 expression was significantly correlated with T3, 4 stage, flt-1 expression in colon cancer (*$P < 0.05$, Fisher's test) and tumour stage 3, 4 (†$P < 0.05$, χ^2 test).

VEGF189 was 43.5 ± 12.1 per ×200 field (range 27–64). The mean vessel density with VEGF189 was 13.2 ± 4.7% per ×200 field (range 9.4–22.9). The RCCs without VEGF189 showed a vessel density of 6.8 ± 1.2% per ×200 field (range 4.4–8.7). Significant differences were found between these two groups in vessel counts and vessel density ($P = 0.0002$, Mann–Whitney U test, Table 1, Figure 3).

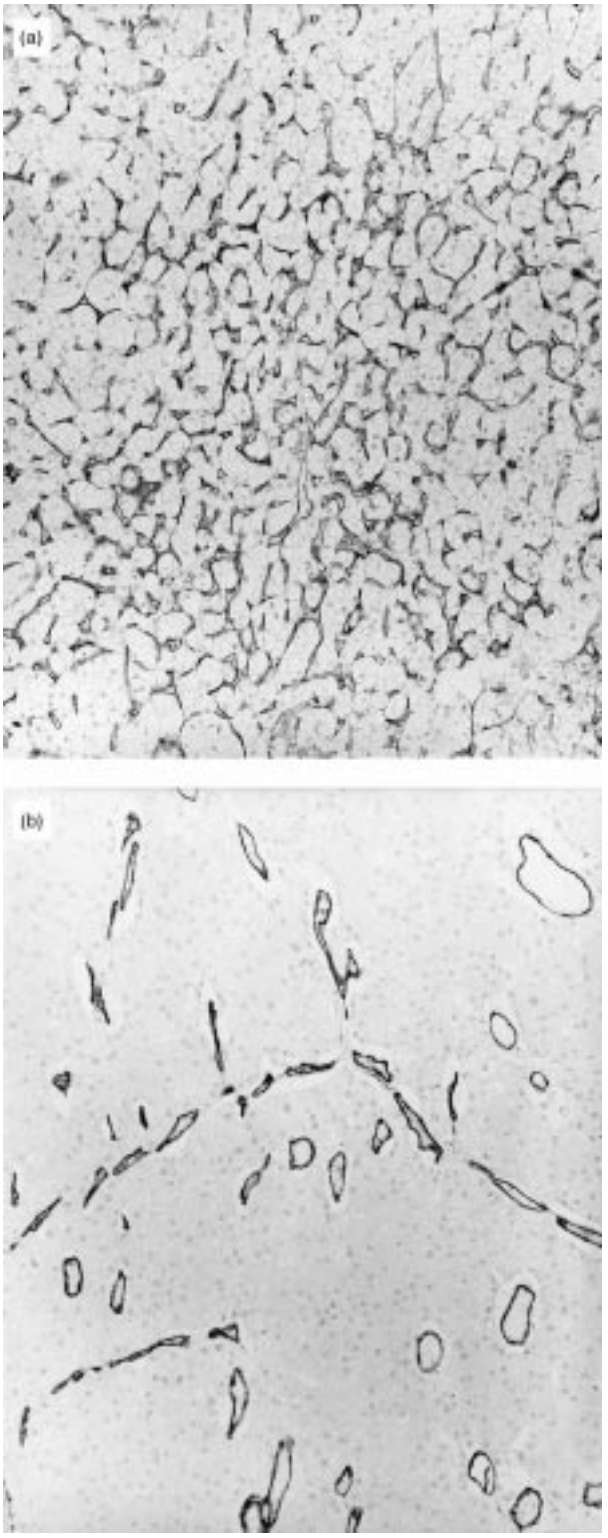

Figure 3. Vascularisation in renal cell carcinoma (RCC) demonstrated by immunostaining for CD34. (a) RCC with VEGF189 showed significantly increased vascular density. (b) RCC without VEGF189 showed moderate vascular density (×130).

Expression of VEGF receptors (flt-1 and KDR)

Forty-two of the 47 (89%) RCCs expressed one of the VEGF receptor mRNAs (flt-1), whereas all the RCCs expressed KDR mRNA. flt-1 expression was confirmed in 32 of 33 RCCs expressing VEGF189, whilst this VEGF receptor was expressed in 10 of 14 RCCs expressing VEGF121 or VEGF121 + 165. There were no correlations between VEGF receptor expression level estimated by Northern blotting and any other clinicopathological features. The levels of VEGF receptor expression were faint in the extraneoplastic tissue compared with RCC, whilst all the extraneoplastic tissue specimens expressed these two VEGF receptors.

DISCUSSION

Angiogenesis plays an important role in the growth, progression and metastasis of solid epithelial neoplasms. VEGF has been studied as an angiogenic factor [1, 2]. In this study, all primary RCCs and corresponding extraneoplastic tissues expressed the VEGF gene, but expression was more intense in RCC than in normal kidney tissue. These results are in agreement with those of previous studies [14–16].

Five different isoforms of human VEGF mRNA are derived from alternative splicing of the primary transcript of a single gene. All the patients with RCC expressed VEGF121, and 70% of patients showed expression of VEGF189 (VEGF121 + 165 + 189) in this study. Neither VEGF165 nor VEGF189 was expressed alone in RCCs. However, in a recent study, all RCCs and matched normal kidney tissue specimens examined expressed VEGF121, VEGF165 and VEGF189 (VEGF121 + 165 + 189) [16]. They examined the VEGF isoform expression pattern by simple agarose gel electrophoresis of RT–PCR products amplified with a single set of primers, and did not confirm the PCR fragments by specific endonuclease digestion or Southern blot hybridisation with specific probes. We analysed the patterns of VEGF isoform expression by RT–PCR with two independent sets of primers amplifying different portions of the VEGF mRNA, and confirmed each fragment by Southern blotting with specific oligonucleotide and cDNA probes. These methods have been verified in our previous studies on VEGF isoforms in colon and lung cancer [11, 18].

Both colon and lung cancer demonstrated higher frequencies of VEGF189 expression than extraneoplastic tissue [11, 18]. We have reported the localisation of VEGF189 in colon or lung cancer using *in situ* hybridisation and xenograft systems, which preserved the corresponding primary cancer cell function in contrast to replacement of stromal elements by murine tissue. Several colon and lung cancer xenografts showed expression of VEGF189 isoform. Moreover, we confirmed VEGF189 expression in lung cancer cells by *in situ* hybridisation. VEGF189 expression was found at a significantly higher incidence in RCC (33/47) than in extraneoplastic tissue (18/47) ($P = 0.002$, χ^2 test). Cultured proliferating vascular endothelial cells showed no apparent expression of VEGF by RT–PCR, whilst vascular endothelium adjacent to the tumour showed VEGF expression by immunochemistry [16]. These results suggest that renal cell cancer tissue may express VEGF189 more frequently than extraneoplastic tissues. Further morphological analyses of the VEGF189 localisation in the RCC are required.

Increased expression of VEGF receptors including flt-1 and KDR was demonstrated in endothelial cells adjacent to RCC by *in situ* hybridisation [14]. KDR but not flt-1 expression was correlated with tumour progression, and vascularity in colon cancer [19]. We examined VEGF receptor (flt-1, KDR) gene expression by RT–PCR and Northern blotting in this study. There was no correlation between VEGF expression level and VEGF receptor gene expression, but the VEGF receptor gene was expressed at higher levels in RCC than in corresponding extraneoplastic tissues. It is possible that the VEGF receptor gene is associated with progression of RCC.

Various factors affect the prognosis of RCC. Tumour stage estimated by pTNM is one of the major prognostic parameters [20–22]. In this study, tumour stages 3 and 4 were significantly correlated with VEGF189 isoform expression. In particular, tumour size (pT3 and pT4) showed the most significant correlation to VEGF189 expression. We also demonstrated a significant correlation between increased vascularisation and expression of this VEGF189 isoform. These results suggest that tumour progression through angiogenesis in RCC may be associated with the cell associated isoform VEGF189.

In various neoplasms, vessel density has been reported to be associated with progression and prognosis [23–26]. We did not find any significant correlation between isoform pattern of VEGF mRNA expression and prognosis of RCC in this study, since the follow-up period was insufficient to evaluate survival rates in association with VEGF189 (the mean follow-up period of RCC patients was 614 days). Our previous study demonstrated that expression of the VEGF189 isoform was correlated not only with liver metastasis, poor prognosis, and xenotransplantability in colon cancer [11, 27], but also with malignant progression in non-small cell lung cancer [18]. It was also suggested that the upregulation of VEGF189 might result in increased angiogenesis, tumour growth and metastasis in a colon cancer cell line [28].

This cell associated VEGF189 expression might be correlated with the vascularisation of RCC. Analysis of the VEGF mRNA isoform pattern may be important for predicting the progression of RCC.

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