The Differential Expression of Vascular Endothelial Growth Inhibitor Isoforms, VEGI251, VEGI174 and VEGI192 in Human Clear-cell Renal Cell Carcinoma

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Abstract. *Vascular endothelial growth inhibitor (VEGI) is a recently identified antiangiogenic cytokine that belongs to the tumour necrosis factor (TNF) superfamily, and may be essential for many physiological and pathological processes. However, the expression of VEGI and in particular its isoforms, VEGI251, VEGI192 and VEGI174, in clear-cell renal cell carcinoma (CCRCC) remain unknown. In the current study, we investigated the expression of the three isoforms of VEGI in CCRCC. The expression of VEGI was examined in paired human normal renal and CCRCC specimens (n=73). The transcripts of the three isoforms of VEGI were all detected in human renal normal and tumour tissues. Levels of VEGI174 and VEGI192 transcripts in normal renal specimens were higher than those in CCRCC (p=0.021 and p=0.038, respectively). Levels of VEGI251 were similar in normal and tumour specimens (p=0.67). The numbers of VEGI174 and VEGI192 transcripts in T1a+T1b tumours were higher than those in T2+T3 tumours (p=0.006 and p=0.018, respectively). Moreover, VEGI192 transcript levels were negatively correlated with pathological nuclear grade (r=–0.216, p=0.022). In immunohistochemical staining, VEGI192 staining in normal and CCRCC tissues differed significantly (100% vs. 39.7%, p<0.0001). VEGI192 staining intensity was also negatively correlated with pathological nuclear grade (r=–0.781, p=0.002). Conclusion: Transcripts of VEGI isoforms were detectable in normal and tumour renal tissues. VEGI192 and VEGI174 expressions markedly decreased in CCRCC and are linked to pathological grade and stage. VEGI192 and VEGI174 are more likely to be putative tumour suppressive factors and a potential therapeutic target in CCRCC.*

Angiogenesis, the development of new blood vessels from the existing vasculature, is essential in the normal developmental processes and is a hallmark of over 50 different disease states, including cancer, diabetes, rheumatoid arthritis and psoriasis (1). The process of angiogenesis is tightly regulated by a balance of pro- and antiangiogenic molecules (2). Physiological angiogenesis is a highly-organized sequence of cellular events comprising vascular initiation, formation, maturation, remodelling and regression, which are controlled and modulated to meet tissue requirements. In contrast, pathological angiogenesis is less well-controlled and although the initiation and formation stages occur, the vessels rarely mature, remodel or regress in disease (3, 4). Therefore, antiangiogenic therapy has been shown to be an effective treatment strategy for angiogenesis-dependent diseases, especially for cancer (1, 2, 5).

It is well-established that the growth of solid tumours is angiogenesis-dependent (6-9). Moreover, multiple studies have demonstrated that the degree of tumour vascularity correlates positively with disease stage, the likelihood of metastases, and cancer recurrence (10). Although there is a long list of angiogenic factors which occur in the body (5, 11-16), naturally-occurring anti-angiogenic factors are less common, with Vascular endothelial growth inhibitor (VEGI) being one of the few reported. VEGI belongs to the tumour

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Table I. *Primer sequences for the current study.*

Primer	Forward	Reverse
GAPDH	5'-AGCTTGTCATCAATGGAAAT	5'-CTTCACCACCTTCTTGATGT
hGAPDH (Q-PCR)	5'-CTGAGTACGTCGTGGAGTC	5'-ACTGAACCTGACCGTACA-CAGAGATGATGACCCTTTTG Z-sequence
*VEGI*174 (Q-PCR)	5'-CAAAGTCTACAGTTTCCCAAT	5'-ACTGAACCTGACCGTACA-TGATT TTTAAAGTGCTGTGTG Z-sequence
*VEGI*192 (Q-PCR)	5'-TTTCAGTCACCCTTTGTCTC	5'-ACTGAACCTGACCGTACA-GCTTATCTCCGTCTGCTCTA Z-sequence
*VEGI*251 (Q-PCR)	5'-GACTCACCACATACCTGCTT	5'-ACTGAACCTGACCGTACA-AGGTGCATAAACTTGCTGAT Z-sequence

necrosis factor superfamily (TNFSF) and has been shown to be a potent antiangiogenic factor (17-19). It inhibits the proliferation of endothelial cells. *VEGI* transcripts have been found to be expressed in the placenta, lung, skeletal muscle, pancreas, spleen, small intestine, prostate and colon. Previous studies have demonstrated an intricate relationship between VEGI and carcinoma *in vitro* and *in vivo*. Systemic administration of VEGI, markedly inhibited tumour growth and increased survival time in a Lewis lung cancer (LLC) murine tumour model (20). Moreover, VEGI has been shown to suppress the growth of colonic carcinoma cells (murine colon cancer cells, MC-38) both *in vitro* and *in vivo* (18, 23). Additional studies also indicate that the antitumour activity of VEGI is mainly dependent on interference with the development of tumour-associated vasculature (3, 23). VEGI thus represents a potent endogenous inhibitor of angiogenesis. To date, three isoforms of VEGI have been reported, named VEGI251, VEGI192 and VEGI174, based on the length of the protein. All the isoforms share a common 151-C-terminal amino acid sequence but differ in their N-terminal regions (19-22). The function and the expression pattern of the isoforms are however less clear. The expression pattern of the isoforms is largely unknown in solid tumours.

In the current study, the expression of all three isoforms of VEGI, and correlations between VEGI and pathological grade and stage of the tumours were examined in renal cell carcinoma specimens.

Materials and Methods

Materials. Human clear-cell renal cell carcinoma (CCRCC) specimens. A total of 73 (46 males and 27 females) pairs of CCRCC and normal renal tissue samples were snap-frozen in liquid nitrogen, immediately after open radical nephrectomy. The average age of patients was 57.7±11.4 (range=29-79) years. The pathologist verified between normal and carcinoma specimens. Each tumour underwent pathological staging based on the Union Internationale Contre le Cancer/American Joint Committee on Cancer (UICC and AJCC) 2002 classification of primary RCC and Fuhrman nuclear grading. In the cohort, 28 (38.4%), 31 (42.5%), 9 (12.3%) and 5 (6.8%) cases were staged as T1a, T1b, T2, and T3, respectively. Fuhrman grades of the cohort were G1 in 16 cases (21.9%), G2 in 36 (49.3%) and G3 in 21 (28.8%). All protocols were reviewed and approved by the local Ethical Committee and all patients gave written informed consent.

Total cellular RNA and cDNA preparation. Total cellular RNA was isolated from the homogenized renal samples using the Trizol method (Triagent, Sigma-Aldrich, Poole, Dorset, England). The concentration of RNA was determined by spectrophotometric measurements (WPA UV 1101; Biotech Photometer, Cambridge, UK). cDNA was prepared using 0.5 μg of the RNA sample and a reverse transcription kit (Sigma, Poole, Dorset, UK). The quality of cDNA was verified through the amplification and detection of the GAPDH house keeping gene. *GAPDH* forward and reverse primers were 5'AGCTTGTCATCAATGGAAAT3' and 5' CTTCACCA CCTTCTTGATGT3', respectively (Table I).

Real-time quantitative polymerase chain reaction (Q-PCR) (24, 25). The iCycler IQ system (BioRad, Camberley, UK) was employed to quantify the levels (shown as copies/μl from internal standard) of VEGI in the renal tissues. cDNA samples of renal tissues were then examined for VEGI expression using the forward and reverse primers (Table I), which were designed based on the human *VEGI* sequence (GeneBank Accession number: VEGI174-BD131562, VEGI192-AY434464, and VEGI251-NM_005118.2). *GAPDH* was used as a house-keeping control. The Q-PCR technique utilised the Amplifluor system (Flowgen, Oxford, England) and the Q-PCR master mix (ABgene, Surrey, England), in conjunction with a universal probe (UniPrimer™, Merck-Millipore, Watford, England).

Real-time QPCR conditions were 95˚C for 15 min, followed by 65 cycles at 95˚C for 15 s, 55˚C for 30 s and 72˚C for 15 s. The results of the test molecules were normalised against the levels of *GAPDH*.

Immunohistochemical staining of human renal tissues. Frozen specimens of CCRCC (n=73) and normal renal tissues (n=73) were cut at a thickness of 6 μm using a cryostat (Leica CM 1900, Leica Microsystems UK Ltd., Milton Keynes, Buckinghamshire, UK). The nature of the samples was independently-verified by two pathologists. After fixation, the sections were blocked with horse serum and probed with or without the VEGI192 antibody (LS-C40892; LifeSpan Bioscience, Seattle, WA, USA) for one hour. Secondary biotinylated antibody and Avidin Biotin Complex were subsequently applied to detect VEGI expression in accordance with the Vectastain Universal Elite ABC kit protocol (Vector Laboratories, Peterborough, UK). After developing colour with 3,3'-Diaminobenzidine or Diaminobenzidine (DAB), the sections were counterstained with Gill's Haematoxylin. Staining was independently assessed by the Authors.

Statistical analysis and software. The mean optical density (MOD) of VEGI192 immunohistochemical staining was analysed using the Image-Pro Plus 6.0 software package (Media Cybernetics, Rockville, MD, USA). All statistical analyses were performed using the SPSS 16.0 software (SPSS Inc., New York, NY, USA). Two-sample *t*-test was used for normally distributed data. Fisher's exact test was used for analysing immunohistochemical staining in renal tissues. Spearman's test was used to evaluate the correlations between the expression of VEGI and pathological stage and grade. Differences were considered to be statistically significant at $p<0.05$.

Results

Transcripts of three VEGI isoforms in CCRCC and normal renal tissues. The transcript levels of three isoforms of VEGI were examined in human renal tissues using Q-PCR. The copies of *VEGI*174 and *VEGI*192 transcripts in normal renal tissues were significantly higher than those in CCRCC specimens ($7.15\times10^{10}\pm1.96\times10^{10}$ *vs*. $3.73\times10^{9}\pm1.41\times10^{9}$, $p=0.021$; $6.67\times10^{13}\pm1.17\times10^{13}$ *vs*. $2.35\times10^{12}\pm1.63\times10^{12}$, $p=0.038$, respectively) (Figure 1). The copies of *VEGI*251 transcripts in normal renal tissues were also higher than that in CCRCC specimens, but there was no significant difference ($7.48\times10^{12}\pm1.31\times10^{12}$ *vs*. $6.44\times10^{12}\pm1.04\times10^{12}$, $p=0.670$) (Figure 1). The copies of *VEGI*251 transcripts were also significantly higher than those of *VEGI*174 in both normal and CCRCC specimens ($p<0.0001$), making *VEGI*251 the most abundant isoform in the tissue.

*VEGI*174 and *VEGI*192 transcripts in T1a+T1b tumours were significantly higher than those in T2+T3 carcinoma specimens ($5.46\times10^{9}\pm1.72\times10^{9}$ *vs*. $8.33\times10^{2}\pm2.67\times10^{2}$, $p=0.006$ for *VEGI*174, $2.86\times10^{12}\pm1.78\times10^{12}$ *vs*. $4.95\times10^{3}\pm1.44\times10^{3}$ for *VEGI*192, $p=0.018$, respectively). Although the copies of *VEGI*174 and *VEGI*192 transcripts in G1+G2 were higher than those in G3 carcinoma specimens ($4.35\times10^{9}\pm 1.34\times10^{9}$ *vs*. $3.46\times10^{9}\pm1.56\times10^{9}$, $p=0.75$, $4.61\times10^{12}\pm2.26\times10^{12}$ *vs*.

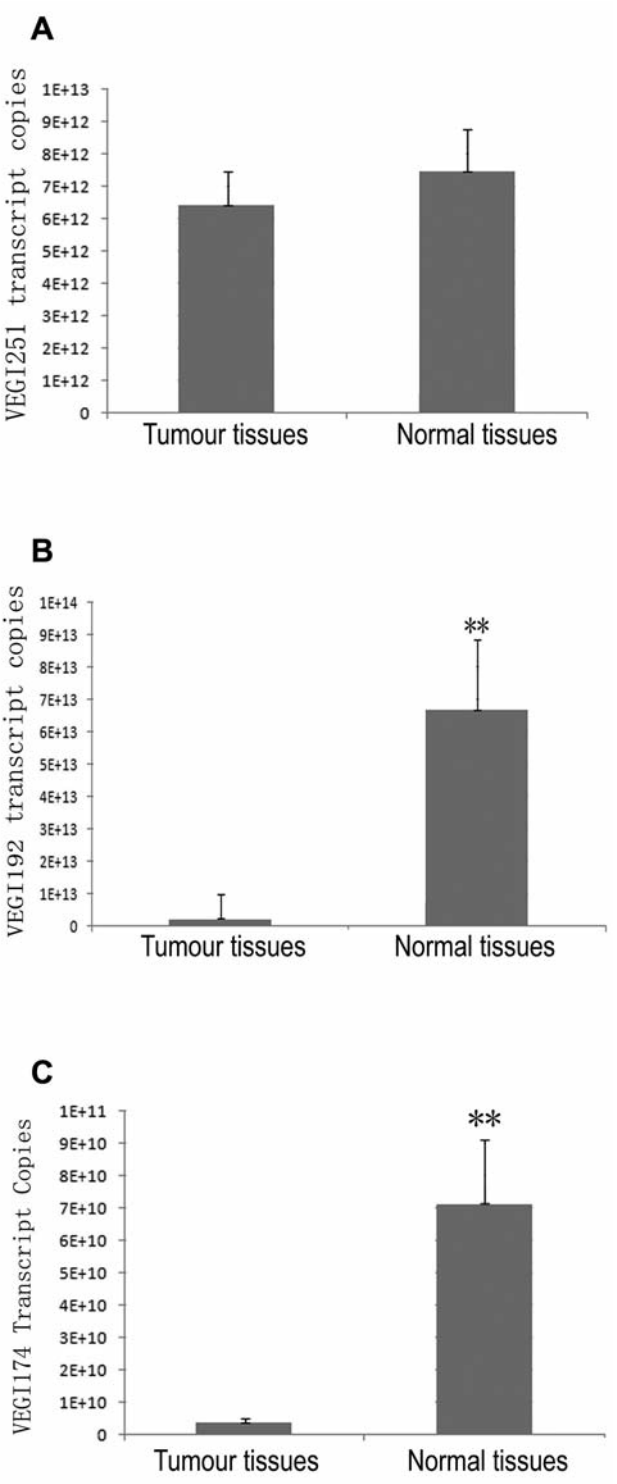

Figure 1. *Transcripts of three isoforms of VEGI in CCRCC and normal renal tissues. A: VEGI251 transcripts in CCRCC and normal renal tissues. There was no significant difference between the tissues p=0.670. B: VEGI192 transcripts in CCRCC and normal renal tissues. VEGI192 expression in renal rumor tissues was significantly lower than in normal renal tissues. C: VEGI174 transcripts in CCRCC and normal renal tissues. Asterisks indicate p<0.05 vs. normal renal tissues.*

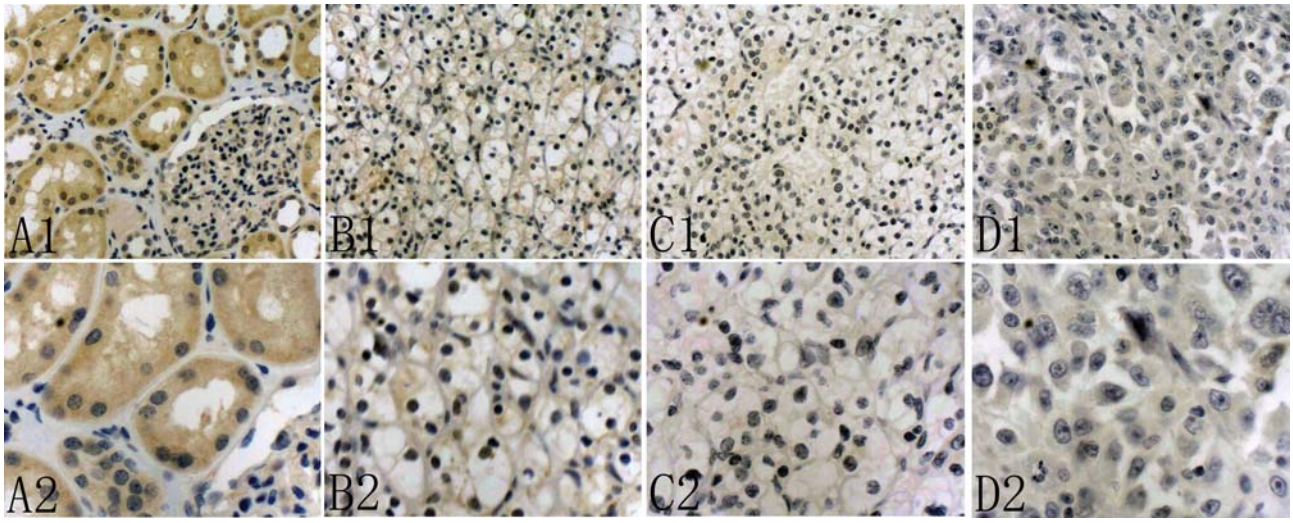

Figure 2. *Immunohistochemical staining of human CCRCC and normal renal specimens. A1 and A2 are normal renal tissues; the VEGI192 protein was found to be intensively stained in the cytoplasmic area of renal tubular epithelia cells. B1 and B2 are CCRCC tissues with Fuhrman nuclear grades of G1; VEGI192 was weakly-stained. C1 and C2 are CCRCC tissues with Fuhrman nuclear grades of G2; the VEGI192 protein staining was weak. D1 and D2 are CCRCC tissues with Fuhrman nuclear grades of G3; the VEGI192 protein staining was almost absent.*

$5.54\times10^3\pm2.50\times10^3$, p=0.159, respectively), there was no statistical difference. Moreover, there was no difference of the copies of *VEGI*251 transcripts between G1+G2 and G3 ($6.57\times10^{12}\pm2.89\times10^{12}$ *vs.* $6.31\times10^{12}\pm1.12\times10^{12}$, p=0.90), nor between T1a+T1b and T2+T3 carcinoma specimens ($5.31\times10^{12}\pm 9.10\times10^{12}$ *vs.* $9.09\times10^{12}\pm1.33\times10^{12}$, p=0.143).

The spearman's rank correlation test was used to evaluate the correlations between the transcripts of the three isoforms of *VEGI* and pathological stage and nuclear grade. It is noteworthy that the number of copies of *VEGI*192 transcript was inversely correlated with pathological nuclear grade (r=–0.216, p=0.022). However, there were no correlations between copy numbers of *VEGI*174 and *VEGI*251 transcripts and pathological nuclear grade (r=0.027, p=0.778 and r=–0.118, p=0.215, respectively). There were no correlations between *VEGI*174, *VEGI*192, and *VEGI*251 and pathological stage (r=–0.049, p=0.639, r=–0.163, p=0.118, r=–0.067, p=0.521, respectively).

Expression of the VEGI92 protein in CCRCC and normal renal tissues. In immunohistochemical staining, VEGI192 was seen in the cytoplasmic area of normal renal tubular epithelia cells, but the staining was lower in or absent from CCRCC cells in tumour tissues, particularly in specimens with higher nuclear grade (Figure 2). The frequency of positive staining of normal tissue (100%, 73/73) was significantly higher than that of CCRCC tissues (39.7%, 29/73), p<0.0001. The mean density of staining in normal renal tissues (0.51±0.11) was also significantly higher than that of CCRCC specimens (0.12±0.06) at p=0.016.

To evaluate the correlation between VEGI192 protein and pathological nuclear grade and stage of renal cell carcinoma, we compared the staining density in the different grades and stages. It is interesting that the MOD of VEGI192 immunohistochemically was negatively correlated with pathological nuclear grade (r=–0.781, p=0.002). VEGI192 staining in G1+G2 carcinoma specimens was higher than that in G3 carcinoma specimens (0.13±0.05 *vs.* 0.04±0.02, p=0.001). However, there was no significant difference between T1a+T1b and T2+T3 tumours (0.11±0.08 *vs.* 0.07±0.07, p=0.322). Furthermore, there were no correlations between VEGI192 staining and pathological stage (r=0.113, p=0.68).

Discussion

VEGI (also known as TL1, TNFSF15 and TL1A), was first reported in 1999, in human umbilical vein endothelial cells, and was identified as an endothelial cell-specific gene and a potent endogenous inhibitor of endothelial cell proliferation, angiogenesis, and tumour growth (17-19, 26-29). The initially reported VEGI protein is composed of 174 amino acids, of which residues 1-25 at the N-terminus are the predicted intracellular and transmembrane domain and the 26-174 residues at the C-terminus form an extracellular domain (17). The intracellular domain is released after a cleavage. These features are consistent with characteristics of type II transmembrane proteins (19, 30). The full length of the *VEGI* gene is ~17 kb, which consists of four exons and three introns, and is mapped to human chromosome 9q32.

Two other isoforms, VEGI251 and VEGI192, were discovered subsequently (10, 18, 19). It should be noted that more than one isoform can be expressed in the same cell type, for example VEGI251 and 192 are both detectable in human coronary artery endothelial cells (HCAE), human umbilical vein endothelial cells (HUVEC), and human microvessel endothelial cells (HMVE) (31-34). The VEGI isoforms also have different tissue expression patterns. A 7.5 kb VEGI251 transcript was detected in placenta, kidney, lung, and liver, whereas the 2-kb VEGI174 transcript was observed in the liver, skeletal muscle, and heart (21, 27). All three isoforms share a common region of 453 bp, which encodes a domain of 24-174 amino acids at the *C*-terminus of VEGI174. However, the three isoforms differ in their *N*-terminal regions, due to alternative exons (27). Previous studies documented that only the solubilised extracellular domain of the three isoforms of VEGI is responsible for its biological activity (19-22, 35). It is interesting that these isoforms displayed various degrees of antitumour activities when tested in their natural or recombinant forms (36).

For the first discovered VEGI, it was demonstrated that the recombinant human VEGI174 inhibits angiogenesis *in vitro*, as well as in animal models bearing tumours. In 1999, Zhai *et al*. found that the VEGI174 protein markedly inhibited the growth of breast and colon xenograft tumours and suggested that the effect may be through indirect inhibition of capillary-like structures and cell growth (18, 19). It has also been suggested that VEGI174 may inhibit the growth of human tumour cell lines, including human histiocytic lymphoma (U-937), human breast carcinoma (MCF-7), human epithelial carcinoma (HeLa) and human myeloid lymphomas ML-1a (28). Moreover, forced expression of VEGI174 can directly reduce the motility and adhesion of prostate and bladder cancer cells (DU-145, PC-3, and T24) (37, 38). In 2006, Parr *et al*. reported that patients with breast tumours expressing reduced levels of VEGI174 had a higher local recurrence rate, shorter survival time and a poorer overall prognosis than patients with tumours expressing high levels of VEGI (24).

Subsequently, VEGI251 and VEGI192 have also been found to have anticancer activities. Of the three isoforms, the longest and most abundant form of VEGI protein is VEGI251 (30). Ectopic expression of VEGI251 in tumour cells causes apoptosis of endothelial cells in the tumour vasculature, reduction of microvessel density, and inhibition of tumour growth, although purified recombinant VEGI-251 protein lacks antiangiogenic activity (21, 31, 32). Moreover, VEGI251 serves as an anticancer factor through its activation of T-lymphocytes (22, 30, 39, 40). VEGI251 binds T-cell receptors. Ligand-receptor binding creates co-stimulatory signals for T-cells, increases interleukin-2 (IL-2) responsiveness and secretion of interferon-alpha (IFN-α) and granulocyte macrophage colony-stimulating factor (GM-CSF), both *in vitro* and *in vivo* (32). These reports suggest that the anticancer activities of VEGI251 depend on both the stimulation of T-cells and antiangiogenesis.

In 2001, Chew *et al*. reported a new isoform of VEGI, VEGI192. Previous experiments showed that VEGI192 was approximately 20-fold more potent than endostatin in its antiangiogenic effect *in vitro* (21). In 2005, Hou *et al*., using a Lewis lung cancer (LLC) murine tumour model, demonstrated that systemic administration of VEGI gave rise to a marked inhibition of tumour growth and to an increase in survival time of the treated animals (20). Recently, Wu *et al*. reported that the recombinant human RGD-VEGI192 exhibited a higher level of antiangiogenic activity, as compared with the parental rhVEGI192, and leading to significant antitumour effect in breast tumour-bearing animals (36). Collectively, these recent studies have indicated that all three isoforms of VEGI have anticancer activity through antiangiogenesis and other functions.

CCRCC has been shown to be an angiogenesis-dependent and immune-regulated disease (41-46), however there are few studies on the expression of VEGI in the CCRCC until now. The present study revealed that the transcripts of the three isoforms of VEGI were all detected in most of normal renal specimens, and in some CCRCC specimens. The levels of the VEGI251 transcripts in normal and tumour specimens were the highest in three isoforms. However, a statistical significant difference between normal and tumour tissues was only found with VEGI251 and VEGI174. Furthermore, our results showed that the number of copies of VEGI251 transcripts in normal and tumour specimens had no statistical difference. It is argued, therefore, that VEGI251 may play a less important role in the development of CCRCC.

VEGI192 and VEGI174 transcripts were significantly lower in tumour specimens than that in normal specimens. Moreover, our results also showed that the higher the pathologic nuclear grade or stage, the lower were the number of copies of VEGI192 and VEGI174 transcripts. This inverse correlation has an important biological meaning. It is implicated that VEGI192 and VEGI174 are negative regulators for aggressiveness during the development and progression of CCRCC.

Owing to the significant difference in the VEGI192 transcripts in CCRCC, we further evaluated the VEGI192 protein, using immunohistochemical staining. Significantly high levels of VEGI192 staining in normal renal tubular epithelia cells, and the negative correlation with pathological nuclear grade, further confirmed that VEGI192 expression was negatively correlated with the aggressiveness of CCRCC. Taken together our data show that expression of VEGI isoforms, particularly VEGI192 and VEGI174 is progressively reduced/lost in aggressive renal tumours, when using tumour grade and staging as indicators of aggression. The absence or reduction of tumour VEGI expression suggests that there may

be a shift in the balance between pro- and antiangiogenic factors with tumour progression. The loss of balance may subsequently produce a microenvironment that is conducive to tumour growth and survival (2, 26). Together with other anticancer activities of VEGI which had been reported, our results suggest that VEGI may be a negative regulator for aggressiveness during the development and progression of CCRCC.

In conclusion, three isoforms of VEGI are expressed in normal and tumour renal tissues and the expression of VEGI192 and VEGI174 is decreased in renal cell carcinoma specimens, particularly in tumours with higher pathological nuclear grade and stage. In normal and tumour renal specimens, VEGI251 is the most abundant form of VEGI protein, whereas there is no difference in the expression of VEGI251 transcript expression between them. It is interesting to note that copies of VEGI192 and VEGI174 transcripts are inversely correlated with pathological stage, and the VEGI192 protein is also inversely correlated with pathological nuclear grade. Overall, our results indicate that VEGI192 and VEGI174 are more likely to be putative tumour suppressive factors and potential therapeutic targets to prevent from development of renal cell carcinoma.

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