Vascular endothelial growth factor in renal cell carcinoma

Jan Jacobsen

Umeå 2006
This thesis is dedicated to
Ida and Heini


Robert Storm Petersen
Danish artist (1882 - 1949)
ABSTRACT

VASCULAR ENDOTHELIAL GROWTH FACTOR IN RENAL CELL CARCINOMA

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Background. Angiogenesis is essential for tumour growth. Vascular endothelial growth factor (VEGF) and its isoforms were investigated in relation to the clinical course in a large number of patients with renal cell carcinoma (RCC).

Methods. RCC subtypes and behaviour were established by clinicopathological criteria and surveillance. VEGF expression was analysed in serum by enzyme-linked immunosorbent assay (ELISA) and in tumour tissue by reverse transcription polymerase chain reaction (RT-PCR), immunohistochemistry (IHC), and Western blot (WB).

Results. Serum VEGF (S-VEGF) was increased in RCC compared to control group. S-VEGF correlated with tumour stage and grade and was associated with survival in men but not in women. S-VEGF correlated with blood platelet counts, which were inversely correlated to increasing age in women, and they were decreased in chronically medicated patients, particularly in men. In contrast to S-VEGF, platelet counts associated with survival only in patients free of medication and chronic diseases. RT-PCR showed a correlation between VEGF_{121}/VEGF_{165} mRNA and between VEGF_{165}/VEGF-R1 mRNA. There was no association between different VEGF mRNA isoforms and S-VEGF. Conventional renal cell carcinoma (CRCC) had higher VEGF_{165}, VEGF_{121}, and VEGF-R1 mRNA levels compared with papillary renal cell carcinoma (PRCC). IHC VEGF staining was strong in kidney cortex. Kidney tumour showed a considerable variation in cytoplasmatic VEGF expression, which correlated with tumour size. Although, there was no difference in VEGF expression between the RCC types, VEGF expression was associated with survival only in CRCC. WB showed a strong protein expression of both VEGF_{189} and VEGF_{165} in kidney cortex. In kidney tumour, expression of VEGF_{189} varied the most, VEGF_{165} less so, and VEGF_{121} was rarely detected. Both CRCC and PRCC expressed low levels of VEGF_{189} and VEGF_{165} compared with kidney cortex. Chromophobe renal cell carcinoma (ChRCC) expressed VEGF_{189} levels comparable to those from kidney cortex, while VEGF_{165} was lower. In PRCC and ChRCC, VEGF_{189} levels correlated inversely with advancing tumour stage, and in PRCC, VEGF_{165} levels correlated inversely with increasing tumour size. VEGF_{189} was an independent prognostic factor for survival in patients with PRCC.

Conclusions. S-VEGF has a stronger association to progression in RCC than platelet count. CRCC showed high levels of VEGF mRNA isoforms and VEGF-R1 mRNA compared to PRCC. VEGF mRNA isoforms expression decreased with advancing stage. IHC demonstrated VEGF expression in cell cytoplasm related to tumour growth, particular in CRCC. Different VEGF isoform patterns were found in different RCC types. Protein VEGF_{189} expression was associated with tumour stage and was an independent prognostic factor in PRCC. Protein VEGF_{165} expression was generally low and had no prognostic value. The trend for decreasing levels of VEGF isoforms in advanced tumour stages may indicate that angiogenic activity is an early event in tumour growth induced by VEGF, but that during later tumour progression the role of VEGF is less clear.

Key words: VEGF, isoforms, quantitative RT-PCR, immunohistochemistry, tissue microarray, Western blot, stage, survival, renal cell carcinoma.
This thesis is based on the following papers, which are referred to in the text by their roman numerals:

I  Jacobsen J, Rasmuson T, Grankvist K and Ljungberg B.  
Vascular endothelial growth factor as prognostic factor in renal cell carcinoma.  

II  Jacobsen J, Grankvist K, Rasmuson T, Ljungberg B.  
Prognostic importance of serum vascular endothelial growth factor in relation to platelet and leukocyte counts in human renal cell carcinoma.  

Tumour vascular endothelial growth factor (VEGF) mRNA in relation to serum VEGF protein levels and tumour progression in human renal cell carcinoma.  


V  Jacobsen J, Grankvist K, Rasmuson T, Ljungberg B.  
Different isoform patterns for vascular endothelial growth factor between clear cell and papillary renal cell carcinoma.  
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ABBREVIATIONS

AJCC American Joint Committee on Cancer

Bcl-2 Gene encodes inhibitors of apoptosis

Bcl-2 Protein encoded of Bcl-2 gene

bFGF (basic) Fibroblast Growth Factor

BHD Birt Hogg Dub’e syndrome

cDNA (complementary) Deoxyribonucleic Acid

ChRCC Chromophobe Renal Cell Carcinoma

CRCC Conventional Renal Cell Carcinoma

CT Computerised Tomography

Cyclins D and E Promote cyclin-dependent kinases to activate cell cycle

DNA Deoxyribonucleic Acid

ECM Extra Cellular Matrix

EGF Epidermal Growth Factor

EGF-R Epidermal Growth Factor Receptor

ELISA Enzyme Linked Immuno Sorbent Assay

erb-B Gene encodes EGF-R

erb-B Protein encoded by erb-B gene

ESR Erythrocyte sedimentation rate

FGF Fibroblast Growth Factor

FH Gene encodes Fumarate Hydratase involved in citric acid cycle

FH Protein encoded by FH gene

HER-2 Synonymous to erb-B gene encodes EGF-R

HER-2 Synonymous to erb-B

HIF Hypoxia Inducible Factor

HLRCC Hereditary Leiomyoma Renal Cell Carcinoma

HPRC Hereditary Papillary Renal Cell Carcinoma

IGF Insulin like Growth Factor

IHC Immunohistochemistry

IS Internal Standard

bp Base Pairs

kDA (kilo) Dalton

met Gene encodes receptor with tyrosine kinase activity

met Protein encoded by met gene

MMP Matrix Metallo Proteinase

MRI Magnetic Resonance Imaging

mRNA (messenger) Ribonucleic Acid

MVD Micro Vessel Density

myc Gene encodes transcription activators and stimulating growth signals
myc Protein encoded by myc gene

p27 Gene encodes transcription inhibitors of cell cycle

p53 Gene encodes transcription inhibitors of cell cycle and activates apoptosis

p53 Protein encoded by p53 gene

PAD Pathological Anatomical Diagnosis

PBS Phosphate Buffered Saline

PDGF Platelet Derived Growth Factor

pRb Gene encodes nuclear transcription factor once activated stimulates cell cycle

PRCC Papillary Renal Cell Carcinoma

pVHL (protein) VHL

RCC Renal Cell Carcinoma

RNA Ribonucleic Acid

RT-PCR Reverse Transcription Polymerase Chain Reaction

SDS Sodium Dodectyl Sulfate

TGF Transforming Growth Factor

TMA Tissue Micro Array

TNF Tumour Necrosis Factor

TNM Tumour Node Metastasis

TS Tissue Section or Slide

UICC Union Internationale Contre le Cancer

VEGF Vascular Endothelial Growth Factor

VEGF-R1 VEGF receptor 1 (flt-1)

VEGF-R2 VEGF receptor 2 (KDR/flk-1)

VHL von Hippel-Lindau

VHL Gene encodes VHL promoting transcription of VEGF

VHL Protein encoded by VHL gene

WB Western Blot
INTRODUCTION

Background
Renal cell carcinoma (RCC) is a heterogeneous group of tumours, which demonstrates wide variation in histopathological features, as well as clinical diversity, with unpredictable tumour behaviour. The mortality-incidence ratio is higher in RCC than in other urological malignancies [1]. In only 50-60% of RCC tumours which are localized to kidney is surgical treatment curative [2, 3]. New approaches in cancer treatment are needed, and through genetic and molecular biological research novel molecular therapeutic targets have been identified [4, 5]. Tumours seem to be influenced by serial gene mutations and methylation with phenotypical expression as dysfunctional cell signalling, cell proliferation, inhibition of apoptosis, and promotion of angiogenesis during the growth [6]. Molecular targeting of dysfunction in cell signalling or angiogenesis is a potential novel therapy, and the knowledge is essential in order to design new anti-angiogenic therapies for patients with RCC.

Incidence
RCC constitutes 2-3% of adult malignancies [7]. Almost 900 new cases of RCC are observed annually in Sweden, with 600 deaths each year attributed to RCC. Men are affected more commonly than women, with a ratio of 3:2. RCC is primarily a disease of the elderly, and is usually detected in the sixth or seventh decade of life [1]. Worldwide, there is improved and earlier detection of localised tumours, largely due to expanded access to better quality in radiological imaging techniques such as ultrasonography, computerised tomography (CT), and magnetic resonance imaging (MRI) [8-10].

Environmental risk factors
Several epidemiologic studies have demonstrated an association between RCC and tobacco smoking and obesity [11, 12], factors which have been associated in 40% of RCC cases in our high-risk society [13]. In rat experiments, the constituents from tobacco smoke, which are excreted to the urine are thought to cause RCC by VHL-mutations [14, 15]. Moreover, gene-environment interactions have been recognized among smokers who have slow acetylator genotype for N-acetyl-transferase, where there is an increased risk for development of RCC [16]. A high body mass index or high body weight has been consistently linked to RCC [17, 18], predominantly in women [19]. Obesity itself or factors associated with obesity may have a stressful effect on renal cell metabolism including through insulin resistance, elevated growth factors (EGF), free circulating estrogens, and insulin-like growth factor (IGF-I), all of which may be involved in development of RCC [20, 21]. Hypertension seems to be associated with RCC, but difficulties in separate possible side effects from medication prevents classifying hypertension as an independent risk factor [22, 23]. Use of diuretics as well as beta-blockers have been implicated as promoting the development of RCC, though it remains unclear whether the medications or hypertension itself is the active factor [22, 23]. The combination of
obesity and hypertension [24] might stress the kidney and lead to renal damage with proliferation. Metabolic or functional changes in renal tubules may result in genetic alteration caused by exposure and increasing susceptibility to carcinogens. Other environmental risk factors are less well defined, including occupational exposure to trichloroethelene, nutritional factors, chronic renal failure, urinary tract infection, use of analgesics or reproductive hormones, and alcohol [13, 20, 22, 25-29].

**Inherited genetic vulnerability**

The genetic alterations in RCC may be a consequence of activation of oncogenes or inactivation of tumour suppressor gene. Since both copies of genes must be distorted in order for a tumour to develop, a series of events are necessary [6].

**Oncogenes** are mutated proto-oncogenes that normally code for proteins involved in control of cell division and differentiation. Intensified signalling in these pathways can lead to uncontrolled cell division and make the cell become cancerous. An activated oncogene that codes for erbB receptors, for example, can cause an over-receptive condition for growth factors as an early stage event in RCC [30]. The myc oncogene facilitates signal transcription in the nucleus and have been found to be elevated as a late stage event in RCC [6]. On the other hand, lack of growth factors lead to apoptosis [31]. The oncogene that code for met tyrosine kinase receptors generate anti-apoptotic signalling by mitogen-activated protein kinase pathways [32-36]. Several oncogenes involved signalling pathways for cell growth control have been reported in RCC [37-40].

**Tumour suppressor genes** are genes that code for proteins involved in recognition and restitution of the damaged DNA. They also initiate cell apoptosis. Otherwise, continuous cell division would transmit genetic defects, which could be intensified by increasing susceptibility due to carcinogenic exposure. The p53 gene codes for the protein p53, which when inactivated or impaired makes cells less able to restore damaged DNA [41, 42]. Inactivation of the VHL gene induces mRNA transcription of growth factor, which generates anti-apoptotic signalling and promotes angiogenesis [43, 44].

New cytogenetic and molecular genetic data have established the association between genetic characterisation and histopathological classification of RCC [45]. Four histopathological types of RCC have been described and linked to genetic diseases such as von Hippel-Lindau (VHL) disease, hereditary papil-lary renal carcinoma (HPRC), Birt-Hogg-Dubé (BHD) syndrome, and hereditary leiomyoma RCC (HLRCC) [46]. Hence, RCC can be divided into predominantly sporadic and non-inherited form or an hereditary form, which accounts only a fraction 2-5% of diagnosed RCC [47-50].

**Clear cell or conventional renal cell carcinoma** (CRCC) is the most predominate renal tumour and accounts for about 70% of diagnosed RCC tumours [45, 51]. The sporadic type of CRCC is genetically characterised by deletion of chromosome 3p, which is believed to be the early-onset event in renal carcinogenesis [45]. Inactivation of both VHL alleles is mandatory for development of CRCC [50]. Studies have shown that this mutation occurs in about 50% followed soon after by loss of heterozygity 10-20% and then, as a late event, silencing of the second VHL allele by hypermethylation.
20% [51-53]. Other genetic alterations, 5q duplication as well as 6q, 8p, 9p, and 14q deletion, have been implicated in carcinogenesis of sporadic CRCC [53-55]. Loss of the tumour suppressor gene (VHL) increases expression of transforming growth factor (TGF-α and β) and vascular endothelial growth factor (VEGF). Increased expression of onco-gene myc with abnormal receptor signalling, despite the lack of growth factors, has also been related to CRCC [56]. The age-related incidence of CRCC increases after 35 years of age and peaks in the sixth decade. Early-onset of CRCC indicates an hereditary cause [57]. A familiar form of CRCC has been described where there is early-onset with balanced 3p translocation but no traceable VHL changes, which excludes mutations in the VHL gene as the mechanism for cancer development [56, 58-60]. However, a majority of familiar CRCC is associated with von Hippel-Lindau syndrome. Inheritance is autosomal-dominant with high penetrance, with an incidence of 1 in 36,000 live births [61, 62]. The mutation affects the tumour suppressor gene (VHL) located on the short arm of chromosome 3. Up to 45% of these patients develop CRCC, and often the early-onset form [52].

Papillary renal cell carcinoma (PRCC) is the second most frequent type of carcinoma, and accounts for about 10-15% of diagnosed RCC [45, 51]. Papillary tumours are characterised by chromosomal trisomies, most often 3q, 7, 8, 12, 16, 17, and 20, with loss of the Y chromosome [45, 51, 53, 56, 63]. Hereditary PRCC is a very rare, and is characterized as an autosomal dominate inheritance with high penetrance [64, 65]. The mutation affects proto-oncogene (met) located on chromosome 7q31. About 80% of families have mutations in this proto-oncogene with late-onset. Histopathologically, the familiar and commonly sporadic form of PRCC are both referred to as type I PRCC [53]. The hereditary leiomyomatosis syndrome (HLRCC) was recently characterised as an autosomal dominant tumour syndrome caused by germline mutations in the fumarate hydratase (FH) gene on 1q42 [66]. Benign leiomyomas of the skin and uterus are often seen with predisposition to early-onset type 2 PRCC in addition to uterine leiomyosarcoma [67]. In contrast to other PRCCs, the HLRCC tumours are usually solitary and unilateral. The morphological characteristics of type 2 PRCC are independently associated with poorer survival [68, 69].

Chromophobe renal cell carcinoma (ChRCC) is a third carcinoma originated from tubular epithelium, and accounts for 5% of diagnosed RCC [45, 51]. It has a better prognosis than other variants of RCC [70]. A low chromosome number is characteristics of ChRCC, due to frequent occurrence of loss of chromosomes 1, 2, 6, 10, 13, 17, and 21 [45, 55, 71]. The Birt-Hogg-Dubé (BHD) syndrome shows autosomal dominant inheritance for benign cutaneous tumours with predisposition for ChRCC and oncocytomas [72]. Pulmonary cysts are seen causing spontaneous pneumothorax in 25% of patients [73]. The germline mutation gene is located at chromosome 17p11 [74].

Renal cell carcinoma, unclassified, is a diagnostic category used when a RCC does not fit into one of the others categories. This group represent about 4-5% of diagnosed RCC [45, 51]. No consistent pattern of genetic abnormalities has been established [53]. Due to variability in morphological patterns this might also
include the collecting duct carcinoma, which accounts for less than 1% of renal neoplasms [45, 51, 53].

**Clinical and pathological prognostic factors**

*Patient-related factors* such as gender and age have no predictive importance [6]. Inherited genetic diseases that predispose for RCC are rare [49]. Biological parameters and serum factors such as ESR, C-reactive protein, haptoglobin, and ferritin have been correlated to survival, but only ESR has been shown to have independent prognostic value [75, 76]. In some studies abnormal hemoglobin values, thrombocytosis, and elevated liver function tests have been correlated to a worse prognosis [77]. Patient performance status together with tumour presentation at the time of diagnosis is more relevant, however, for prediction of prognosis [76, 78].

*Tumour related prognostic factors* include macroscopic and histologic features. The use of TNM (tumour-node-metastasis) classification as proposed by Union International Contre Le Cancer (UICC) and American Joint Committee on Cancer (AJCC) [79] is the most important tool to predict clinical behaviour and outcome for RCC [77]. It takes into account the size of the tumour, growth beyond the renal capsule, venous involvement, adrenal metastasis, lymph node metastases, and distant metastases (Table 1). After partial or radical nephrectomy [76, 80-83], the prognosis for survival at 5 years is 90-100% for TNM stage I, 75-95% for TNM stage II, 60-70% for TNM stage III, and less than 10% for distant metastatic RCC [2, 3]. Although the 5-year-survival rate is very favourable for patients within TNM stage I, a significant number of late recurrences was observed when 10- and 15-years survival rates are taken into account [84]. After such a long follow-up periods, no significant differences in survival has been observed between TNM stages I and II or II and pT3b [81, 84]. The clinical (TNM) and pathological (pTNM) classification was modified in 2002 [85] so as to better stratify patients with favourable prognosis [86] for the clinical setting of nephron-sparing surgery [87]. The T1 category was then subdivided into T1a (less than 4 cm) and T1b (4 to 7 cm) [87, 88]. However, tumour size alone is not a definite predictor of prognosis. Sarcomatoid variants seen in all histological subtypes of RCC are associated with a markedly worse prognosis, even if the primary tumour is small [51, 89]. In ChRCC, the probability of metastatic spread is very low, and in PRCC the lower stages tends to have a better prognosis than CRCC [90].

**Histopathological grading and classification**

*The nuclear grading system* in multivariate analysis has proved to be the second most important classification (after TNM) for predicting the clinical outcome for RCC. Particular in low stages (T1-T2) the nuclear grading system was found to be an independent prognostic factor for survival [91-95]. Different grading systems have been proposed for RCC [91, 96]. Most evaluations emphasize the nuclear features and not the morphology of cytoplasm or histological formation. The worst area of the tumour, rather than over-all impression, defines the grade. Both approaches are taken into account in the Skinner and Fuhrman systems [91, 96] but the Fuhrman grading
system is currently the most used system [91, 97] (Table 2). Both systems suffer from interobserver variability and problems in reproducibility [98-100]. The 5-year survival rates for Fuhrman grades 1–4 are 65–76% in grade 1, 30–72% in grade 2, 20–50% in grade III and 10–35% in grade IV [3].

New cytogenetic and molecular genetic data have established the histologic classification of RCC [51, 79, 101] which was presented at a consensus meeting in Heidelberg in 1996 [45] and approved by the UICC and AJCC in 1997 [51].

Table 1. TNM classification and stage grouping of RCC [85]

<table>
<thead>
<tr>
<th>Primary Tumor (T)</th>
</tr>
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<tbody>
<tr>
<td>TX</td>
</tr>
<tr>
<td>Primary tumor cannot be assessed.</td>
</tr>
<tr>
<td>T0</td>
</tr>
<tr>
<td>No evidence of primary tumor.</td>
</tr>
<tr>
<td>T1</td>
</tr>
<tr>
<td>Tumor 7 cm or less in greatest dimension, limited to the kidney.</td>
</tr>
<tr>
<td>T1a</td>
</tr>
<tr>
<td>Tumor 4 cm or less in greatest dimension, limited to the kidney.</td>
</tr>
<tr>
<td>T1b</td>
</tr>
<tr>
<td>Tumor more than 4 cm but not more than 7 cm in greatest dimension; limited to the kidney.</td>
</tr>
<tr>
<td>T2</td>
</tr>
<tr>
<td>Tumor more than 7 cm in greatest dimension; limited to the kidney.</td>
</tr>
<tr>
<td>T3</td>
</tr>
<tr>
<td>Tumor extends into major veins or invades adrenal gland or perinephric tissues, but not beyond Gerota’s fascia.</td>
</tr>
<tr>
<td>T3a</td>
</tr>
<tr>
<td>Tumor directly invades adrenal gland or perirenal and/or renal sinus fat, but not beyond Gerota’s fascia.</td>
</tr>
<tr>
<td>T3b</td>
</tr>
<tr>
<td>Tumor grossly extends into the renal vein or its segmental (muscle-containing) branches, or vena cava below the diaphragm.</td>
</tr>
<tr>
<td>T3c</td>
</tr>
<tr>
<td>Tumor grossly extends into vena cava above diaphragm or invades the wall of the vena cava.</td>
</tr>
<tr>
<td>T4</td>
</tr>
<tr>
<td>Tumor invades beyond Gerota’s fascia.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Regional Lymph Nodes (N)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NX</td>
</tr>
<tr>
<td>Regional lymph nodes cannot be assessed.</td>
</tr>
<tr>
<td>N0</td>
</tr>
<tr>
<td>No regional lymph node metastases.</td>
</tr>
<tr>
<td>N1</td>
</tr>
<tr>
<td>Metastases in a single regional lymph node.</td>
</tr>
<tr>
<td>N2</td>
</tr>
<tr>
<td>Metastasis in more than one regional lymph node.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Distant Metastasis (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MX</td>
</tr>
<tr>
<td>Distant metastasis cannot be assessed.</td>
</tr>
<tr>
<td>M0</td>
</tr>
<tr>
<td>No distant metastasis.</td>
</tr>
<tr>
<td>M1</td>
</tr>
<tr>
<td>Distant metastasis.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Stage grouping</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
</tr>
<tr>
<td>T1 N0 M0</td>
</tr>
<tr>
<td>Stage II</td>
</tr>
<tr>
<td>T2 N0 M0</td>
</tr>
<tr>
<td>Stage III</td>
</tr>
<tr>
<td>T1, T2 N1 M0</td>
</tr>
<tr>
<td>T3 N0, N1 M0</td>
</tr>
<tr>
<td>Stage IV</td>
</tr>
<tr>
<td>T4 N0, N1 M0</td>
</tr>
<tr>
<td>Any T N2 M0</td>
</tr>
<tr>
<td>Any T Any N M1</td>
</tr>
</tbody>
</table>

* Not affected by laterality. Regional lymph nodes includes renal hilar, paracaval, aortic (para-aortic, peri-aortic).
Table 2. Nuclear grading systems

**Fuhrman grading system** [91]

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>Nuclei are small, round and uniform (10 μm), with inconspicuous or absent nucleoli.</td>
</tr>
<tr>
<td>G2</td>
<td>Nuclei are slightly irregular (15 μm), with small nucleoli.</td>
</tr>
<tr>
<td>G3</td>
<td>Nuclei are very irregular (20 μm), with large and prominent nucleoli.</td>
</tr>
<tr>
<td>G4</td>
<td>Nuclei exhibit large and pleomorphic often poly-lobed and bizarre (&gt; 20 μm).</td>
</tr>
</tbody>
</table>

**Skinner grading system** [96]

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>Nuclei are small, indistinguishable from those seen in normal tubular cells</td>
</tr>
<tr>
<td>G2</td>
<td>Nuclei are slightly irregular and frequently pyknotic without abnormal nucleoli</td>
</tr>
<tr>
<td>G3</td>
<td>Nuclei are irregular, enlarged and pleomorphic with prominent nucleoli</td>
</tr>
<tr>
<td>G4</td>
<td>Nuclei are extremely giant and bizarre</td>
</tr>
</tbody>
</table>

**Clear cell or conventional renal cell carcinoma** (CRCC) that appears in light microscopy with a lucid cytoplasm appears more or less empty after staining with hemotoxylin and eosin (H&E). This is an effect of intense intracytoplasmatic accumulation of glycogen and phospholipids due to increased glucose-6-phosphate levels caused by activated glycolysis and reduced gluconeogenesis [102, 103]. The nuclei of well-differentiated tumour cells are condensed, while in less differentiated tumour cells the nuclei demonstrate polymorphism and prominent nucleoli [104]. Another variant of CRCC is the eosinophilic or granular appearance of the cytoplasm, due to augmentation of mitochondria. The architecture is characteristically variable within the same tumour, with acinar or tubular growth patterns associated with poor defined stroma despite being surrounded by rich branching of delicate vasculature. [51, 89, 105]. There is a positive correlation between the extent of lymphocytic infiltration and increasing grade of malignancy [106].

**Papillary renal cell carcinoma** (PRCC) is characterized by a distinct papillary growth pattern that can become solid in undifferentiated tumour areas. The papillary structure consists of delicate fibrovascular cores with focal lipid-loaded macrophages which are covered by single layer of neoplastic cells [90, 107]. Type 1 PRCC exhibits a faint basophilic stained cytoplasm with small centrally located nuclei [108]. The cytoplasm is predominantly surrounded by endoplasmic reticulum. Type 2 PRCC represents a more aggressive form [68] with enlarged and prominent nucleoli in addition to a eosinophilic and granular cytoplasm related to accumulation of mitochondria [109]. This histological sub-typing of PRCC has prognostic value since the morphological characteristics of type-2 is independently associated with poor survival [69, 109].

**Chromophobe renal cell carcinoma** (ChRCC) is characterised by large polygonal cells with transparent cytoplasm and prominent cell membranes [110, 111]. The cytoplasm is filled with glycogen deposits and numerous microvesicles. Some tumour cells have an eosinophilic or granular cytoplasm due to an accu-
mulation of mitochondria [111]. Nuclear size and shape varies, with lack of cytoplasm colouring by routine dyes, which have been described as perinuclear ‘halos’. The microvesicles can be stained blue by the Hale colloidal iron technique [89]. The architectural pattern is usually solid [51]. ChRCC has a better prognosis than other variants of RCC [89, 105, 107, 110].

Collecting duct RCC has a tubular structure combined with a microcystic, pseudopapillary, and solid growth patterns associated with an indefinite stroma and granulocytic infiltration. It has a clinically aggressive course, often demonstrating metastases at presentation and rapid progression [112, 113]. No specific genetic alterations have been described in the rare collecting duct RCC [113]. Unclassified RCC is a category containing a heterogeneous group of tumours that cannot be classified as any of the previously mentioned subtypes [51]. Sarcomatoid changes can occur in all subtypes of RCC [51, 89].

The distinct histological subtypes have different biological and clinical behaviour [114-116]. The most common type is CRCC, which has a 55-60% 5-year survival rate [117, 118]. PRCC has a 80-90% 5-year survival rate [90, 107, 109]. ChRCC is less common and therefore survival data are limited, but ChRCC is overall associated with favourable prognosis [70]. Collecting duct RCC show an extremely aggressive behaviour with no reported 5-year survivors [112, 113]. However by using multivariate analysis, the histological subtype was not found to be an independent prognostic factor for survival [119, 120]. Recent studies indicated a clinical utility for classifying RCC in different histological subtypes since they may respond differently to treatment [121].

Angiogenesis in cancer

Angiogenesis is the formation of new capillaries by outgrowth of endothelial cells from pre-existing blood vessels. Folkman and coworkers demonstrated that tumours could only grow up to 1.75 mm when nutrition was obtained solely by diffusion. Growth beyond this size required a supply by new blood vessels [122]. The onset of angiogenesis depends on a shift in the equilibrium in extracellular matrix (ECM) created by numerous of inhibitors and stimulators towards an activation of angiogenesis [123]. Once activated, a sequence of events is required in order for formation of new blood vessels to occur [124]. First, a local degradation of basement membrane is required to facilitate the migration of endothelial cells, and this is followed by alignment of proliferated endothelial cells which form new capillaries towards an angiogenic stimulus [124]. A growing tumour needs to recruit additional blood supply in order to maintain sufficient oxygen and other nutrient availability for rapidly dividing cells. This onset of angiogenesis may be brought about by a hypoxic or hypoglycaemic condition, or may be caused by increased angiogenic stimulators due to genetic faults [123, 125]. Several growth factors with angiogenic signalling have been identified [126]. These include basic fibroblast growth factor (bFGF) and platelet-derived growth factors (PDGF), which have signalling activity for several different types of cells, whereas vascular endothelial growth factor (VEGF) affects primarily vascular endothelial cells [127].
**Tumour secreted VEGF-A**

**VEGF** is a disulfide-bonded dimeric glycoprotein with a molecular mass 34-46 kDa, and it is the most potent of all growth factors that stimulate vascular permeability and endothelial cell proliferation [128-131]. Structurally, VEGF exhibits an sequence amino acid homology to platelet-derived growth factor (PDGF) [132-134]. In cultured vascular smooth muscle cells, there are three natural occurring isoforms of VEGF-A that have been identified through analysis of isolated cDNA clones to predict sequences of 189, 165 and 121 amino acids [135]. Applying the reverse transcription polymerase chain reaction (RT-PCR) technique to different carcinoma cell lines has helped to identify additional VEGF isoforms [136]. To date, 7 different mRNA forms (VEGF$_{121}$, 145, 165, 183, 189, and 206) have been described [136-139]. All VEGF isoforms are generated from the same gene, which is located on the short arm of chromosome 6 [140]. Recently, Bates et al. described a variant of VEGF with 165 amino acid isoforms termed VEGF$_{165b}$ [141]. The VEGF$_{165b}$ demonstrated inhibitory properties on proliferation, migration, and vasodilatation in endothelial cells. This indicates that alternative spliced variants of VEGF have different functions or activities. All VEGF isoforms exhibit exons 1-5 and a terminal exon 8 although altered in VEGF$_{165b}$ [141]. Exons 1-5 and terminal exon 8 encodes VEGF$_{121}$, Inclusion of exon 7 or exons 6 and 7 encodes VEGF$_{165}$ and VEGF$_{189}$, respectively [139]. The biological differences between VEGF isoforms are dependent on incorporated exons. Exon 1 encodes the signal sequence, exon 2 the N terminus, and exon 3 the dimerisation domain [142]. Exons 3 and 4 encode binding domain to VEGF-R1 and VEGF-R2, respectively [138]. Exons 6 and 7 provide the binding affinity to heparin or heparan sulphate proteoglycans [143]. In addition, exon 7 encodes the binding domain to receptor of Neurophilin-1 [144]. Exon 8 are required for the stimulation of mitosis [141,145]. Thus, alternative splicing of VEGF mRNA generates forms that differ in solubility, and longer forms are retained by proteoglycans at cell surface or extracellular matrix [143].

Alterations in the extracellular environment can cause affected cells to release VEGF, and extracellular stimuli include, for example, hypoxia [146], and hypoglycaemia [147], the presence of inflammatory cytokines [148], platelet-derived growth factor (PDGF) [149], basic fibroblast growth factor (bFGF) [150], epidermal growth factor (EGF) [151], and insulin-like growth factor (IGF) [152, 153]. The mechanisms behind these different growth factors leading to VEGF promotion of angiogenesis are unknown. Uncontrolled cell proliferation is a causal event to produce VEGF and allow cell survival. Uncontrolled cell proliferation may be driven by an activated oncogene [154] or though series of genetic events due to inactivated or impaired tumour suppressor gene [155], by abnormal cell signalling in response to growth factors (myc) [6, 153], amplifying growth recaptor signalling (erb-B/EGF-R, met) [30, 32-36], interfering in cell cycle regulation (Cyclins D and E, p27, p53, pRb) [37-40, 156, 157], or preventing apoptosis (p53, Bc12)[158, 159]. In contrast, an inactivated or impaired tumour suppressor gene (VHL) leads tumours to consistent production of VEGF [160]. These tumours have an impaired von Hippel-Lindau protein (pVHL), which cause an accumulation of hypoxia indu-
cible factor (HIF-1α), and, as a consequence, release of several growth factors such as VEGF, PDGF and TGF-α into the extracellular matrix [150]. Co-expression between growth factors might have an autocrine role as well as a paracrine function [161].

All isoforms of VEGF-A are able to stimulate endothelial cells through two receptor tyrosine kinases VEGF-R1 [162, 163] and VEGF-R2 [164, 165]. Structural dimerisation of VEGF is essential for biological activity, and particularly for VEGF165, where the ligand pairing has a high affinity to VEGF-R2 [142, 144]. Several members of the VEGF family (VEGF-A, -C, -D, and -E) can activate VEGF-R2 and promote endothelial cells to proliferate, migrate, and differentiate [166-169]. On the other hand, VEGF-R1, activated by VEGF-A, -B, and PDGF stimulates endothelial cells to migrate [170-172]. VEGF and PDGF demonstrate an amino acid sequence homology which may in heterodimerisation provide a potential diversity in signal transduction through ligand-mediated paring with receptors VEGF-R1 or PDGF-R [132, 171]. The functional consequences of heterodimerisation between the growth factors are not quite understood. Thus, VEGF-R1 regulates structural organisation and stability of new-formed vessels, whereas VEGF-R2 regulates proliferation of endothelial cells and appears to have a more prominent role of promoting angiogenesis [144, 169, 170]. The other family members in VEGF (VEGF-C, and -D) are involved in regulation of endothelial cells during formation of new lymphatic vessels [166, 172, 173]. VEGF, PDGF, and bFGF stimulates endothelial cells to release proteases such as matrix metalloproteinases (MMP), which degrade the EMC and basement membrane, creating a window through which endothelial cells can migrate [174-176].

**Synergistic role of bFGF**

bFGF (18 kDa) was earlier described as an important angiogenic factor involved in the angiogenic switch during early tumourigenesis [177], though recently VEGF has been advocated as responsible [178]. bFGF is produced by a variety of cells including tumour cells, endothelial cells, fibroblasts, and macrophages [179, 180]. Most bFGF that is secreted from these cells is sequestered by heparan sulphate in the extracellular matrix, with the matrix serving as a reservoir for growth factors [174, 179-181]. bFGF activates additional proteases from endothelial cells to dissolve the basement membrane (ECM) and thus facilitate and induce proliferation and migration of endothelial cells [127, 179, 182]. bFGF is not as potent as VEGF in stimulating endothelial cells [127]. There is evidence that VEGF and bFGF act synergistically to promote angiogenesis [182]. In serum, bFGF has been found at significantly higher concentration levels in patients with RCC than in patients without tumours [183-185]. RCC somehow leads to secretion of bFGF [186], but no differences in expression could be observed between tumour cells and normal renal tissue [187, 188]. bFGF was found localised to ECM, whereas a small number of tumour cells expressed bFGF [189]. The expression of bFGF in general has been associated with poor survival, but was not an independent factor related to survival in RCC [189].

**Miscellaneous role of PDGF**

PDGF (30 kDa) is stored in the plate-
let granules, and is released with platelet activation [190]. It can also be produced by a variety of cells, including activated macrophages, endothelial cells, smooth muscle cells as well as tumour cells [191]. PDGF is responsible for migration and proliferation of fibroblasts, smooth muscle cells, and monocytes [192]. Although PDGF does not appear to be important for early formation of blood vessels, it may play a role at later stages through its ability to recruit pericytes and stimulate development of vascular smooth muscle cells [4, 191].
AMIS OF THE INVESTIGATION

I. To analyse the prognostic value of VEGF in preoperatively collected sera from patients with RCC.

II. To investigate whether intercurrently diseases and medications affects VEGF in serum as a prognostic factor in patients with RCC.

III. To investigate the prognostic impact of VEGF mRNA isoforms and VEGF receptor mRNA in RCC

IV. To evaluate the clinical impact of immunohistochemical detected VEGF expression in RCC.

V. To investigate the prognostic impact of VEGF protein isoforms in different RCC types.
MATERIAL AND METHODS

Patients

The study included a total of 265 patients with histopathologically verified RCC after surgery between August 1982 and December 1999. Each patient participated after informed consent and the ethics committee at the Umeå University approved the studies. The patients were randomly assigned to the individual studies as indicated in figure 1. There were 156 men and 109 women, with a median age of 66.0 years (mean 65.4; range 25 – 87 years). Among the 265 patients, 256 were operated with nephrectomy, seven with partial, and two with combined partial and radical nephrectomy due to bilateral RCC. None of the patients had been treated with radiation, chemotherapy, or immunotherapy before surgery. Most of patients were followed-up according to clinical routine [83] with clinical and radiological examinations at regular intervals. Survival was determined from time of nephrectomy to the latest follow-up, where information was obtained from hospital records or from the Cancer Registry of Sweden [1].

At the latest follow-up, 65 patients were alive with a median follow-up time 133 months, (range 26 – 236 months), 138 had died of RCC with median survival time of 17 month (range 1 – 168 months), and 62 had died of unrelated causes with 55 month median survival time (range 1 – 203 months). After radical or partial nephrectomy the survival rate at 5-years was 95% for TNM stage I, 80% for TNM stage II, 45% for TNM stage III, and 5% for TNM stage IV. The distinct histological subtypes showed different biological and clinical behaviour [116]. CRCC had a 35% 15-year survival rate, PRCC had a 47% 15-year survival rate and the less common ChRCC had an 80% 15-year survival rate. Histological unclassified RCC had an extremely aggressive behaviour as no survival could be reported at 10-year follow-up. The 15-year survival rates for Skinner grades 1– 4 was 88% in grade 1, 75% in grade 2, 32% in grade III, and 18% in grade IV.

Tumour staging

Tumour staging was performed according to the TNM stage classification system 1997 (Paper I-III) and 2002 (Paper IV-V) [85]. TNM classification included microscopic evaluation for tumour presence in regional lymph node and perinephric tissues as well as tumour invasion through the renal capsule into peri-renal fat or into major renal veins at the renal hilum. There were 78 stage I tumours (29.4%), 37 stage II (14%), 57 stage III (21.5%), and 93 stage IV tumours (35.1%). Among the 57 stage III tumours, 12 invaded peri-nephric tissue or adrenal gland (pT3a, pN0, M0), 23 major veins only (pT3b-c, pN0, M0) and 22 invaded peri-nephric tissues and major veins (pT3a-b, pN0-1, M0). One patient with stage III tumour had metastasis in a single regional lymph node. There was no apparent shift in tumour stage distribution during the 20 years of sample collection. Tumour size was measured as the maximal diameter on the surgical specimen or by computerized tomography. The median tumour diameter was 75 mm (mean 80; range 10 – 220 mm).

Morphologic classification

Histopathologic nuclear grading was performed according to Skinner and co-
workers [96], characterized on the worse histologic features, by pathologists at Umeå University Hospital (Table 3). Eleven (4%) tumours were classified as grade 1, 55 (21%) were grade 2, 132 (50%) were grade 3, and 67 (25%) were grade 4 (Table 4). RCC tumour type was histopathologic examined according to the Heidelberg classification system [45]. More than 75% tubulopapillary architecture was used for designation of tumour as a papillary RCC [193, 194]. There were 203 (77%) conventional (clear cell), 36 (13%) papillary and 15 (6%) chromophobe RCCs while 6 (2%) tumours were not amenable to morphological classification. Five (2%) tumours were missing and not available for second microscopic review according to Heidelberg classification system by Professor G Kovacs [45].

Methods

The methods used in this thesis are described in material and methods sections of the individual papers. A more general description is presented below.

Blood analysis

Preoperative blood samples were obtained as a routine before 10 a.m. The blood cell composition was analysed in the Clinical Chemistry Laboratory with standard Coulter counter technique (Coulter counters S-Plus STKR, Coulter Electronics, Luton, England, or Sysmex SE-9000, Toa Medical Electronics Co., Kobe, Japan). Patients receiving preoperatively intravenous treatment were excluded.
Table 3. Pathologists who participated in the clinical examination of microscopic sections in this thesis.

<table>
<thead>
<tr>
<th>Nuclear grading according to Skinner [96]</th>
<th>Grade 1</th>
<th>Grade 2</th>
<th>Grade 3</th>
<th>Grade 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>R Stenling</td>
<td>9</td>
<td>44</td>
<td>113</td>
<td>58</td>
</tr>
<tr>
<td>L Boquist</td>
<td>1</td>
<td>3</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>U Gerdes</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>O Hassler</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>J Vasko</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>S Cajander</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>F Bergman</td>
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<td></td>
</tr>
<tr>
<td>B Lundskog</td>
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<tr>
<td>L Bjersing</td>
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<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B Bozoky</td>
<td>1</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>G Hallmans</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A Bergh</td>
<td>1</td>
<td></td>
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</table>

Table 4. Clinicopathological characteristics of different RCC types involved in this thesis.

<table>
<thead>
<tr>
<th>Conventional Papillary</th>
<th>Chromophoe</th>
<th>Not classified</th>
<th>Not accessible</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skinner</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Grade 1</td>
<td>5</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Grade 2</td>
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<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Grade 3</td>
<td>101</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>Grade 4</td>
<td>62</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>TNM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>57</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td>Stage II</td>
<td>23</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Stage III</td>
<td>47</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Stage IV</td>
<td>76</td>
<td>13</td>
<td>2</td>
</tr>
</tbody>
</table>

Serum analysis

The samples were routinely obtained from peripheral veins in patients. The recommendation was 5 min horizontal bed resting and food restriction before sample taking [195]. Samples were collected in tubes without additive and allowed to coagulate for 30 min and centrifuged (1500 x g) for 15 min at room temperature. Obtained serum samples were stored at -80°C until analysis. Serum VEGF was analysed by quantitative sandwich enzyme immunoassay technique (Quantikine, Human VEGF immuno-assay, R & D Systems, Minneapolis, MN). A microplate precoated with mouse monoclonal antibody against human VEGF, immobilized free VEGF in samples. After a new wash, an enzyme-linked secondary goat polyclonal antibody against VEGF was added to the wells. After additional wash, a substrate solution was added to the wells and colour developed in
proportion to the amount of VEGF bound in initial step. Optical density was determined on a microtiter plate reader (Multiskan MCC/340, Lab Systems, Stockholm, Sweden). The results were measured in duplicate, and a deviation more than 10% between measurements led to re-measurement.

**Tissue collection and preparation**

Tumour and kidney cortex tissue samples were obtained from the surgical specimen as described by Ljungberg et al [196]. Each sample was divided into smaller pieces. One part was snap frozen in liquid nitrogen and stored in −80°C and the other part was formalin fixed and paraffin embedded for immunohistochemical staining and morphologic examination.

**Competitive quantitative RT-PCR**

Frozen tumour samples were homogenized and total RNA was isolated using the TRIZol method (Life Technologies, Stockholm, Sweden). Total RNA concentrations were measured spectrophotometrically at 260 nm (Lambda 2 UV/VIS, Perkin Elmer, Stockholm, Sweden). The mRNA levels of all isoforms of VEGF-A, VEGF-R1 and cyclophilin were quantified by competitive RT-PCR, as described previously [197]. Primer sequences were designed to quantify all isoforms simultaneously from the human genes of VEGF-A (5´-ATC TTC AAG CCG TCC TGT GTG C-3´ and 5´-TCA CCG CCT CGG CTT GTC ACA T-3´) and flt-1 (5´-AGG AGA GGA CCT GAA ACT GTC TT-3´and 5´-ATT CCT GGC TCT GCA GGC ATA G-3´). Briefly, 50 ng of total RNA was reverse-transcribed together with appropriate amounts of internal RNA-standards (IS), meaning truncated RNA of VEGF, flt-1, and cyclophilin. Each RNA sample was titrated with three different concentrations of IS for the respective gene (in duplicates). During 30 cycles of PCR (94°C, 30 s; 61°C, 30 s; 72°C, 45 s) templates were competitively amplified with cDNA for corresponding IS. VEGF primers used in the PCR reaction were designed for the simultaneous amplification of all VEGF isoforms (Figure 2). The PCR products were analysed by a laser fluorescence system (ABI PRISM 377 DNA sequencer, Perkin Elmer), and processed by the ABI PRISM GenScan software (Perkin Elmer) (Figure 3). Messenger RNA levels were calculated from the linear regression by extrapolation at equivalent templates to IS signals as previously described. The VEGF and flt-1 values were corrected for the corresponding cyclophilin values in each RNA sample and expressed as relative levels (fmol/amol cyclophilin). The measurements were performed twice, and a deviation more than 10% between measurements led to re-measurement.

VEGF-A is a 34-46 kDa peptide formed by two subunits (monomers) organized in anti parallel (homodimeric) manner by disulfide linked bridges. VEGF exists in different isoforms due to alternative splicing after signal sequence cleavages of transcript encode, mRNA [198]. The human VEGF gene is structured as 8 exons, separated by 7 introns. The basic sequence of mRNA consists of 8 exons and encodes the longest variant of VEGF189 with 189 amino acid. The other isoforms arise from alternative splicing of mRNA regarding sequence of exons 6 and 7. VEGF165 lacks exon 6 and VEGF121 lacks exons 6 and 7. Sense primer 5´-ATC TTC AAG CCG TCC TGT GTG C - 3´ corresponds with exon 3, and anti-
Reverse Transcription

Polymerase Chain Reaction

The primers formed (sense/antisense primer) double strands with the complementary sequences of desired site of DNA amplification (annealing). Substrate and DNA polymerase were added and from primers site a DNA fragment was synthesised (extension). A fluorescence was marked at sense primer (-5') and PCR products were analysed by a laser fluorescence system.

Figure 2. Schematically illustration on reverse transcription polymerase chain reaction (RT-PCR).

Figure 3. Chromatogram on VEGF analysis. Three isoforms of human VEGF were detected after RT-PCR and following analysis by ABI PRISM 377 DNA sequencer. Adapted from Häggström S [197].
sense primer 5′ - TCA CCG CCT CGG CTT GTC ACA T -3′ with exon 8 of human cDNA.

**Immunohistochemistry**

Representative paraffin tumour blocks were selected by primary evaluation of haematoxylin/eosin-stained slides before tissue microarray (TMA) preparation. Two tissue cores were taken with a sample needle (0.6 mm in diameter) from each tumour block and placed in a new recipient paraffin-block containing 98 tissue cores with 0.8 mm space between (Beecher Instruments, USA). For microscopic evaluation 4-μm thick paraffin sections was sliced. The slides were treated with standard procedures for deparaffinating, rehydrating, microwave heating and immunohistochemical (IHC) staining. Different antibodies were tested and a mouse monoclonal anti-human VEGF antibody (NeoMarkers, Lab Vision Corporation, Fremont, CA; VEGF Ab-3) was selected. The remains of antibody bound to antigen through washing were visualized through a colour reaction with biotinylated multilink secondary antibody (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlingame, CA). Most favourable antibody was chosen on three tissue slides selected by presence of kidney tissue, adjacent to tumour tissue, which contained poor, intermediary or strong IHC VEGF expression. (Table 5).

<table>
<thead>
<tr>
<th>Applications</th>
<th>Antibody</th>
<th>Manufacture</th>
<th>Reactivity/Epitope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Mouse, monoclonal antibody, anti-VEGF</td>
<td>Quantikine, Human VEGF immunoassay, R &amp; D Systems, Minneapolis, MN</td>
<td>Human VEGF165 and VEGF121 homodimer (VEGF165/hPDGF heterodimer exhibit 20 % cross-reactivity)</td>
</tr>
<tr>
<td></td>
<td>Goat polyclonal antibody, anti-VEGF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WB</td>
<td>Rabbit, polyclonal antibody, anti-VEGF (A-20)</td>
<td>Santa Cruz, Biotechnology Inc, Santa Cruz, CA</td>
<td>Human (Mouse and Rat) VEGF / N-terminus of VEGF</td>
</tr>
<tr>
<td>WB*</td>
<td>Rabbit, polyclonal antibody, anti-VEGF (Ab-1)</td>
<td>NeoMarkers, Lab Vision Corporation, Fremont, CA</td>
<td>Human (Mouse, and Rat) VEGF / Not determined</td>
</tr>
<tr>
<td>IHC</td>
<td>Mouse, monoclonal antibody, anti-VEGF (Ab-3)</td>
<td>NeoMarkers, Lab Vision Corporation, Fremont, CA</td>
<td>Human (Rabbit) VEGF / Not determined</td>
</tr>
<tr>
<td>IHC*</td>
<td>Rabbit, polyclonal antibody, anti-VEGF (147)</td>
<td>Santa Cruz, Biotechnology Inc, Santa Cruz, CA</td>
<td>Human (Mouse, and Rat) VEGF / Antibody raised against a peptide corresponding to amino acids 1-140 of VEGF</td>
</tr>
</tbody>
</table>

* Not applicable to this study.
Figure 4. These images illustrate the evaluation of different profiles using a special grid to calculate percentage of fields with positive IHC staining in cells emphasized by presence of nucleus and the overlaying chosen area. Profiles intersected by inclusions-edges were counted in addition to enclosed ones. A. Cytoplasmic VEGF staining. B. Cell surface VEGF staining.

The positive and uniform IHC VEGF expressions in normal kidney tubular cells were used as control for adequate immuno-histochemical application for evaluation positive IHC VEGF expression in tumour series. An additional control to confirm IHC VEGF expression was performed with the absence of the primary antibody and by a neutralizing primary VEGF antibody with recombine VEGF peptide
(Recombinant human VEGF, 293 VE; R&D Systems Inc., Minneapolis, MN) before incubation of the slides. For quantifying IHC VEGF staining, a square-magnitude mounted in the eyepiece of a light microscope was used to evaluate the density of VEGF stained tumour cells. Within framework of 121-point eyepiece graticule at 200x magnification, the density was defined as number of manually counted fields with cellular stained VEGF and overlaying chosen area (Figure 4). In TMA the sampling area was demarcated within framework of a 100-square graticule at 200x magnification, and area density of VEGF expression was defined as maximal score of two samples from each tumour. For tissue section (TS), the slides were examined at low magnification (100x), and three areas containing the highest VEGF expression were chosen as hot spot areas and then evaluated at 200x magnification. The VEGF expression in the tissue sections was defined as mean score of the volume density from the three hot spot areas. In TS, the staining intensity of IHC VEGF was assessed according to a three-graded scale: weak staining- when visible at 200x magnification, moderate intensity- when visible at 100x, and strong staining- when visible at 20x. Staining intensity was evaluated separately and independently three times, and the quantification of volume density was also evaluated three times by one observer without knowledge to preceding results. Any discordance was resolved by re-examined by a pathologist.

**Protein extraction**

Imprints were made from frozen tissue and stained with Giemsa to verify presence of tumour cells. Frozen tissue samples were homogenized with a micro-dismembrator for 2 x 10 sec., and after slight thawing, the samples were suspended in chilled Tris lysis buffer containing protease inhibitors. The homogenates were then centrifuged (13000 x g) for 30 min at 4°C. Protein in the supernatant was quantified by bicinchoninic acid assay (Pierce, Illinois). A standard curve created on absorbance of the control series was used to set the concentration of protein extractions. These measurements were performed in duplicate, a deviation between measurements of more than 10% led to re-measurement.

**Western blotting**

A Western blot (WB) procedure was performed on 30 µg protein extracts to which was added an equal volume of electrophoresis sample buffered with reducing agents to accomplish a monomeric form of VEGF. The samples were electrophoresed on 12% SDS poly-acrylamide gel and then transferred to a nitrocellulose membrane. Staining with Ponceau-red was performed to ensure a total transfer of protein extract to membrane. The membrane was either blocked in 10% horse serum or in 5% milk powder and 2.5% bovine calf serum with PBS, 0.1% Tween-20 prior to incubation with antibodies. The membrane was primarily incubated with a polyclonal rabbit antibody to human VEGF, and secondarily incubated with horseradish peroxidase (HRP) labeled anti-rabbit antibody. VEGF was visualised by enhanced chemiluminescence (ECL Plus, Amersham Int., England) and recorded on Hyperfilm-ECL (Amersham Int., England) (Figure 5). Protein expression was quantified using Fluor-S Multi Imager scanning and Quantity One software analysis (Bio-Rad Laboratories,
**Figure 5.** The membrane was soaked with ECL Plus detection reagent. Where the HRP-labeled secondary antibody was bound, a peroxidase-catalyzed oxidation of luminol was induced leading to chemiluminescence. This resulting light was detected on hyperfilm ECL Western.  

Hercules, California). Relative protein expression was evaluated against a linear standard curve obtained by different dilutions on extracts of a standard tumour used in each gel (Figure 6). Detected protein bands were controlled by not adding primary antibody or by primary antibody was blocked with human VEGF peptide before incubation. At start of the study, different primary VEGF antibodies were evaluated (Table 5). The molecular weights of detected proteins were verified by comparison with pre-stained SDS-PAGE standards (161-0372, Bio-Rad Laboratories, Hercules, California). These measurements were performed in duplicate, and a deviation of more than 10% led to re-measurement. Equal loading of protein extracts was confirmed by the actin expression. The band at 28-kDa was defined as VEGF189, the band at 22-23-kDa as VEGF165 and the band at 18-kDa was defined as VEGF121 [21, 22].

**Statistical methods**

The Mann-Whitney U test was used to identify differences in non-parametric variables for two independent groups, and the Kruskal-Wallis test was used for comparison of more than two groups. Spearman rank correlation test was used to compare relationship between sets of non-parametric variables that did not demonstrate a linear relation. Chi-Square test was used to evaluate differences in proportions of observations between independent groups. Fisher’s exact test was used when the sample size was too small to use the chi-square test. The median value was chosen as the cut-off value. Survival data was analysed using the Kaplan-Meier method, and comparison of survival times for groups was performed with the log-rank test. The variables were dichotomously tested, and analysed as continuous in a univariate Cox regression analysis. Multivariate analysis was performed according to Cox proportional hazard model. Survival time was measured from the date of nephrectomy to date of death, or latest follow-up. In all tests, a two-tailed significance level was set to $\leq 0.05$.  

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Figure 6. Using Fluor-S Multi Imager scanning and Quantity One software analysis facilitate the determination of protein density from Hyperfilm ECL. The relative protein concentration was evaluated against a linear standard curve obtained by different dilutions and extracts of tumour loaded in each gel.
RESULTS AND COMMENTS

Paper I

Serum VEGF concentration (S-VEGF) was significantly increased in patients with RCC compared to a control group. No alteration in S-VEGF was observed due to storage time of the samples. There were no differences in distribution of tumour stage, grade, and size related to gender or age. Increasing S-VEGF correlated with higher tumour stage and grade. Localized tumours (stages I-II) had low S-VEGF compared to tumours with vein invasion or extracapsular dissemination (stage III) as well as tumours with distant metastasis (stage IV). There was no relation between S-VEGF and extent of tumour-thrombus in vena renalis (pT3b) or vena cava (pT3c). A weak correlation was observed between S-VEGF and size of localized tumours (stages I-II). There was no difference in S-VEGF between PRCC and CRCC, although ChRCC results showed lower S-VEGF compared to CRCC but not PRCC. Increasing S-VEGF correlated with shorter survival in male patients, but not in female patients. In multivariate analysis, S-VEGF was not an independent prognostic factor.

Comments on Paper I

Considerable efforts have been made to find biomarkers for progression of disease in order to monitor effect of cancer treatment. Measuring circulating biomarkers in peripheral blood samples has practically appeal since access is easy and results reliable when uniform handling of blood samples is performed [199]. We demonstrated a significant correlation between S-VEGF levels and tumour stage, and that high S-VEGF levels were associated with poor outcome in patients with RCC. When Paper I was accepted for publication, there was discussion regarding the value of measuring VEGF in sera [199-201]. Peripheral venous blood samples showed higher concentration of VEGF in serum compared with matched plasma samples [200, 202-207] due to VEGF release related to platelet activation and some contribution from white cells [200, 202-204, 207, 208]. RCCs are thought to affect the bone marrow leading to increased platelet counts [208, 209]. Platelet activation in tumours [210-213] may lead to elaboration of several angiogenic growth factors, including VEGF [200]. The theoretical advantage of analysing VEGF in plasma has limitations since circulating free VEGF in vivo is subject to degradation as well as binding to VEGF-binding protein levels, and may not directly reflect tumour secretion of VEGF [201]. S-VEGF levels differ from plasma VEGF also due to the fact that platelets deliver VEGF to tumours [214-216]. It has been suggested that PDGF reflects tumour biological activity [217], and that serum samples are more useful for measurement circulating growth factors [205]. VEGF165 is the predominant isoform in serum [218] and is also the isoform predominantly secreted by a variety of cancer types [219]. In Papers I-II, analysed levels of S-VEGF165 reflect also the biological effects of VEGF121 and VEGF165/PDGF heterodimer occurring in serum after platelet agglutination.

Paper II

Approximately two-thirds of patients with RCC were concurrently treated with long-term medication because of chronic
diseases. No differences in distribution of tumour stage, grade, or size were observed related to gender, age group, or presence of long-term medication. The proportion of patients with long-term medication increased with age for both genders. No difference in S-VEGF was found in relation to gender, age group or presence of long-term medication. Increasing S-VEGF was correlated with higher tumour stage and grade, as well as higher platelet and leukocyte counts. No difference in blood platelet counts was found between male and female patients. However, platelet counts showed an inverse correlation to age, particularly in women. In male patients, platelet counts were lower in those on long-term medication compared to those without. Univariate analysis showed that increased S-VEGF and platelet counts correlated with shorter survival especially in male patients. In contrast to S-VEGF, higher blood platelet counts were only associated to shorter survival in patients with RCC with no intercurrent chronic disease or long-term medication. Leukocyte counts were not associated with prognosis. Age, gender, or long-term medication had no association with survival time in patients with RCC. Using Cox proportional hazard step-wise elimination, VEGF levels were the last variable to be excluded, and only stage and grade remained as independent prognostic factors.

**Comments on Paper II**

This paper demonstrated a correlation between VEGF levels and blood platelet and leukocyte counts, as reported in other malignancies [202]. Platelets have been revealed as the main source of VEGF in serum [200, 207, 210, 211, 220]. However, the lack of relationship between S-VEGF levels and blood platelet counts in survival curves may be due to peripheral distribution of larger immature platelets with higher quantities of VEGF [207] or caused by other factors as acquired dysfunction of platelets commonly associated with medication in elderly people [221]. Moreover, decreased blood platelet counts have been associated with medication and advancing age [222-224]. Epidemiologic studies have shown that established risk factors such as obesity and cigarette smoking can be confounding factors when examining effects of medication [225]. In addition, anti-hypertensive drugs and analgesics have been proposed as independent risk factors for RCC development [225, 226]. This paper showed that patients with RCC had decreased platelet counts that were associated with intercurrent chronic disease, presence of long-term medications, and advancing age. In women, blood platelet counts were not affected by chronic diseases or medications but decreased with advancing age. In addition blood platelet counts had no predictive value for survival for women, regardless of presence or absence of chronic decease or long-term medications. In men with chronic diseases and long-term medications, blood platelet counts were decreased, and no predictive value for survival was found in those subgroups of patients. It is noteworthy that RCC is most common in patients at their sixth to seventh decades of life [1], but this ratio between gender tends to decrease with increasing age [227]. Whether or not other health risk factors may initiate conversion of tumour dormancy to angiogenic phenotype is not clear [228]. S-VEGF levels in peripheral blood samples suggest that S-VEGF has much of its origin from platelets and to some extent
from white blood cells. However, the tumour angiogenesis process may be dependent on platelets that deliver VEGF to tumours. Still, S-VEGF levels have been reported to be significantly higher in samples from tumour-bearing renal veins compared with samples from peripheral veins [229]. We found that S-VEGF levels were unaffected by intercurrent chronic diseases or medications. In addition, there were no differences in S-VEGF levels in relation to age or gender. In contrast to men, S-VEGF levels had no predictive value for survival in women with RCC, and the reason for this is unclear. Our results indicate that S-VEGF is more useful as a prognostic indicator or biomarker, than platelet or leukocyte counts. Measurement of VEGF in serum may have value for monitoring of therapeutic effect or choice of therapeutic modality [230].

Paper III

The RCC samples demonstrated a wide range of mRNA levels for VEGF121, VEGF165, and Flt-1 (VEGF-R1). Other VEGF isoforms were not be detected. A significant correlation was observed between mRNA levels for VEGF121 and VEGF165 and between mRNA levels for VEGF165 and VEGF-R1. RCC confined to the kidney (pT1-2 N0 M0) had higher mRNA levels for VEGF121 compared with locally advanced tumours (pT3 N0-1 M0). On the other hand, S-VEGF was lower in localised tumours (pT1-2 N0 M0) compared with locally advanced tumours (pT3 N0-1 M0). There was no association between different isoform VEGF mRNA levels and S-VEGF. CRCC had significantly higher VEGF121 and VEGF-R1 mRNA levels compared with PRCC. In patients with locally advanced tumours (pT3a-c N0-1 M0) increased VEGF121 mRNA levels correlated with adverse survival. For VEGF165 and VEGF-R1 mRNA levels no predictive information was found.

Comments on Paper III

RT-PCR method allows detection and quantification of small amounts of specific mRNA in RCC samples. Inappropriate preparation of tissue samples can lead to invalid results due to tiny amounts DNA introduced through external contamination. Optimal preservation, storing and handling of samples are important to avoid mRNA degradation. Despite adequate execution of the RT-PCR method, the interpretation of analysed mRNA involves a mixture of cells from different origin that includes tumour cells. The surgical procedure, with ligation of the blood supply during nephrectomy and time to excision of tumour samples, inevitable effects VEGF expression. However, previous studies have shown that when handling tissue samples, HIF-1α levels remain constant up to 60 min after clamping of the renal artery [43]. Moreover, no significant up-regulation of mRNA VEGF or changes in protein VEGF content has been demonstrated during 20 minutes of ischemic conditions in kidney tissue [231]. Although the patient sample sizes was small in Paper III we demonstrated that VEGF mRNA and VEGF-R1 mRNA levels were higher in CRCC compared with PRCC, and the appearances of diversity between VEGF mRNA and S-VEGF levels should be further examined. VEGF-R1 mRNA levels had no predictive information, which could be explained by endothelial cell activation of VEGF-R1 brought about by several growth factors, including VEGF-A, VEGF-B as well as PDGF released at different stages in RCC.
**Paper IV**

Positive immunohistochemical (IHC) VEGF staining was observed in both RCC and kidney cortex samples. Kidney cortex usually showed a strong IHC VEGF expression in cytoplasm of tubular cells. While samples from RCC showed considerable variation in IHC VEGF expression on cell membranes as well as in cytoplasm of tumour cells. IHC VEGF expression at cell membrane was affected by the storage time of paraffin embedded tumour specimens. Hence, membranous VEGF expression was not further evaluated. There were no differences in cytoplasm VEGF expressions between gender or different age groups. A significant correlation was found between VEGF expression in cytoplasm and the size of tumours. In tumours smaller than 7 cm, increasing VEGF expression was observed between stage I (pT1 N0 M0), stage III (pT3a-c N0-I M0), and stage IV (N2 M1), but no such difference was found in VEGF expression at tumours larger than 7 cm. Tumours invading through the renal capsule (pT3a, N0-1, M0) had higher VEGF expression compared with tumours confined to kidney (pT1-2, N0, M0) or tumours invading veins only (pT3b-c, N0, M0). No difference in VEGF expression was recognized between different RCC types. In univariate analysis, increased VEGF expression correlated with adverse survival in patients with CRCC. No correlation was found between VEGF expression and survival time in PRCC or in ChRCC. In multivariate analysis the cytoplasmatic IHC VEGF expression was not an independent prognostic factor.

**Comments on paper IV**

Immunohistochemistry allows identifying also the localization of protein expressed within tissue sections. However, the outcome of immunohistochemical examination is dependent on the quality of the biopsy sample, presence of tissue necrosis, antigen loss due to delay in fixation of samples, type of fixative, duration of fixation, processing of samples, choice of antibody, and sensitivity of detections method for antigen-antibody reaction [232]. Fixation prevents the degradation of tissues, preserves the morphology, and stabilizes cells and tissues. On the other hand, fixation causes a cross-linking within protein molecules, which alters the structure of the proteins, possibly altering the epitope and masking the antigen against potential antibody binding. Heat-induced antigen retrieval may disrupt these cross-links but recovery of native molecule structure may depend on achieved temperature and length of incubation. Complete recovery of native molecule structure is particularly important for monoclonal antibodies, which recognise a single epitope, whereas polyclonal antibodies recognise a number of different epitopes. Polyclonal antibodies may express increased sensitivity to target but may also contain increased risk for cross-reactivity. Monoclonal antibodies on the other hand are targeted at a single epitope on the antigen and are therefore highly specific. However, a monoclonal antibody might fail to bind the epitope if the antigen is vulnerable and unstable due to the fixation process. It has been demonstrated that the antigenicity is inversely correlated to duration of exposure to formaldehyde [233]. Quantification of immunoreaction intensity is an error filled process, since no linear relationship exists between amount of antigen and the expressed antigen-antibody reaction. A
more valid form of quantification is determining the percentage of estimated antigen-antibody specific cellular reaction. Identifying hotspot areas and estimating relative area with immunoreactions is a highly operator-dependent process, and other investigators have emphasized the advantage of using the methods described by Chalkey [234]. In Paper IV, variation in results obtained from different methods was shown, but nevertheless a correlation between observations could be noted. Thus, the established VEGF expression in cytoplasm as being predictor for survival led us to further examine the role of VEGF location in RCC. Cytoplasmic VEGF was demonstrated to be of prognostic significance only in smaller tumours. Furthermore, in solid tumours, several other growth factors have been proposed as candidate to persuade the growth when tumours reached a certain volume [235].

**Study V**

Both RCC and kidney cortex samples expressed immunoreactive VEGF bands. Samples from kidney cortex expressed VEGF\textsubscript{189} and VEGF\textsubscript{165} isoforms, but not VEGF\textsubscript{121}. Both VEGF\textsubscript{189} and VEGF\textsubscript{165} isoforms were strongly expressed in kidney cortex, compared to RCC samples. In RCC samples, VEGF\textsubscript{189} had highest immunoreactive expression and VEGF\textsubscript{165} intermediate, while VEGF\textsubscript{121} was rarely detected. VEGF\textsubscript{121} expression was considered positive when a band was visually detected on the Western blot. There was considerable variation in VEGF\textsubscript{189} expression between the different RCC types, in contrary to VEGF\textsubscript{165} expression, which was usually low. No differences in VEGF isoforms were found according to gender, age, or due to storage time of tumour samples. CRCC had lower levels of both VEGF\textsubscript{189} and VEGF\textsubscript{165} isoforms compared with kidney cortex. There were no correlations between isoforms and tumour stage or grade in CRCC, although VEGF\textsubscript{189} was verified as low in tumours with renal capsule invasion (pT3a, N0-1, M0) compared with those without invasion (pT1-2, N0, M0). VEGF\textsubscript{121} was more frequently detected in smaller (pT1) and localized tumours (stage I-II) compared with larger (pT2) and advanced tumour stages (stage III-IV). PRCC showed also lower levels of VEGF\textsubscript{189} and VEGF\textsubscript{165} compared with kidney cortex. In PRCC, protein VEGF\textsubscript{165} level correlated inversely with tumour size, and VEGF\textsubscript{189} levels correlated inversely with tumour stage and grade. In ChRCC, protein level of VEGF\textsubscript{189} was comparable to that in kidney cortex, while VEGF\textsubscript{165} level was significantly decreased. Also in ChRCC, the VEGF\textsubscript{189} levels were lower in advanced tumour stages (stage III-IV) compared with tumours confined to kidney (stage I-II). VEGF\textsubscript{121} was not detected in chromophobe RCC. In univariate analysis, VEGF\textsubscript{165} showed no predictive information. However, low VEGF\textsubscript{189} levels were associated with shorter survival in PRCC. In Cox multivariate analysis, the VEGF\textsubscript{189} expression remained an independent prognostic factor in patients with PRCC, while TNM stage was the last variable to be omitted after stepwise elimination analysis.

**Comments on paper V**

The WB-technique is based on protein extractions separated in SDS-PAGE gel. An equal loading of samples is crucial when WB is used for quantification. Assessment of protein extract concentration was reproduced at least twice, and the loaded protein was controlled by
Ponceau-Red staining, and sample protein concentration re-assured through actin detection by WB. Each sample was analysed in duplicate, and in this paper with different blocking solutions, which were used due to the EU import restriction of hoarse serum from New Zealand in 1995. Any discordance in duplicate results led to re-analysis with available blocking solutions. Advantages with WB-technique are the option to evaluate different VEGF protein isoform by their molecular weight separated in SDS-PAGE gel, as well as the possibility to quantify expression of each VEGF isoform. A weakness with the WB method is that protein extractions are based on expression from a mixture of cells of different origin including some other than tumour cells. Therefore imprints were made to verify all samples in fact contained tumour tissue. The findings in this paper support the idea that VEGF_{189} may be the primary isoform reflecting tumour angiogenesis. Although mRNA VEGF_{189} was barely detectable in Paper III, the high VEGF_{189} protein expression in tumour tissue can be interpreted that VEGF_{189} protein is produced and most likely stored locally in tumour cells. Expression of mRNA VEGF_{189} has been demonstrated in tumours stage T3-T4, and furthermore is associated with higher microvessel density in contrast to other isoforms [236]. Angiogenesis is induced by released VEGF and enhanced by increased VEGF receptor density on endothelial cells. In addition, angiogenesis is thought to be promoted by release of bioactive different growth factors such as VEGF_{165}, VEGF_{189}, and bFGF during proteolytic degradation of ECM [143, 237].
Molecular biomarkers are expected to have an important impact on future diagnosis, prognostication and selection of therapeutic targets for RCC. Several potential drugs appear promising to inhibit tumour angiogenesis in RCC. These include targeting VEGF with monoclonal antibody (Bevacizumb) [238, 239] and inhibition of VEGF-R2 and PDGF-R (BAY 43-9006 / Sorafenib) [240, 241], or multitargeted tyrosine kinase inhibitor of VEGF-R1, VEGF-R2, Flt-3 and PDGF-R (SU11248) [240, 241], whereas Thalidomide [242] and TPN-470 [243] have provided less promising clinical results. Novel targeted therapies such as matrix metalloproteinase inhibitors (AG3340 / Primo-mastat), (BB2516 / Marimstat), AE-941 / Neovastat) have undergone clinical testing with positive results [244]. Unique approaches with the aim of selectively inhibiting angiogenesis is provoking apoptosis in tumour stimulated endothelial cells in order to undermine growth and support of existing tumour vessels and cause tumour cell death from ischaemia (ZD6126 / Combretastatin) [245]. Considering the number of different genetic defects in RCC, it is unlikely that a single drug will be effective in treatment of advanced RCC. More likely, these new angiogenesis inhibitors can be expected to be most effective when used as adjuvant therapy to surgery where risk for recurrences of RCC exist or in combination with drugs having different mechanisms of action. Currently no prognostic biomarkers are available to independently validate the therapeutic effect in individuals with RCC. These evaluations still depend on traditional clinical surveillance. The complexities of multiple different growth factors are not completely understood, and their role and relationship remains important as a subject of future investigation in RCC.

There are several issues, which need to be further investigated, as whether cancer cells have receptors for VEGF and what significance this existence of autocrine effect might have. Do different VEGF isoforms have an auxiliary effect on potential target cells i.e. causing vascular permeability to macromolecules and oedema, hemodynamic alteration by vasodilatation, and incitation of angiogenesis? Despite evidence of vascular dilatation and increased permeability the effects and regulation are not clear in pathological angiogenesis. Does VEGF independently correlate to increased microvessel density, or are there other factors that counteract or counterpart VEGF? Hypoxy inducible factor (HIF) affects transcription level of VEGF, however do there exist other factors that could be considered as regulatory of VEGF in RCC?
Figure 7. Antiangiogenic drugs currently under evaluation in cancer therapy.
CONCLUSIONS

Paper I

In RCC, S-VEGF level was significantly correlated to tumour stage and grade. Increased levels of S-VEGF were associated with shorter survival. Although, S-VEGF did not remain as an independent prognostic factor in multivariate analysis, the levels of S-VEGF were found useful for the identification of patients with potentially progressive disease especially those with vein invasion.

Paper II

S-VEGF showed a stronger association to prognosis than blood platelets and leukocyte counts. Blood platelet count seems to be affected in patients with intercurrent chronic disease and long-term medication, and in this large group of patients blood platelets counts are not associated with survival. Blood leukocyte count had no prognostic value.

Paper III

No correlation was found between VEGF_{165} mRNA and S-VEGF levels. Both VEGF_{121} and VEGF_{165} mRNA isoforms and Flt-1 mRNA (VEGF-R1) levels were higher in CRCC compared with PRCC. The trend of decreased levels of isoforms VEGF_{121} and VEGF_{165} mRNA in locally advanced RCC indicates that initiation of angiogenic activity by VEGF might be an early event and during tumour progression other factors are more relevant.

Paper IV

IHC VEGF staining was observed at cell membrane and cytoplasm in RCC cells. There was no difference in cytoplasm VEGF expression between the different RCC types. Associations between expression of cytoplasm VEGF and tumour size as well as prognosis indicate the significance of VEGF in growth and progression. The expression of IHC VEGF in cytoplasm might be useful for identification of potentially progressive RCC but was not an independent prognostic factor in multivariate analysis.

Paper V

Different VEGF isoform patterns were found between the specific RCC types. VEGF_{165} protein expression had no predictive value for survival and was observed in low levels in RCC samples. The protein VEGF_{189} expression was associated with tumour stage and grade and was an independent prognostic factor in PRCC.
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