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# Prognostic Factors in Renal Cell Carcinoma



ACADEMIC DISSERTATION

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## ACADEMIC DISSERTATION

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*To Marja,  
Joel and Lauri*

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## LIST OF ORIGINAL PUBLICATIONS

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II Kallio J, Tammela TLJ, Marttinen AT and Kellokumpu-Lehtinen P (2001): Soluble immunological parameters and early prognosis of renal cell cancer patients. *J Exp Clin Cancer Res* 20(4): 523-528.

III Kallio JP, Hirvikoski P, Helin H, Kellokumpu-Lehtinen P, Luukkaala T, Tammela TLJ, Martikainen PM (2003): Membranous location of EGFR immunostaining is associated with good prognosis in renal cell carcinoma. *Br J Cancer* 89: 1266-1269.

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V Kallio JP, Mikkelsen J, Tammela TLJ, Karhunen P, Kellokumpu-Lehtinen P (2004): Genetic variation in platelet integrin  $\alpha_{IIb}\beta_3$  (GPIIb/IIIa) and metastatic potential of renal cell carcinoma. Submitted to *European Urology*.

## ABBREVIATIONS

AgNOR	silver stained nuclear organizer region
AJCC	the American Joint Committee on Cancer
CGH	comparative genomic hybridization
CT	computed tomography
EGFR	epidermal growth factor receptor
GM-CSF	granulocyte macrophage –colony stimulating factor
GMTV	gene-modified tumour vaccines
HLA	human leukocyte antibody
ICAM	intercellular adhesion molecule
IFN	interferon
IG	immunoglobulin
IL	interleukin
LAK	lymphokine activated killer cells
LIF	leukaemia inhibitory factor
MDR	multidrug resistance
MRI	magnetic resonance imaging
p	short arm of the chromosome
PCNA	proliferating nuclear cell antigen
PCR	polymerase chain reaction
q	long arm of the chromosome
RCC	renal cell carcinoma
RFLP	restriction fragment length polymorphism
TGF	tumour growth factor
TIL	tumour infiltrating lymphocytes
TNF	tumour necrosis factor
UICC	Union Internationale Contre le Cancer
VEGF	vascular endothelial growth factor
VHL	von Hippel-Lindau –syndrome



## ABSTRACT

The prognosis in renal cell carcinoma (RCC) varies widely, from very good in small local and non-aggressive tumours to poor, only a few months' survival, in aggressive and widely disseminated cases. More efficient prognostic factors would be desirable for patient information, planning of follow-up and adjuvant treatments, and for evaluation of response to different chemo-immunotherapy schedules. This study examined the prognosis of renal cell carcinoma in five different aspects: 1) The number of DNA losses found in comparative genomic hybridization (CGH) and proliferation activity. 2) Pretreatment and postoperative serum cytokines and short-term survival. 3) The cellular location of EGFR immunostaining. 4) Expression of apoptotic regulators Bax and Bcl-2. 5) The genetic polymorphisms in platelet fibrinogen receptor and possible associations with metastatic disease.

In prospective studies CGH evaluation was made in 20 patients, cytokines were studied in 24 patients and EGFR, Bax and Bcl-2 –assays performed in 138 patients with RCC. In addition, a cross-sectional survey of fibrinogen receptor status was made in a group of 128 male patients. All patients were treated in the departments of Urology and/or Oncology at Tampere University Hospital.

CGH detected genetic aberrations in all tumours. Losses of genetic material were more common (85%) than gains (65%). Most common was the loss in the short arm of chromosome 3, found in 70% of tumours. The number of deleted chromosomal areas varied from none to six. The number of deleted areas in common type carcinomas had no association with prognosis, but high proliferation activity was associated with poor outcome.

Serum IL-6, sIL-2R and sICAM-1 levels before nephrectomy were significantly higher in non-local than in local tumours. In contrast, IL-12 levels were higher in local tumours and the levels increased significantly ( $p \leq 0.005$ ) after removal of the primary tumour in patients with local disease. All patients with local tumours had normal IL-6 values, while only one with non-local tumours had an IL-6 level below 10 pg/ml. In addition, IL-6 and sICAM-1 levels before operation were significantly higher in patients with short (less than one year) survival (in IL-6  $p=0.007$  and in

sICAM-1  $p=0.006$ ). In contrast, patients with shorter survival had significantly lower IL-12 ( $p=0.03$ ).

Evaluation of patients with positive EGFR immunostaining (77%) showed prominent membranous staining to be associated with a good prognosis. Their overall survival was significantly longer ( $p=0.004$ ) than that of patients with either EGFR-negative tumours or with mainly cytoplasmic staining. Membranous location of EGFR was associated with low Fuhrman grades. Only disease stage reached statistical significance in Cox multivariate analysis

Apoptotic regulators Bax and Bcl-2 reached maximal statistical power when their scores were handled as two classes, negative and positive. Surprisingly, both regulators evinced high apoptotic activity associated with poor prognosis. Possibly other factors influence fast progression of the tumour more intensively, high apoptosis being only a compensatory phenomenon. Low MIB-1 values, low Fuhrman grades (I-II) and low stage (stage I-II) associated with good survival. Only stage and Fuhrman classification reached statistical significance in multivariate analysis.

The different alleles of GPIIIa  $PI^{A1/A2}$ , GIB-IX-V C3550T and GPIa/IIa C807T integrins were evaluated and their associations with metastatic disease were recorded. The frequency of presence of the GPIIIa  $PI^{A2}$  allele among patients with metastatic RCC was 43.6% and with local disease 24.7%,  $p=0.024$ . The frequencies of the different alleles of the GIB-IX-V C3550T and GPIa/IIa C807T polymorphisms did not differ between groups. In stepwise logistic regression (metastatic vs. local RCC) the  $PI^{A2}$  allele remained a significant risk factor,  $p=0.02$ .

According to these results good prognosis for a patient with RCC is associated with: 1) low proliferation activity, 2) low preoperative IL-6 and ICAM-1 and high IL-12 values and marked postoperative elevation of IL-12, 3) membranous location of EGFR in tumour cells, 4) low apoptotic activity (estimated by Bax and Bcl-2 –expression) and 5) absence of the prothrombotic  $PI^{A2}$  allele of platelet fibrinogen receptor GPIIb/IIIa associating with the risk of metastases.

## 1. INTRODUCTION

Kidney cancer was the 10<sup>th</sup> commonest malignoma in men (390 new cases) and 12<sup>th</sup> in women (311 new cases) among newly detected cancers in Finland in 2002 (Finnish Cancer Registry, April 2004). The incidence rises with age, reaching a maximum at 60 to 70, and men are affected slightly more often than women. The incidence varies considerably in different countries; for example the male incidence in Iceland is 15.5 and in Angola 0.3 (Pantuck et al. 2001).

There has been an increase in the number of asymptomatic incidental tumours detected as a result of the widespread use of non-invasive new imaging modalities such as ultrasound, computerized tomography (CT) and magnetic resonance imaging (MRI) (Jayson and Sanders 1998). A shift towards smaller, lower-stage lesions has been noted and better survival reported (Pantuck et al. 2001).

Surgery remains the most effective treatment in local renal cell cancer, including principles of radical nephrectomy (Robson 1963), open partial nephrectomy (Fergany et al. 2000) and more recently laparoscopic nephrectomy, which is coming into wider use (Cadeddu et al. 1998). The results of new ablative therapy such as cryotherapy and radiofrequency interstitial tumour ablation are as yet only preliminary (Gill and Novick 1999; Walther et al. 2000).

In metastatic renal cell carcinoma the role of nephrectomy has been palliative. However, in two recent prospective randomized studies the survival benefit of nephrectomy was demonstrated when nephrectomy combined to interferon- $\alpha$  therapy was compared with interferon- $\alpha$  therapy alone (Flanigan et al. 2001; Mickisch et al. 2001). In chemoimmunotherapy the combination IL-2 and interferon- $\alpha$  seems so far to be the most effective (Atzpodien et al. 2004; Negrier et al. 1998).

The prognosis in renal cell carcinoma (RCC) varies considerably, from very good in small local and non-aggressive tumours to poor, with only a few months' survival, in aggressive and widely disseminated cases. For example, 5-year survival rates in different TNM classes are generally: organ confined (T1-2): 75-100%, perinephric or adrenal invasion (T3): 40-78%, lymphatic node metastases (N1): 30-50% and distant metastases (M1): 0-10% (Javidan et al. 1999). More efficient prognostic factors would be desirable for patient information, for the planning of follow-up and

adjuvant treatments, and for evaluation of response to different chemo-immunotherapy schedules. An appropriate new prognostic marker should be statistically significant and independent and clinically relevant. In spite of several newer candidates, stage, histological grade, histological type and performance status have remained the main prognostic factors in renal cell carcinoma. Newer candidates (VEGF, cyclin D1, p27, nm23 etc.) have often shown clear prognostic association, but in multivariate analysis have lost their significance as independent prognostic factors. Combination of several factors has been an attempt to achieve greater statistic power. When advanced statistical methods are combined with new information on tumour and patient, better prognostic results will be obtained.

The purpose of the present study was to evaluate the significance of a number of potential prognostic factors in RCC, including the number of DNA losses found in comparative genomic hybridization (CGH) and proliferation activity, pre-treatment and postoperative serum cytokines and short-term survival, the cellular location of EGFR immunostaining, expression of the apoptotic regulators Bax and Bcl-2, the genetic polymorphisms in platelet fibrinogen receptors and possible associations with metastatic disease.

## 2. REVIEW OF THE LITERATURE

### 2.1. Epidemiology of renal cell carcinoma

#### 2.1.1. Incidence and prevalence

Kidney cancer was the 10<sup>th</sup> commonest malignoma in men (390 new cases, incidence 9.5/ 100,000) and 12<sup>th</sup> in women (311 new cases, incidence 5.6/ 100,000) among newly detected cancers in Finland in 2002. The predicted figures for the year 2004 are clearly higher (426 for men, 319 for women, Finnish Cancer Registry, April 2004). The incidence increases with age, reaching a maximum at 60 to 70, and men are affected slightly more often than women. The incidence varies considerably in different countries; for example the male incidence in Iceland is 15.5 and in Angola 0.3 (Pantuck et al. 2001).

Since 1950 there has been in the United States a 126% increase in incidence accompanied by a 36.5% increase in annual mortality. This increase occurred in all age groups, the greatest increase being seen in patients with local tumours, suggesting a shift toward earlier stages (Pantuck et al. 2001). This may be explained partially by the increased number of asymptomatic incidental tumours detected as a result of the widespread use of new non-invasive imaging modalities such as ultrasound, computerized tomography (CT) and magnetic resonance imaging (MRI) (Jayson and Sanders 1998). A tendency towards smaller, lower-stage lesions has been noted and better survival reported (Pantuck et al. 2001). On the other hand, scrutiny of a population-based cancer registry, The Surveillance, Epidemiology and End Results (SEER) Program of the National Cancer Institute (NCI), revealed no significant difference in stage presentation of renal cancer in different time periods. The lack of a decrease in metastatic disease despite the increased detection of regional and localized renal cancer implies that a proportion of innocuous renal cancer cases may be detected by increased abdominal imaging. The growing incidence of RCC in all stages indicates that other contributing factors may also be implicated. Large single institution studies do not necessarily represent whole populations; there may be a selection bias in favour of localized (and operable) tumours (Hock et al. 2002).

### 2.1.2. Aetiology

A well-known risk factor associated with renal cell carcinoma is cigarette smoking. Approximately 30% of renal cell carcinomas in men and 24% in women are thought to be due to smoking (McLaughlin et al. 1984). Many case-control studies have shown that smoking clearly increases the risk of renal cell carcinoma (Martel and Lara 2003), with estimated relative risks ranging from 1.24 to 2.3. Most studies have demonstrated a dose-response relationship, resulting in heavier smokers being at greater risk than light smokers. The risk decreases after smoking cessation, with a 25-30% fall in risk by 10-15 years after quitting.

Obesity has emerged as a clear risk factor for renal cell carcinoma in many studies (Martel and Lara 2003). The effect is more pronounced in women and no good explanation has been offered for this association. Hormonal factors have been suggested, but the evidence for this remains limited (Newsom and Vugrin 1987).

Some medications have also been associated with an elevated risk of RCC. Phenacetin-containing analgesics are implicated in transitional cell carcinoma and there may also be a mild risk of RCC. Acetaminophen has proved to involve an elevated risk in one study, but the finding was not confirmed. Some studies have associated diuretics with an elevated risk of RCC (Martel and Lara 2003).

The role of diet in renal cell carcinoma risk remains unclear. Some studies find association between elevated risk and increased consumption of coffee and alcohol, but other studies do not (Yu et al. 1986). A diet rich of fruits and vegetables has consistently been associated with decreased risk of renal cell carcinoma, but what specific nutrients are responsible for this remains obscure (Wolk et al. 1996).

Evidence for an association between occupational exposures and renal cell carcinoma is relatively weak (McLaughlin and Lipworth 2000). Some studies have suggested an increased risk with exposure to the following compounds: asbestos, polycyclic aromatic hydrocarbons, oil, gasoline, the solvents trichloroethylene and perchloroethylene, and the heavy metals lead, cadmium and arsenic. However, none of these associations has been consistently found in all studies (Martel and Lara 2003).

Ionizing radiation has been reported to increase the risk of renal cell carcinoma in patients exposed to high doses for therapeutic indications (e.g. as therapy for cervical cancer), but the level of increase appears to be fairly low (Boice et al. 1988).

A 100-fold increase in the risk of RCC has been reported in patients with end-stage renal disease (Doublet et al. 1997). This may be related to acquired cystic disease of the kidneys. Nearly 50% of long-term dialysis patients will develop these cystic changes in the kidneys and approximately 6% of these patients will develop renal cell carcinoma (Brennan et al. 1991). This gives a special indication to imaging follow-up of these patients.

### 2.1.3. Genetics

The von Hippel Lindau tumour suppressor gene (VHL) is mutated or inactivated in most sporadic clear cell carcinomas (CC-RCC); this is an early event in carcinogenesis of this tumour. The normal functions of the VHL protein include the regulation of the oxygen-dependent expression of genes which regulate the cellular response to hypoxia. These include genes associated with angiogenesis, erythropoiesis and resistance to hypoxia, for example hypoxia-inducible factors (HIF). VHLp regulates the ubiquitin-mediated destruction of HIF. In the absence of VHLp high levels of HIF prevail and this induces high expression of endothelin-1, vascular endothelial growth factor (VEGF), ceruloplasmin, erythropoietin, cyclin G2, transforming growth factor (TGF) etc., leading to tumour progression (Maxwell et al. 1999; Maynard and Ohh 2004). Gene expression profiling has yielded abundant new information on genetic changes in CC-RCC. Highly expressed genes are for example ceruloplasmin (iron-copper metabolism), lysine oxidase (copper metabolism), VEGF, fibronectin, caveolin-1. Down-regulated genes are many tumour suppressor genes, the metallothionein family, aldolase-B, glypican-3, etc. (Takahashi et al. 2003). Histologically divergent RCC types also often have typical chromosomal/ genetic changes (see below “Classification of tumours”).

Some genetic syndromes are associated with renal cell carcinoma: Von Hippel-Lindau (VHL) disease is caused by a mutation of the von Hippel-Lindau gene, which encodes a tumour suppressor protein. The gene is also important in sporadic RCC, as both gene copies are inactivated in 75% of sporadic clear cell renal carcinomas. Phenotypically the von Hippel-Lindau syndrome is characterized by multiple retinal and central nervous system hemangioblastomas,

pheochromocytoma, and less commonly by pancreatic cysts and cystadenomas, islet cell tumours, tumours of the endolymphatic sac in the inner ear and papillary cystadenomas of the epididymis or adnexae (Iliopoulos and Eng 2000). Patients develop hundreds of bilateral renal cysts and 40-60% of them develop clear cell renal carcinoma. These tumours develop at a younger age than do sporadic cases and they tend to be multicentric and bilateral (Walther et al. 1995).

Hereditary leiomyomatosis and renal cell cancer (HLRCC) is an autosomal dominant tumour syndrome caused by germline mutations in the fumarate hydratase (FH) gene (1q42.1) (Tomlinson et al. 2002) It is characterized by a predisposition to benign leiomyomas of the skin and the uterus and a subset of patients will develop RCC with specific papillary type 2 histology (Launonen et al. 2001).

Hereditary papillary renal cell carcinoma (HPRC) is characterized by an early onset of papillary renal carcinomas, which tend to be multifocal and bilateral. These disorders are autosomal-dominant. About 80% of affected families have mutations in the MET proto-oncogene located on chromosome 7q31. The MET protein is a tyrosine kinase receptor, increased activity of which leads to increased cell proliferation and motility and an increased ability to invade the extracellular matrix. The mutations associated with papillary renal carcinoma generate increased receptor activity (Schmidt et al. 1997).

Tuberous sclerosis is an autosomal dominant disorder which results from a mutation in one of two different genes: TSC 1 on chromosome 9q34 and TSC 2 on chromosome 6p13. Both genes encode tumour suppressor proteins. Phenotypically, tuberous sclerosis is characterized by the formation of multiple benign hamartomas in the CNS and in the kidney (angiomyolipomas). Patients with tuberous sclerosis evince an increased incidence of RCC (Iliopoulos and Eng 2000).

Hereditary non-polyposis colorectal cancer (HNPCC) is caused by germline mutations in the mismatch repair genes the most common manifestations being colorectal and endometrial cancers. Patients also run an elevated risk of urothelial carcinoma (Lynch and Lynch 2000).

Birt-Hogg-Dubé syndrome is a dominantly inherited predisposition to benign fibrofolliculomas and other benign tumours of the skin and soft tissue as well as colon polyps and lung cysts. The gene for the syndrome (BHD) is located at 17p11. There is also an increased incidence of renal tumours (Toro et al. 1999).



Hyperparathyroidism-jaw tumour syndrome (HPT-JT) is a rare autosomal dominant disorder (gene 1q21-31) with parathyroid adenomas, fibromas of the jaw and various renal lesions including RCC (Haven et al. 2000).

Familial renal cell carcinoma syndrome has been identified in one extended family with a germline reciprocal translocation between chromosomes 3p and 8p, the 3p site not involving the von Hippel-Lindau gene (Li et al. 1993).

## 2.2. Diagnostic methods

The differential diagnosis of a renal mass lesion is broad and includes benign cysts, pseudotumours, vascular malformations, angiomyolipomas, lymphoma, sarcoma, Wilm's tumour and metastases. However, percutaneous biopsy of a solid renal mass should not be undertaken, as more than 80% of such masses are RCC (Silver et al. 1997), benign lesions and metastases being rare. There is also a high rate of false negative biopsies and a theoretical risk of bleeding or seeding the biopsy tract (Campbell et al. 1997). Biopsy of a renal mass should therefore be done only to establish a diagnosis in a patient with clear evidence of metastatic disease. A suspicious renal lesion in an individual without obvious metastatic disease should be managed by surgical resection only.

According to the National Comprehensive Cancer Network (NCCN) guidelines for the management of RCC, further evaluation of patients with known or suspected renal cell carcinoma should include routine laboratory studies (complete blood count, chemistry panel, urinalysis, prothrombin time, partial thromboplastin time), a computer tomography (CT) scan of the abdomen and pelvis, chest X-ray and CT of the chest if the chest X-ray is abnormal or if there is extensive disease. Further studies such as a magnetic resonance imaging (MRI) of the brain and a bone scan should be undertaken only if clinically indicated.

## 2.3. Classification of tumours

### 2.3.1. Histopathology

The latest proposal for the classification of renal cell carcinoma is that issued by the Union Internationale Contre le Cancer (UICC) and the American Joint Committee on Cancer (AJCC) in 1997 (Storkel et al. 1997), table 1. Both histological and genetic factors are used in the classification.

**Conventional (clear cell) renal carcinoma** is the most common malignant tumour in the kidney, accounting for approximately 70% of cases in surgical series. The great majority of these comprise predominantly cells with clear cytoplasm in routine sections, although foci in which the cells have an eosinophilic cytoplasm are common. These carcinomas have a characteristic delicate, branching vasculature and commonly have solid and cystic architectural patterns. Only rarely do the cysts dominate and only a small population of clear cells is present. Genetically, these tumours are characterized by loss of genetic material in 3p; 50% show somatic mutation in the von Hippel-Lindau (VHL) gene and an additional 10-20% of clear cell carcinomas show inactivation of the VHL gene by epigenetic changes comprising hypermethylation.

**Papillary renal carcinoma** comprises 10-15% of cases. Papillary architecture predominates and is present, at least focally, in almost all. The cells covering the papillae range from small to large and show variable cytoplasmic staining. Psammoma bodies, foamy macrophages and oedema fluid are common in the papillary cores. Genetically, these tumours are characterized by trisomies (chromosomes 3q, 7, 12, 16, 17 and 20) and loss of the Y chromosome.

**Chromophobe renal carcinoma** accounts for approximately 5% of cases. Here the cells have variable amounts of pale or eosinophilic cytoplasm which stains blue with Hale's colloidal iron stain and contains many microvesicles. In routine sections, the cytoplasm tends to condense near the cell membrane, producing a halo around the nucleus. The cells vary widely in size and there is often a concentration of the largest cells along small blood vessels. Solid architecture is most common. Genetically, chromophobe renal carcinoma is characterized by monosomy of multiple chromosomes (1, 2, 6, 10, 13, 17 and 21) and hypodiploidy.

**Collecting duct carcinoma** accounts for <1% of RCCs and the term has been applied to carcinoma with a wide variety of appearances. The best accepted morphology is one of irregular channels lined by a highly atypical epithelium which sometimes has a hobnail appearance. The channels are set in an inflamed desmoplastic stroma. The main difficulty in assigning tumours to this category is demonstrating origin in the collecting ducts. Small tumours which can be observed to arise from a medullary pyramid are good candidates. Associations with dysplastic changes in neighboring collecting ducts and affinity for *Ulex europaeus* lectin also support the conception of collecting duct origin. No consistent pattern of genetic abnormalities has been established for this form.

**Renal cell carcinoma, unclassified** is a diagnostic category to which renal carcinomas should be assigned when they do not fit readily into any of the other categories (4-5% of cases). Possible features which might prompt assignment to this category are: apparent composites of recognized types, sarcomatoid cells without recognizable epithelial elements, mucin production, mixtures of epithelial and stromal elements and unrecognizable cell types. Genetic lesions are variable. Sarcomatoid features are regarded as a manifestation of high-grade carcinoma, but not as a distinct histological type in itself.

### 2.3.2. TNM-classification (-97) and staging

**Table 1.** UICC and AJCC TNM classification and staging of renal cell cancer (1997)

<i>Primary tumour (T)</i>	
T1	diameter $\leq$ 7cm, limited to the kidney
T2	diameter $>$ 7cm, limited to the kidney
T3a	invasion of adrenal gland or perinephric tissues, not Gerota's fascia
T3b	extension into renal veins or vena cava below the diaphragm
T3c	extension into the vena cava above the diaphragm
T4	extension beyond Gerota's fascia
<i>Regional lymph nodes (N)</i>	
N0	no lymph node metastases
N1	metastasis to one regional lymph node
N2	metastases to more than one regional lymph node
<i>Distant metastases (M)</i>	
M0	no distant metastases
M1	distant metastases
<i>Stage grouping</i>	
Stage I	T1N0M0
Stage II	T2N0M0
Stage III	T1-2N1 or T3N0.N1
Stage IV	T4 (any N or M) or N2 (any T or M) or M1

## 2.4. Treatment of renal cell carcinoma

### 2.4.1. Surgical treatment

Surgery remains the most effective treatment of local RCC and the principles proposed by Robson in 1962 have improved results (Robson 1963). The standard of care remains radical nephrectomy, which includes removal of Gerota's fascia and all its contents, including the adrenal gland and local lymph nodes. There have been no randomized studies demonstrating the need for lymph node dissection or adrenalectomy. The adrenal gland can be left in place if it appears to be uninvolved at the time of surgery and the tumour is low-risk for adrenal involvement, i.e. small and not involving the upper pole of the kidney (Wunderlich et al. 1999). Resection of the regional lymph nodes might be of benefit in removing lymph nodes with microscopic metastases, but results of nonrandomized studies have been conflicting (Minervini et al. 2001; Schafhauser et al. 1999). The randomized European study by Blom and colleagues showed a very low incidence (3.3%) of metastases in unsuspected lymph nodes; survival data are not yet available (Blom et al. 1999). If there is extension of the tumour into the vena cava or even into the right atrium or ventricle, this can also be resected, with cardiopulmonary by-pass if necessary (Russo 2000). If the tumour invades directly into the wall of the vena cava, then this portion of the wall should be resected (Hatcher et al. 1991). Subsequently open partial nephrectomy showed its effectiveness in properly selected patients (Fergany et al. 2000). With better imaging modalities the majority of patients can undergo adrenal-sparing nephrectomy and with long-term follow-up data laparoscopic nephrectomy is coming into wider use (Cadeddu et al. 1998). The results of ablative therapy such as cryotherapy and radiofrequency interstitial tumour ablation are still preliminary (Gill and Novick 1999; Walther et al. 2000).

Surgical resection of metastases does produce occasional long-term survivors and is therefore reasonable in selected patients. For example, Kavolius and colleagues reported a 54% 5-year survival rate for patients who had had a solitary metastasis resected (Kavolius et al. 1998). Operation of a primary renal tumour in patients with known systemic disease has been questionable, but two randomized studies have shown its favourable effect when combined with interferon therapy (Flanigan et al. 2001; Mickisch et al. 2001). It is important to note, that patients with a favourable response had a good performance status and interferon therapy especially was used.

The results of treatment vary markedly with disease stage. Patients with disease limited to the kidney are cured with radical nephrectomy in 95% of cases. Those with tumours extending beyond the capsule, but remaining within Gerota's fascia have a 5-year survival of 60%, while those with cancers which migrate into the inferior vena cava or local nodes have a 5-year survival of 40%. Only 5-10% of patients with metastatic disease will be alive at 5 years.

### 2.4.2. Immunotherapy

Spontaneous regression of renal cell carcinoma metastases is well documented but rare. The phenomenon occurs in probably less than 1% of patients overall and it appears to be of short duration, with a range of 2-13 months being reported (Gleave et al. 1998; Snow and Schellhammer 1982).

#### **Interferon**

There are three categories of interferons:  $\alpha$ ,  $\beta$ , and  $\gamma$ . Interferon  $\alpha$  appears to be the most active in the treatment of renal cell carcinoma. Interferon  $\alpha 2a$  and  $\alpha 2b$  are probably equivalent, although  $\alpha 2a$  induces higher levels of neutralizing antibodies. Potential mechanisms of action are direct inhibition of tumour cell proliferation, augmentation of the lytic activity of natural killer (NK) cells and an increased expression of the human leukocyte antibody (HLA) class I on tumour cells. As a consequence tumour cells are likely to be better recognized and killed by cytotoxic T-cells (van Herpen et al. 2000). Several studies with interferon  $\alpha$  have shown response rates of 10-15%. Among responders the response duration is usually 4-6 months; rarely, responses can last for several years. Patients are more likely to respond if they have good performance status, a long interval between initial diagnosis and development of metastases, low erythrocyte sedimentation rate, and the presence of only lung or lymph node metastases. Principal side-effects include flu-like symptoms such as fever, myalgia and asthenia. Sometimes confusion, depression, nausea, diarrhoea, liver dysfunction, leucopenia and thrombocytopenia may occur. Toxicity necessitates dose reduction or discontinuation in 20-40% of patients (Fossa 2000). Pegylated interferon is a potential alternative in treatment in that it can be given once weekly and there seem to raise fewer side effects (Motzer et al. 2001). Interferon- $\alpha$  therapy has also been used in studies demonstrating the survival benefit of nephrectomy in metastatic cases (Flanigan et al. 2001; Mickisch et al. 2001).

## **Interleukin-2**

Interleukin-2 (IL-2) is a cytokine which activates T cells and natural killer cells, stimulating them to produce a variety of other cytokines such as interferon  $\gamma$ , granulocyte-macrophage colony-stimulating factor and tumour necrosis factor (TNF)  $\alpha$ , which in turn stimulate the activity of other cells in the immune system, for example the monocyte-macrophage lineage. IL-2 was approved for use in renal cell carcinoma by the Food and Drug Administration in 1992. This approval was based on studies using high-dose bolus regimens with response rates of approximately 15% (Fisher et al. 2000). One half of responders had a complete remission, and most of these have never relapsed, even after more than 10 years of follow-up, and thus appear to have been cured. Marked side-effects are a problem when using high-dose IL-2 therapy. It causes hypotension, renal toxicity and alterations in mental status, fluid shifts, pulmonary oedema, coronary events, thrombocytopenia, and an increased incidence of infection. Attempts to modulate the toxicity with steroids or anti-TNF agents have been unsuccessful (Margolin 2000). Continuous infusion of IL-2 likewise proved no less toxic.

## **Cytokine combinations**

There have been a number of studies exploring the effect of combining IL-2 and interferon. Overall, the response rates seem to be similar to those with interferon or IL-2 alone, although individual studies have reported either higher (Atzpodien et al. 2004) or lower response rates for the combinations (Negrier et al. 1998).

### **2.4.3. Other oncological treatments**

#### **Other biologic therapies**

Lymphokine-activated killer cells (LAK), tumour-infiltrating lymphocytes (TIL) and dendritic cell vaccines have been tested in RCC therapy, but none of these has shown any convincing benefit thus far (Kugler et al. 2000; Law et al. 1995; Mulders et al. 1998). Radioimmunotherapy has also been tested in RCC, but results have not been promising.

Antiangiogenic agents have yielded promising results in preclinical studies, and clinical studies are ongoing. Thalidomide evinces antitumour activity via inhibition of the expression or release of several angiogenic factors, and may also cause immunomodulation. Reported response rates have

been 0-10%, with stable disease rates of approximately 10-30% (Motzer et al. 2002). Studies with thalidomide in combination with other agents are ongoing. SUO11248 is an oral multi-target tyrosine kinase inhibitor with antitumour and anti-angiogenic activity. In a phase 2 study in metastatic RCC, partial response was achieved in 24% (Motzer et al. 2004). An anti-VEGF antibody, bevacizumab (Avastin<sup>®</sup>), has been combined with the EGFR antagonist erlotinib (Tarceva<sup>®</sup>); here 25% evinced partial response in metastatic RCC treatment, supporting the conception of combined inhibition of critical pathways (Hainsworth et al. 2004).

The cyclin-dependent kinase (cdk) inhibitors have been widely investigated, also in the treatment of RCC. Flavopiridol, the first cdk inhibitor to enter human trials, induces cell cycle arrest via the inhibition of several different cdks. In a study with prolonged intravenous infusion in RCC the objective response rate was only 6% (Stadler et al. 2000). However, bolus administration may have significantly greater antitumour effects than prolonged infusion, and further studies are clearly called for.

### **Chemotherapy**

A wide range of chemotherapeutic agents have been tested in the treatment of RCC, but results have been relatively poor; almost all trials have yielded response rates of less than 10 %. Initial reports on vinblastine were better, but subsequent studies failed to confirm the findings. Floxuridine, 5-fluorouracil and gemcitabine have also been tested (Amato 2000). The reason for the relative resistance of renal cell carcinoma to chemotherapy is probably at least in part the high level of expression of the drug transporter P-glycoprotein, which transports many chemotherapeutic agents out of cells (Fojo et al. 1987). Attempts to enhance the chemosensitivity of RCC by using inhibitors of P-glycoprotein such as calcium channel blockers have been disappointing, possibly in part because renal carcinoma cells also express other drug transporters such as the multi-drug resistance-associated proteins (Samuels et al. 1997). High expression of the multidrug resistance 1 (MDR1) gene has been associated with good survival (Oudard et al. 2002), but there have also been studies failing to show this correlation.

### **Allogeneic stem cell transplant**

Allogeneic stem cell transplants have been used to rescue the recipient from the myeloablation caused by high-dose chemotherapy. However, it is now understood that high-dose chemotherapy may in fact be less important than immunologic rejection of the malignancy by donor T-cells in the allograft. Because immunologic mechanisms seemed to have a central role in the treatment of



metastatic RCC, the allogeneic stem cell transplant was also a logical target for further investigations. In a study of 19 patients both complete remissions lasting over a year and durable partial responses were achieved; preliminary reports are thus promising in this highly selected patient population (Childs et al. 2000).

### **Tumour vaccines**

Since there is clear responsiveness to immunotherapy in RCC and results of this therapy have been relatively poor, there has been great interest in the development of tumour vaccines. The major objective in the use of cancer vaccines has been the generation of a T-cell response against antigens expressed by tumours. Two major strategies have been the focus of investigation: gene-modified tumour vaccines (GMTV) and dendritic cell-based therapies.

In GMTV cultured tumour cells are transfected with cytokine genes or malignant cell are engineered to express the cytokine GM-CSF (granulocyte macrophage –colony stimulating factor) with or without costimulatory molecules. Although the results of small early-phase pilot trials have been encouraging, many questions still remain in the field of safety, technical problems, antigen presenting function and in measuring immune responses.

In the immune system dendritic cells have a pivotal role in the induction and regulation of all T-cell and B-cell responses. Class I-restricted antigens must be channelled through dendritic cells to activate the T-cell arm of the immune system. These cells have the exceptional ability to prime and activate CD4+ and CD8+ T cells, an activity important in inducing potent and long-lasting antitumour immunity. Antigen loading can be accomplished by coculturing dendritic cells with different antigens, transfecting the cells with antigens in the form of RNA and tumour cell-dendritic cell hybrids. The optimal administration route for these vaccines seems to be intradermal or intralymphatic. Preliminary reports have been encouraging. Adjuvant strategies have been developed to enhance the efficacy of these vaccines (costimulatory and adhesion molecules, cytokine cocktails, CD40 ligand) (Vieweg and Dannull 2003).

### **Radiation therapy**

There is no proper role for radiation therapy in the management of localized renal cell carcinoma. However, it can play a marked palliative role for patients with symptomatic brain or bone metastases. Unfortunately renal cell carcinoma is relatively radioresistant and requires higher doses of radiation than many other tumours (DiBiase et al. 1997).

### **Ablative techniques**

Cryotherapy, where the tumour is frozen in controlled conditions, has been used to a small extent, the follow-up is still short and results are preliminary (Gill and Novick 1999). The clinical role of radiofrequency interstitial tumour ablation is much the same (Walther et al. 2000). In high-intensity focused ultrasound (HIFU) the treatment can be given as an entirely extracorporeal tissue ablation. Limitations with HIFU treatment are difficulties in imaging lesions with ultrasound for precise targeting of tissue destruction (Wu et al. 2002).

## 2.5. Prognostic factors

### 2.5.1. Stage, grade and histology

Tumour stage, which reflects the anatomical spread of the disease, is recognized as the most important prognosticator for renal cell carcinoma, with the greatest influence on the clinical course of the disease (Robson et al. 1969; Thrasher and Paulson 1993). Currently Robson staging (Robson et al. 1969) and TNM staging (Guinan et al. 1997) are the systems most commonly used. In the TNM system in 1997 T1 stage was expanded from less than 2.5 to less than 7cm. Also the classification of tumour thrombus was changed: T3b for renal vein and below the diaphragm and T3c for above the diaphragm. By these changes the stages achieved statistically more significant differences in terms of survival. In several studies tumour diameter is an independent prognostic factor (Grignon et al. 1989), but there is some discrepancy as to the diameter at which the maximal prognostic power lies (Pantuck et al. 2001).

In clinical series histological grade almost invariably follows stage in statistical power with regard to prognosis (Fuhrman et al. 1982; Nurmi 1984; Thrasher and Paulson 1993). The first report of histological grade associated with prognosis was made by Hand and colleagues in 1932. In 1971 Skinner and co-workers noted nuclear features correlating to prognosis (Skinner et al. 1971). Fuhrman then introduced a 4-grade nuclear grading system which has since been widely used (Fuhrman et al. 1982).

Histological type of tumour has likewise some impact on prognosis. Over 70% of malignant renal tumours are clear cell type (common type). Papillary carcinoma has had a better prognosis in some studies (Amin et al. 1997) and there are obviously two different types of this carcinoma with different prospects (Delahunt et al. 2001). Chromophobe carcinoma seems to carry a better prognosis (Akhtar et al. 1995), while collecting duct carcinoma is associated with poor outcome (Chao et al. 2002). Sarcomatoid structures in the histology turn the prognosis to the worse (Lanigan 1995).

### 2.5.2. Routine laboratory tests, symptoms and signs

The Karnovsky or Eastern Co-operative Oncology Group (ECOG) patient performance status can predict prognosis in RCC and has been used especially with metastatic disease when evaluating the clinical situation before initiating chemoimmunotherapy (Zisman et al. 2001). Weight loss, sedimentation rate (Fossa et al. 1994) and anaemia (Yasunaga et al. 1998) have been identified as independent prognostic factors. Many parameters ( $\beta_2$ -microglobulin, serum albumin, serum calcium, lactate dehydrogenase, alkaline phosphatase) have correlated with stage and grade but do not constitute independent prognostic factors (Mejean et al. 2003).

### 2.5.3. Acute phase reactants

Several reactions are started and regulated by factors both in the tumour and in the host. C-reactive protein, haptoglobin, ferritin, uromucoid, and  $\alpha_1$ -antitrypsin have associations with prognosis in RCC, but no role as independent prognostic factors (Kirkali et al. 1999; Ljungberg et al. 1995)

### 2.5.4. Proliferation markers

Proliferative activity is essential for tumour development and progression and factors associated with this process have been seen as candidate prognostic markers.

**MIB-1 (murine monoclonal antibody -1 against Ki-67 antigen)**

The Ki-67 index has long been one of the cell proliferation markers. The monoclonal antibody Ki-67 detects a nuclear antigen that is present only in proliferating cells (Gerdes et al. 1984). This antigen (nonhistone protein) has been shown to be essential for DNA synthesis. In the cell cycle this product is found in growing concentrations from phase S through phase G2 to mitosis (Gerdes et al. 1991). The corresponding gene (coding Ki-67 antigen) is located in chromosome 10 (10q25) (Schonk et al. 1989). This protein can be identified by immunohistochemistry with Ki-67 antigen and the number of positive nuclei (cells) in tissue section can be counted. The MIB-1 assay shows an equivalent antigen in paraffin-embedded samples and gives so wider possibilities to use this test (Cattoretti et al. 1992; Key et al. 1993). High MIB-1 labelling index values (percentage of positive cells in tumour tissue) are associated with poor prognosis in many tumours, also in RCC (Jochum et al. 1996; Tannapfel et al. 1996). Several studies with multivariate analyses have found that Ki-67 (or MIB-1) index constitutes an independent prognostic factor (Bui et al. 2004; de Riese et al. 1993; Visapaa et al. 2003).

**AgNOR (Silver-stained nucleolar organizing regions)**

Silver-stained nucleolar organizing regions reflect ribosomal RNA transcription activity and cellular mitotic activity. In some studies the AgNOR score has been an independent prognostic factor (Yasunaga et al. 1998), but in others not.

**PCNA (Proliferating cell nuclear antigen)**

Proliferating cell nuclear antigen is a protein synthesized during the late G1 and S phases of the cell cycle. Hofmockel and colleagues found it to be associated with disease-free survival, but not overall survival (Hofmockel et al. 1995). In most studies association with survival has been found (Rini and Vogelzang 2000).

**p27 (Kip1)**

Cyclin dependent kinases and sets of activating and inhibitory molecules control the regulation of the cell cycle. p27 is a negative regulator of cell cycle. Low p27 levels have been associated with poor prognosis in RCC, and it also reached the status of an independent prognostic factor (Migita et al. 2002).

### 2.5.5. Angiogenesis

Neovascularization is necessary to all progressive tumours and their metastases. Tumours can grow to a distance of only 1mm without vasculature.

#### **Vascular density**

The density of intratumoral microvessels is still a matter of controversy as an independent prognostic factor: some studies show high predictive value (Yoshino et al. 1995), others not (MacLennan and Bostwick 1995).

#### **VEGF (Vascular endothelial growth factor)**

Vascular endothelial growth factor (VEGF) is one of the most important factors in angiogenesis. Hypoxia as a result of tumour growth induces a cascade of growth factors, including VEGF. The tumour itself also evinces autocrine secretion of endothelial growth factors. Hypoxic conditions also induce elevated levels of HIF leading to elevated expression of VEGF. Increased VEGF protein expression in RCC tumour tissue or in serum has correlated to an adverse prognosis (Jacobsen et al. 2000). In a study by Lee and colleagues VEGF expression was associated with grade and stage, but had no status as an independent prognostic factor (Lee et al. 2001).

### 2.5.6. Growth factors

#### **EGFR (The epidermal growth factor receptor)**

The epidermal growth factor receptor (EGFR) family has been found to play a central role in tumour progression. Ligand binding to the EGFR, receptor dimerization and the activation of downstream signalling pathways are molecular events involved in tumorigenesis (Carpenter 2000; Kim and Muller 1999). High expression of EGFR is considered to be an unfavourable prognostic factor in patients with a variety of tumours (Liebermann et al. 1985; Neal et al. 1990; Slamon et al. 1987), including renal cell carcinoma (Uhlman et al. 1995; Yoshida et al. 1997). However, there are also studies on RCC reporting no association between EGFR and prognosis (Hofmockel et al. 1997). In these studies, in addition to ligand binding and immunohistochemical methods (Yoshida et al. 1997), also Northern blot (Sargent et al. 1989) and Southern blot (Gomella et al. 1989) analyses have been used. The discrepancy in results may be due partly to these various and different assessment methods.

### **TGF- $\beta_1$ (Transforming growth factor)**

Transforming growth factor (TGF- $\beta_1$ ) is a potent growth inhibitor of renal epithelial cells (Gomella et al. 1989). Acquisition of TGF- $\beta_1$  resistance provides a release from negative control of TGF- $\beta_1$  and may be of important prognostic significance (Ramp et al. 1997).

### 2.5.7. Apoptosis

Programmed cell death (apoptosis) is a complex phenomenon associated with the development and maintenance of normal tissue and also with oncogenesis and tumour progression (Thompson 1995).

#### **Bax, Bcl-2 and p53**

In carcinogenesis the most important regulators of apoptosis are the p53 gene (Symonds et al. 1994) and members of the bcl-2 family (Harris and Thompson 2000). The tumour suppressor gene p53 acts as an inducer of apoptosis (Vogelstein and Kinzler 1992). Mutations in the gene encoding p53 have been associated with poorer prognosis (Lanigan 1995). In the Bcl-2 family, the bcl-2 gene has inhibitory effects on apoptosis (Hockenbery et al. 1990), while Bax acts to promote cell death (Miyashita and Reed 1995). Bcl-2 and Bax are able to form homo- and/or heterodimers among themselves and it is obvious that susceptibility to apoptosis is determined by multiple competing dimerizations in which Bax may be a common partner (Sedlak et al. 1995). In general the caspase family of proteases constitutes the ultimate effector of apoptosis and the major intracellular apoptotic pathways can therefore be classified by the type of specific pro-caspase activated (Thornberry and Lazebnik 1998). Activation of the initiator pro-caspase 9 is dependent primarily on mitochondrial signalling pathways involving members of the bcl-2 family (Harris and Thompson 2000).

Results of studies on the relationship between bcl-2 proteins and RCC characteristics and survival have been conflicting. Vasavada and associates found a positive correlation between Bcl-2 and high-grade clear cell carcinomas and between Bax and high-grade RCC in general (Vasavada et al. 1998). In several studies expression of Bcl-2 has evinced associations with the progression of RCCs, tumour type and nuclear grade (Lipponen et al. 1995a; Paraf et al. 1995; Tomita et al. 1995). Hofmockel and colleagues demonstrated a low incidence of Bcl-2 expression in locally confined

RCCs (Hofmockel et al. 1996), and in a study by a group under Oudard Bcl-2 and Bax had no significant correlation with either grade or clinical stage (Oudard et al. 2002).

### 2.5.8. Cytokines and adhesion molecules

Immunological factors have a role in both development and treatment of renal cell carcinoma. A traditional sign of immunological relevance has been the disappearance of lung metastases after nephrectomy. However, the incidence of this is less than 1 per cent, having no real clinical value. Immunomodulators, especially cytokines, have been studied in the treatment of RCC. However, only from 10 to 20% of patients exhibit objectively perceptible responses to interferon (IFN) treatment (Kellokumpu-Lehtinen and Nordman 1990; Pastore et al. 2001; Pyrhönen et al. 1999).

#### **Interleukins**

Interleukins (IL) are a family of immunomodulators also participating in various immunological processes in RCC. Interleukin-2 (IL-2) was discovered as a protein secreted by activated T-cells. It autoactivates T-cells and affects cell division and differentiation of cells sensitive to it. The effects of IL-2 are mediated via the receptor complex (IL-2R) (Mertelsmann and Welte 1986). IL-2 promotes the cytotoxic potential of large granular lymphocytes and induces the production and release of several other cytokines. IL-2 carries a 15-20% clinical response rate in advanced renal cell cancer (McIntyre et al. 1992). IL-6 has been shown to be a paracrine or autocrine growth factor for many tumour cell types (Orr et al. 1993). It is produced by several renal cancer cell lines but only one cell line has shown enhanced growth stimulation in clonogenic assay (Koo et al. 1992). A negative correlation has been demonstrated between high IL-6 concentrations and survival in renal cell cancer patients. In addition, high IL-6 serum levels predict a poor response to IL-2 and IL-2 plus IFN therapy (Blay et al. 1992; Stadler et al. 1992). On the other hand, an increase in sIL-2-receptor levels has been seen to correlate with a poor response when combining IL-2 and IFN. IL-12 is a more multipotent cytokine bearing many similarities to IL-2 in the induction and proliferation of cytotoxic T-lymphocytes and inducing natural killer cell activity (Goey et al. 1996; Yao et al. 1999). IL-12 inhibits angiogenesis in vivo by inducing IFN gamma and other mediators (Yao et al. 1999). It has suppressed tumour growth in many animal studies (Brunda et al. 1993). IL-12 has been shown both to fibrinolyse and to coagulate in a significant proportion of cancer patients (Portielje et al. 2001).

### **Cell adhesion molecules**

Cell surface adhesion molecules play an important role in mediating cell-to-cell interaction in immunologic reactions (Larson and Springer 1990). Elevated levels of ICAM-1 have been measured in the serum of RCC patients (Banks et al. 1993; Vuoristo et al. 2000). Cadherins are major epithelial adhesion molecules. Loss of E-cadherin expression is associated with RCC progression (Fischer et al. 1999). Reduced alpha catenin (a modulator of cadherin function) correlates with shorter survival in RCC (Shimazui et al. 1996). CD44 is a transmembrane glycoprotein which recognizes extracellular hyaluronan. It has several splitting products, CD44 v3 and v6, in addition to the standard form CD44s. CD44s expression correlates with RCC progression and recurrence, but has a strong association with Fuhrman nuclear grade and does not act as an independent prognostic factor (Gilcrease et al. 1999).

## 2.5.9. Chromosomal and genetic markers

### **Individual genes**

Numerous chromosomal abnormalities or over/under expression of genes have been studied and associations with histological grade and stage have been established, without however reaching independent prognostic value (Rini and Vogelzang 2000). In conventional RCC, tumour progression has been associated with the occurrence of additional genetic alterations such as duplication of chromosome region 5q22 and deletions at 6q23, 8p, 9p and 14q (Presti et al. 2002; Schullerus et al. 1997). The fullest information so far has been obtained using microarray techniques for finding gene clusters associated with different parameters, for example with prognosis (Takahashi et al. 2003).

### **Ploidy of DNA**

An aneuploidic DNA as a prognostic factor continues to be debated. For some researchers the diploidic DNA has a favourable impact on survival (Abou-Rebyeh et al. 2001; Ekfors et al. 1987), but in many papers no such association was not found (Ruiz-Cerda et al. 1999; Shalev et al. 2001).

### **CGH (Comparative genomic hybridization)**

Comparative genomic hybridization (CGH) allows rapid screening of a large number of tumours for gains and losses of chromosomes, which might make it a useful tool in clinical practice (Kallioniemi et al. 1992). A high number of DNA losses per tumour has been associated with poor



prognosis in pT3 nonmetastatic clear cell RCCs (Moch et al. 1996). In the study in question deletion of 9p was the only single CGH finding associated with (poor) prognosis. Schraml and colleagues found the exact locus on chromosome 9p13 associated with progression of papillary renal cell carcinoma (Schraml et al. 2000). 3p deletion is an early event in carcinogenesis and has no connections with prognosis. At least three separate regions on chromosome 3p have been implicated by loss of heterozygosity (LOH) studies in RCC: one with the von Hippel-Lindau (VHL) disease gene locus at 3p25-26, the RASSF1A gene at 3p21 and the NRC-1 gene at 3p12 (Lott et al. 1998; Morrissey et al. 2001). The most common DNA losses have involved 3p (61%), 4q (50%), 6q (40%), 9p (35%), Y (38%), 13q (37%), Xq (21%), 8p (18%) and 14 (24%), while the most common gains have been seen on chromosome 7 (38%), 5q (20%) and 17 (20%) (Mertens et al. 1997). RCCs with sarcomatoid transformation are aggressive tumours with poor prognosis (Moch et al. 2000). CGH studies on these tumours have shown most prevalent DNA losses at 13q and 4q, while DNA gains have most often involved chromosomes 17, 7 and 8q. A high-level coamplification involving 11q22-23 and 7p21-22 in one sarcomatoid RCC was not present in the adjacent nonsarcomatous tumour area, raising the possibility of oncogene involvement at these loci for sarcomatoid transformation and also for poorer prognosis (Jiang et al. 1998). In view of the genetic heterogeneity of different RCCs, branching tree and distance-based tree models have been used for further evaluation of CGH findings (Desper et al. 1999, 2000). Using the distance-based tree model Jiang and colleagues found a subgroup of pT3/4 clear cell RCC tumours with poor prognosis having deletions in 9p, 13q and/or 18q (Jiang et al. 2000). The extremely long branch to -8p in the distance-based tree suggests that this event is more likely to be a late effect than an early cause. This is in accordance with findings in a study where 8p loss was relatively common in metastases but rare in primary tumours (Bissig et al. 1999). -8p in RCC primary tumour subclones may be predictive of further metastatic progression.

### **Microarray studies**

Gene expression profiling by microarray serves as a high-throughput and comprehensive means of identifying the molecular signatures of cancer. This method allows viewing of the expression of thousands of genes which probably best reflect the biology of a given tumour. These profiles can serve as the molecular signatures of particular tumours, and different groups of genes may correlate with certain aspects of its behaviour, for example the invasiveness or state of angiogenesis or clinical outcome

Comparative genomic microarray analysis (CGMA) has been demonstrated to be effective in predicting large cytogenetic alterations (Crawley and Furge 2002). Two colour microarray gene

expression profiling data sets are obtained in which tumour cell RNA is compared with adjacent noncancerous normal tissue RNA. For each microarray probe, a ratio of tumour to normal transcript level is calculated. The completion of the human genome now makes it possible to map the microarray probe sequences to their corresponding genomic locations (Lander et al. 2001). Takahashi and colleagues made clustering of clear cell RCC on the basis of 5-year survival and clusters for good and poor prognosis were found. However, it turned out that histological grade and gene expression classification were highly correlated (Takahashi et al. 2003). Larger patient cohorts with multivariate analysis will give more information on the significance of these genetic clusters.

#### 2.5.10. Metastatic potential

**Regulatory factors in metastasis:** A number of systems take part in the metastatic process: MMPs (matrix metalloproteinases), serine proteinases, angiogenesis regulatory factors (bFGF, VEGF, angiostatin, endostatin), the immunoglobulin (IG) superfamily, cadherins, selectins (P-, E-, L-), integrins, chemokines, Rho family GTPases, metastasis suppressor genes, scatter factors and semaforin receptors (Robinson et al. 2004). Approximately 30% of patients with RCC have metastases at the time of diagnosis and another 50% will ultimately develop metastatic disease. Frequent metastatic sites include the lung parenchyma (in 50-60% of patients with metastases), bone (in 30-40%), liver (in 30-40%) and brain (in 5%). Unusual sites of metastases are characteristic of RCC, and virtually any organ site can be involved, including the thyroid, pancreas, skeletal muscles and skin or underlying soft tissue (Padrik 2003).

**Fibrinogen receptors:** Fibrinogen receptors play a central role in the development of haematogenous metastases. Although transport of tumour cells in the bloodstream is an essential component of the metastatic process, the mere presence of tumour cells in the circulation does not inevitably lead to the formation of metastases (Hood and Cheresch 2002). The physiologic conditions of the blood provide an environment hostile to tumour cells (Fidler 1970). Various tumour cell -associated procoagulant activities have been described (Colucci et al. 1981; Curatolo et al. 1979; Dvorak et al. 1983) and fibrin is always found around all cancerous lesions (Blackwell et al. 2000; Rybarczyk and Simpson-Haidaris 2000). These fibrin deposits may influence the progression of a tumour in several ways: serving as a barrier keeping host inflammatory cells from destroying the tumour (Dvorak et al. 1983; Gunji and Gorelik 1988) and enhancing angiogenesis in a metastatic tumour (Olander et al. 1985). Platelets take part in the formation of fibrinogen deposits

around tumour cells. The ability of tumour cells to aggregate platelets was first recognized by Casic and co-workers as far back as 1968 (Gasic et al. 1968). The main platelet fibrinogen receptor is glycoprotein  $\alpha_{IIb}\beta_3$  or GPIIb/IIIa. GPIIIa  $PI^{A1/A2}$  polymorphism is associated with functional differences in this receptor: the presence of 1 or 2  $PI^{A2}$  alleles is associated with increased platelet aggregability (Feng et al. 1999). The same allele may be associated with the risk of acute coronary thrombosis (Weiss et al. 1996) and sudden cardiac death (Mikkelsson et al. 1999). The C3550T polymorphism of GPIB-IX-V platelet von Willebrand Factor receptor and the C807T polymorphism of the GPIa/IIa platelet collagen receptor have also been suggested to be associated with the risk of myocardial infarction in early middle age (Mikkelsson et al. 2001; Moshfegh et al. 1999). These platelet adhesive receptors are assumed to have a central role in the development of haematogenous metastases (Nierodzik et al. 1994). However, clinical studies concerning these platelet receptors, their polymorphisms and the metastatic potential of human cancers are few (Bojesen et al. 2003).

#### 2.5.11. Prognostic indexes

As the power of single prognostic factors is limited, there has been interest in combining several factors into a more effective index. The principles of a prognostic index are demanding: the status of single components must be well established, they must be statistically independent factors and the statistical methods applied must be optimal (Fielding and Henson 1993).

The University of California-Los Angeles integrated staging system (UISS) uses TNM stage, ECOG performance status and Fuhrman grade to build up its prognostic classes (Zisman et al. 2001). The SSIGN score is a candidate prognostic index for clear cell carcinomas; and it is based on stage, size, grade and necrosis (Frank et al. 2002). Some extra accuracy in prognosis has been achieved with these methods.

### 3. AIMS OF THE PRESENT STUDY

This prospective study was conducted to evaluate relationships between clinical findings and candidate prognostic factors in patients with newly diagnosed renal cell carcinoma. The specific aims were:

1. To screen by CGH the most common chromosomal aberrations in unselected RCC specimens and to compare the findings with histopathology and proliferative activity and with the clinical outcome, to evaluate whether the CGH technique yields any additional information on the prognosis of the disease in clinical practice.
2. To evaluate the clinical behaviour of sIL-2R, IL-6, IL-12 and sICAM-1 before nephrectomy for RCC and after three months' follow-up, and their associations with tumour stage and patient's outcome.
3. To examine the associations between the cellular location of the EGFR immunostaining and prognosis in renal cell carcinoma.
4. To evaluate Bax and Bcl-2 levels in RCC and their associations with prognosis, proliferation and traditional prognostic factors.
5. To explore whether genetic polymorphisms in platelet receptors known to be associated with platelet activity have any association with haematogenous metastases in renal cell carcinoma (RCC).

## 4. PATIENTS AND METHODS

### 4.1. Patients

Our prospective (papers I-IV) study population consisted of 138 consecutive patients who underwent radical or palliative nephrectomy for RCC by urologists and thereafter patients with metastases were treated by oncologists, treatment was started with all patients between 1993 and 1999 at Tampere University Hospital. In the CGH study (paper I) the tumour specimens were obtained from 20 unselected patients treated for RCC between 1993 and 1995. In the cytokine study (paper II) twenty-four patients were operated for RCC during the years 1995-1997. Serum samples for cytokine measurements were taken before surgery and at three months' control. The EGFR (paper III) and Bax-Bcl-2 (paper IV) studies had the same patient population, including all 138 patients treated between 1994 and 1999.

Fibrinogen receptor study (paper V) was performed in cross-sectional manner in 128 male patients. During the recruit time of the study 55 men with disseminated disease and 73 men with local disease were included and a single whole blood test was taken for analysis. In addition to below mentioned parameters also antithrombotic/ anti-inflammatory medication (including acetosalicylic acid, dipyridamol, oral anticoagulants, clopidogrel, non-steroidal anti-inflammatory drugs and corticosteroids) were recorded.

Preoperative evaluation included a thoracoabdominal computer tomography scan and routine laboratory tests (ESR, haemoglobin, erythrocyte count and morphology, leucocyte count, alkaline phosphatase, creatinine and urine sediment) in addition to normal physical examination. Radical or palliative nephrectomy was undertaken in every patient and postoperative follow up and treatment was done by oncologists (disseminated disease) or by urologists (local disease). The study was conducted in a prospective manner and follow-up according to a fixed schedule: 0 mo, 3 mo, 9 mo, 15 mo, 2 yrs, 3 yrs, 4 yrs and 5 yrs. The following parameters were recorded: age and gender at operation time, date of operation, TNM classification (UICC 1997), diameter of tumour, histological diagnosis, WHO grade, Fuhrman nuclear grade, date of study sample, corresponding TNM status, date of exitus, cause of death, date of last follow-up visit.

## 4.2. Methods

### 4.2.1. Analysis of chromosomal gains and losses and proliferation activity (Paper I)

The tumour specimens were frozen immediately in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  until analysis. DNA was extracted from frozen tissue by a non-enzymatic method (Lahiri and Nurnberger 1991) and the normal DNA from male and female peripheral lymphocytes by a standard procedure (Kallioniemi et al. 1994). The histopathology of all specimens was confirmed by one pathologist (H.H.). Tumours were classified pathologically according to the classification of UICC (Storkel et al. 1997) and nuclear grading was done by the principles of Fuhrman and colleagues (Fuhrman et al. 1982). Staging was done according to the TNM system (Guinan et al. 1997) and histopathological classification before CGH analysis.

#### **Comparative genomic hybridization and image analysis**

CGH was carried out as previously described by Kallioniemi and associates (1994) (Kallioniemi et al. 1994). Briefly: hybridization was performed using directly fluorochrome-conjugated DNAs. Normal DNA was labeled with Texas Red-6-dUTP (DuPont, Boston, MA) tumour DNA with FITC-12-dUTP (DuPont) using nick translation. We used 600ng labeled tumour DNA and 400ng normal DNA and 10 $\mu\text{g}$  unlabeled Cot-1 DNA (Gibco BRL, Gaithersburg, MD). The normal lymphocyte preparations were denaturated in denaturation solution (70% formamide, 2xSSC, pH7) for 2-3 minutes at  $70^{\circ}\text{C}$ . After two days' incubation, the hybridization was washed and dried and the DNA counterstained with 0.1g/ml 4',6-diamindino-2-phenyldindole (DAPI) in an antifade solution. As a negative control there was a normal male versus normal female DNA hybridization.

From each tumour images were collected from 4-6 metaphases using an Olympus BX 50 epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a Xillix MicroImager 1400 camera (Xillix Inc., Vancouver, BC, Canada) and interfaced to a Sun LX Workstation (Sun Microsystems, Mountain View, CA). Analysis was made with Scil-Image software (National Research Institute, Delft, Netherlands) with Quips extensions (Resource of Molecular Cytogenetics, Lawrence Berkeley National Laboratory, Berkeley, CA). The chromosomes were identified using the DAPI-banding pattern. The green and red fluorescence intensities were calculated along the median axis of each chromosome and exposed as a green to red ratio profile. A copy number

change was considered a gain when the green to red fluorescence ratio exceeded the 1.15 cut-off and as a loss when the ratio fell below the 0.85 line. Heterochromatic areas were excluded from the analysis.

### **MIB-1 assay**

Preparation of sections, microwave processing and immunostaining were done as described elsewhere (Rautiainen et al. 1998). Briefly: the sections were dewaxed in xylene and rehydrated and citrate buffer and microwave processing were used for antigen retrieval. For the immunostaining of Ki-67 antigen, the monoclonal antibody MIB-1 (IgG1, Immuno-tech S. A. Marseille, France) was used at a 1:40 dilution. Counter-staining was done using 0.4 per cent ethyl green in acetate buffer. The staining was evaluated using a computer-assisted image analysis system (CAS-200 Software, Becton Dickinson & Co., U.S.A.). The MIB-1-index was defined as the percentage of cells with immunopositivity in nuclei. Image analysis was made twice; the correlation between the two separate assays was excellent ( $p= 0.98$ , t-test).

### **4.2.2. Analysis of cytokines (Paper II)**

Blood samples were taken at every follow-up visit. The serum was separated from the clotted blood by centrifugation at 2000 x g for ten minutes and stored at -70°C until analysis. Determinations of serum levels of immunological variables were made using specific commercial enzyme immunoassay kits.

IL-2R: a PredictaR interleukin-2 receptor (Genzyme Diagnostics, MA, USA) kit was used to measure the soluble alpha component of the IL-2 receptor complex. The detection limit of the assay was 100pg/ml. The mean sIL-2R level in the serum of 80 healthy individuals was according to the manufacturer 1250pg/ml.

IL-6: a PeliKline™ human IL-6 ELISA kit (Central laboratory of the Netherlands Red Cross, Amsterdam, Netherlands) was used. The sensitivity of the test was 0,3pg/ml and IL-6 values in fresh serum and plasma samples in healthy individuals were below 10pg/ml.

IL-12: a PredictaR interleukin-12 (Genzyme Diagnostics, MA; USA) kit measuring p40 subunit and p70 heterodimer of IL-12 was used. The sensitivity of the assay was 10pg/ml.

ICAM-1: a Parameter human soluble sICAM-1 (RD Systems, Oxon, United Kingdom) kit was used. The sensitivity of the assay was < 0.35ng/ml. In serum from healthy donors the mean ICAM-1 concentration was 210.6 ng/ml.

#### **4.2.3. Immunostaining of EGFR (Paper III)**

Archival formalin-fixed, paraffin-embedded RCC material was used for this study. All tissue blocks were re-evaluated and from a representative area for each tumour a 3-mm core was transferred to a multi-tissue block, which was then used for further analysis. All tumours were classified according to the Heidelberg classification (Kovacs et al. 1997) and graded according to the Fuhrman system (Fuhrman et al. 1982) by two pathologists.

Paraffin-embedded multi-tissue blocks were cut 4-5 µm in thickness and mounted on precoated slides. After deparaffinization, antigen retrieval was performed by heating the sections in a microwave oven for 2 x 7 minutes in 10 mMTris/1mM EDTA (pH9.0) buffer, followed by washes with water. A polyclonal rabbit anti-EGFr variant III antibody (Zymed Laboratories, Inc., San Francisco, CA) was used for EGFR immunostaining at a concentration of 5 µg/ml. A TechMate™ 500 Plus Immunostainer (DAKO a/s, Glostrup, Denmark) was used for the staining procedure and a ChemMate™ peroxidase/DAB detection kit (DAKO a/s, Glostrup, Denmark) for visualization of the antigen-antibody complex. Sections were slightly counterstained with hematoxylin.

#### **Evaluation of immunohistochemical staining**

The optimal titre for EGFR staining was defined as the dilution giving clearly identifiable membrane staining and negligible background on human placental samples. The intensity of the immunostaining in RCC (scale 0-3) was multiplied by the percentage of cells with positive staining to give a score of 0-300. Thus scored, the positive placental control (Neal et al. 1990) in our system gave a score of 100. Five staining patterns were scored: solely (m) or predominantly (m>c) membranous staining, solely (c) or predominantly (c>m) cytoplasmic staining or equal (c=m).



#### **4.2.4. Bax and Bcl-2 assays (Paper IV)**

##### **Specimens**

The same archival formalin-fixed, paraffin-embedded RCC material as in study III was used and all tissue blocks were re-evaluated (PH) and prepared in the same way as in study III (Kallio et al. 2003).

##### **Immunohistochemical staining**

Preparation of sections and immunostaining were done as previously described. The sections were deparaffinized and antigen retrieval was carried out by heating the sections in a microwave oven for 2x7min in 10mM Tris/ 10mM EDTA (pH 9.0) For the immunostaining of Ki-67 antigen, the monoclonal antibody MIB-1 (IgG1, Immuno-tech S. A. Marseille, France) was used in a 1:110 dilution. Counter-staining was done using 0.4 per cent ethyl green in acetate buffer. The staining was evaluated using a computer-assisted image analysis system (CAS-200 Software, Becton Dickinson & Co., U.S.A.). The MIB-1 index was defined as the percentage of cells with immunopositivity in nuclei. Polyclonal rabbit anti-human Bax antibody (PharMingen, Europe) was used at 1:1000 and monoclonal mouse anti-human Bcl-2 oncoprotein clone 124 (Dako, Glostrup, Denmark) at 1:60. Sections were slightly counterstained by hematoxylin. Stainings for Bax and Bcl-2 were analyzed semi-quantitatively: stainings were quantitated by intensity (0-3) and percentage of areal expression (0-100%) and by multiplying to obtain a staining score (0-300)

#### **4.2.5. Determination of fibrinogen receptors in platelets (Paper V)**

##### **Laboratory methods**

DNA was extracted from EDTA blood samples drawn from the patients during their recruitment for the study by a method previously described (Isola et al. 1994). The polymorphism of cytosine/thymine in exon 2 of the GPIIIa gene was detected by polymerase chain reaction and restriction digestion (PCR-RFLP). Genomic DNA (10-30ng) extracted from frozen blood samples was used in each amplification. DNA was amplified with a PTC 100 (Perkin Elmer) for 37 cycles of denaturation at 94°C for 45 seconds, annealing at 53°C for 45 seconds, and extension at 72°C for 60 seconds. The final extension step was at 72°C for 4 minutes. The 266 bp-product was then incubated at 37°C for 1 hour with 10 U of MspI. The resulting fragments were then separated by size in a 2% agarose gel and visualized by ethidium bromide staining. Genotyping was successful in all patients and the genotypes were found to be in Hardy-Weinberg equilibrium.

#### **4.2.6. Statistical methods**

The associations of RCC status and other covariates were tested by cross-tabulation and chi-square test (papers I, III-V). Possible confounding effects were taken into account by including them as covariates in the logistic regression model (paper V). In the cytokine study (paper II) pre- and postoperative values in the same patients were compared using paired Student's T-test; separate patient groups were tested by non-paired Student's T-test. Associations of different EGFR staining patterns with other main prognostic parameters were tested using Fisher's exact test (paper III). Bax and Bcl-2 associations with Fuhrman grade and stage were tested by Fisher's exact test; MIB-1 associations with Bax and Bcl-2 were tested by Mann-Whitney test and with Fuhrman grade and stage by Kruskal-Wallis test (paper V). In all testing for differences in RCC-specific survival Cox proportional hazards regression analysis was used. Deaths from causes other than renal cell carcinoma were considered censored events. Both univariate and multivariate analyses were undertaken. The computation was carried out with SPSS software for Windows (versions 9.0, 10.0 and 11.0 SPSS).

#### **4.2.7. Ethics**

The research plan was approved by the ethical committee of Tampere University Hospital and the principles of the Helsinki Declaration were followed during the study. In studies I-IV patients gave oral informed consent and written consent in study V to confirm their participation. Involvement in the study did not affect the treatment of the patients and no information on the individual findings was given to the participants.

## 5. RESULTS

### 5.1. Chromosomal gains and losses and proliferation activity (Paper I)

#### **Clinical findings**

The mean age of patients at operation was 63 years (range 41-82). In 9 cases the tumour was incidental, whereas in the others the main symptom was pain (6), haematuria (4) or detected during the follow-up of a patient with von Hippel-Lindau syndrome (1). The diameter of the tumours varied from 2.5 to 9 cm. Four of these cases were disseminated, the remaining 16 were local. In disseminated cases the association with poor survival was marked, as expected (RR=16.91, 95% CI 2.96-96.49). Average survival was only 6 months with T4/N+/M+ patients. Six patients died of other illnesses during the mean follow-up time of 36 months (range 6-77).

#### **Histopathology**

Seventeen tumours were of the conventional (clear cell) type, 2 were papillary and 1 of the chromophobe type. In 12 cases the nuclear grade was 1- 2, in 5 cases 3 and in 3 cases 4. T3 local tumours and disseminated cases had higher nuclear grade values and poorer prognosis compared with T1-2 local tumours.

#### **CGH**

Every tumour showed gains or losses of DNA sequences; seventeen (85%) showed losses and 13 (65%) relative DNA sequence gains. High-level amplification was not seen. The number of genetic aberrations per tumour varied between 1 and 10. Deleted areas were found most frequently in the chromosome 3p arm, occurring in 14 of the 20 cases (70%). In addition, a minimal region of loss in the 3p arm was 3p14-25. Other common losses were deletions of 4q (minimal region 4q31-ter), 13q (13cen-qter), 18p (18p), 18q (18q) and Xp (Xp21-pter), each of them found in 4 cases (20%). Deletions of 3q, 8p and 9p were found in 3 tumours (15%). The most common gain was in the long arm of chromosome 7 (7q), seen in 5 tumours, the smallest region of involvement being 7cen-q22. Gains of the entire chromosome 17 were detected in 15% of the tumours (tables 2 and 3). The number of deleted areas in tumours with conventional histology had no association with RCC-specific survival (RR=1.03, 95% CI 0.64-1.67).

### **MIB-1**

The proliferation index (MIB-1) varied from 0 to 39 (median 4.0). The patients were divided into three groups according to proliferative activity: low (MIB-1= 0-2.0), moderate (MIB-1= 2.1-9.0) and high (MIB-1= 9.1-39.0). The corresponding mean numbers of deletions per tumour in each group were 2.5, 1.7 and 3.6. The total number of chromosomal aberrations or the number of deletions per tumour showed no clear association with MIB-1 index or nuclear grade.

However, high MIB-1 values were associated with poor RCC-specific survival (RR=1.14, 95% CI 1.02-1.27) and the presence of metastases, while low MIB-1 values were associated with lower nuclear grades.

**Table 2.** Histological type and nuclear grade and CGH findings in RCCs of study population\*

Patient	Histology	Nucl. grade	CGH losses	CGH gains
1	conventional	3	3p,9pq, 10pq, 17pq, Xp21-pter	12p12-cen-q23, 13q21-q31
2	conventional	1	17pter-cen-q22	13q21-qter, Xpq
3	conventional	2		4q, Xpq
4	chromophobe	3	1p31-pter, 3p, 19q	12p12-cen-qter, 4pq, 7pq,11pq, 14pq, 15pq, 18pq
5	papillary	2		2pq, 3q, 6q, 7pq, 12pq, 17pq
6	conventional	4	3p14-25, 9pq	5pter-cen-q13, 5q31-qter
7	conventional	4		5p, 6pq
8	conventional	1	3p, 8p	
9	conventional	2	3p	
10	conventional	1	3p, 4pq	
11	conventional	4	13pq	
12	conventional	1	3p	
13	conventional	3	2q22-qter, 3pter-cen-q24, 8p, 13q, 18pq, Xp11-pter	1q
14	conventional	2	3p, 8pq, 12pq, 18pq, 21q, Xpq	
15	conventional	2	3pter-cen-q21, 4q31-qter, Xp21-pter, Xq21-qter	5q21-qter
16	conventional	2	3p, 4q	
17	conventional	2	3p, 5p, 6q, 13pq, 14pq	17p11-cen-q22
18	conventional	3	3pter-cen-q13, 4pq, 6q, 9p21-pter, 13pq	7cen-q22
19	conventional	3	3p	7p14-cen-qter, 16p
20	papillary	1	18pq	7pq, 12pq, 16p, 17pq

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**Table 3.** Summary of the most common losses and gains of DNA sequence copy number in renal cell carcinoma\*

The most common losses			The most common gains		
Region	Frequency (%)	Minimal change region	Region	Frequency (%)	Minimal change region
3p	70	3p14-p25	7q	25	7cen-q22
4q	20	4q31-qter	5q	20	5cen-q13,q31-qter
13q	20	13q	7p	20	7p
18p	20	18p	12p	20	12cen-p12
18q	20	18q	12q	20	12cen-q23
Xp	20	Xp21-pter	17p	15	17p
3q	15	3cen-q13	17q	15	17cen-q22
8p	15	8p	4q	10	4q
9p	15	9p21-pter	5p	10	5p
4p	10	4p	6q	10	6q
6p	10	6p	13q	10	13q21-q31
9q	10	9q	Xp	10	Xp
16p	10	16p	Xq	10	Xq
17p	10	17p			
Xq	10	Xq21-qter			

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## 5.2. Adhesion molecule (ICAM-1) and cytokines (Paper II)

The total number of patients in this study was 24. Two patients died of RCC before the first postoperative control at three months. There was a strong association between IL-6, IL-12 and sICAM-1 concentration and survival (Table 4). In contrast, in spite of some very high preoperative sIL-2R values the median concentration of sIL-2R did not differ statistically significantly between short- (less than one year) and long-term survivors.

All local tumour patients (N=12) except one had serum IL-6 levels within the normal range, under 10 pg/ml, the mean being 6.3 pg/ml (range from 0.5 to 30 pg/ml). On the other hand, only one patient out of the 12 with non-local tumours had an IL-6 value under 10 pg/ml. The mean value in these patients was 53 pg/ml (range from 2.5 to 200 pg/ml).

The mean preoperative sICAM-1 value in patients with local tumours was lower (290 ng/ml, range from 240 to 532 ng/ml) than that in non-local cases, (443 ng/ml, range from 280 to 960 ng/ml,  $p < .03$ ). In patients with local tumours the concentration of sICAM-1 remained at the same level after the operation in all but one case. In non-local tumour patients sICAM-1 was over 300 ng/ml in 9 of the 12 patients after nephrectomy.

Preoperative sIL-2R levels were over 1000 pg/ml in all patients except one before nephrectomy (mean 1796 pg/ml, range from 1050 to 3200 pg/ml) and increased in all but one after the operation. The mean value after nephrectomy was 2150 pg/ml (range from 1400 to 3550) in patients with local tumours. sIL-2R levels varied considerably in patients with non-local tumours and were in general much higher (mean 3779 pg/ml, range from 550 to 8300 pg/ml).

Prior to nephrectomy the mean level of IL-12 was 148 pg/ml (range from 33 to 290) in patients with local and 102 pg/ml (range 20 to 460) in those with non-local tumours. This IL-12 concentration in the serum increased significantly from 148 to 250 pg/ml (range 55 to 510),  $p = 0.0006$ , after removal of the primary tumour in patients with local disease.

**Table 4.** Comparative results on mean ( $\pm$ SD) concentrations of IL-6, sICAM-1, sIL-2R and IL-12 in patients with local or disseminated RCC before nephrectomy (A), in patients with local RCC before nephrectomy and at 3 month's control (B) and patients surviving over one year or not (C)\*

	IL-6 (pg/ml)	sICAM-1 (ng/ml)	sIL-2R (pg/ml)	IL-12 (pg/ml)
<b>A: Local vs. dissem</b>				
Local	6.3 $\pm$ 8.0	290 $\pm$ 81	1796 $\pm$ 747	148 $\pm$ 87
Disseminated	53 $\pm$ 70	443 $\pm$ 280	3779 $\pm$ 2307	102 $\pm$ 120
P	p=0.03	p=0.03	p=0.01	p=0.29
<b>B: 0 vs. 3 months</b>				
Local 0 mmonths	6.3 $\pm$ 8.0	290 $\pm$ 81	1796 $\pm$ 747	148 $\pm$ 87
Local 3 months	2.7 $\pm$ 2.2	268 $\pm$ 34	2150 $\pm$ 740	250 $\pm$ 133
P	p=0.142	p=0.36	p=0.08	p=0.0006
<b>C: Surv vs. non-surv</b>				
Survivors	7.4 $\pm$ 8.0	295 $\pm$ 74	2290 $\pm$ 1813	160 $\pm$ 118
Non-survivors	67 $\pm$ 77	486 $\pm$ 255	33616 $\pm$ 2011	67 $\pm$ 40
P	p=0.007	p=0.006	p=0.11	p=0.03

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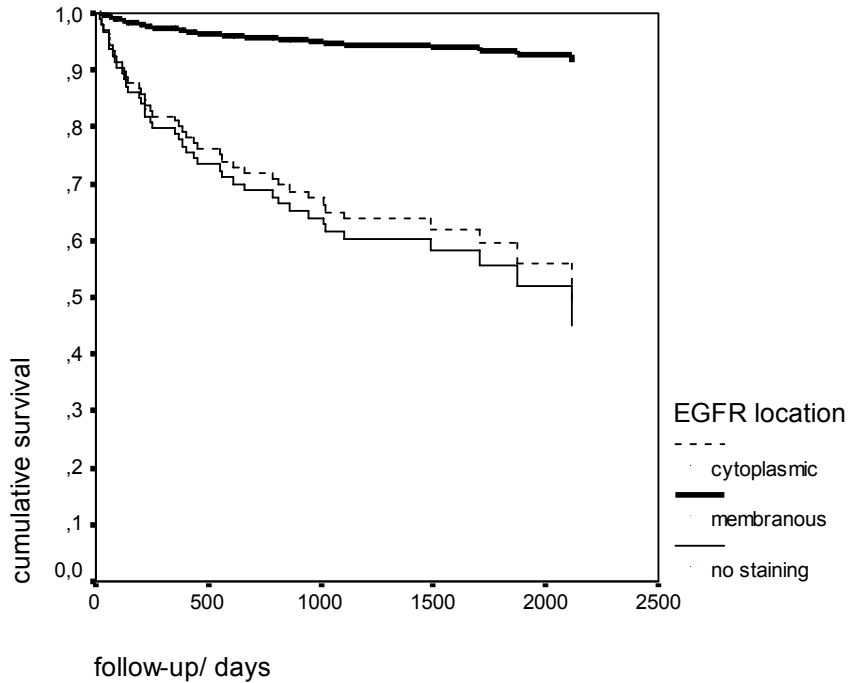
### 5.3. Epidermal growth factor receptor (Paper III)

The total number of patients was 134. Altogether 69 of the tumours here (52%) were local (T1-2, N0, M0), 27 (20%) were locally advanced (T3-4, N0, M0) and 38 (28%) disseminated (T1-4, N+/M+); 122 (89%) of the tumours were of conventional type (clear cell), 6 (5%) papillary, 2 (2%) chromophobe, 2 (2%) collecting duct histology. Two of the tumours (2%) remained unclassified. In nuclear grading 4 (3%) of the tumours were classified as grade 1, 59 (44%) as grade 2, 57 (43%) as grade 3 and 14 (10%) as grade 4.

EGFR scores varied between 0 and 300, mean score 83. Altogether 73% of tumours were positive in EGFR immunostaining with a cut-off score of 20. When placental control (score 100) was used as cut-off score, the percentage of positive tumours was 49%. Papillary tumours had markedly elevated scores (mean  $140 \pm 63$ ) when compared to clear cell (mean  $79 \pm 69$ ).

The distribution of EGFR immunostaining was as follows: no staining 23%, m (membranous) 3%, m>c (cytoplasmic) 11%, m=c 13%, c>m 25% and c 25%. Stratification into three groups was made: 1: no staining (23%), 2: prominent membranous staining (27%, including cases m, m>c and m=c) and 3: predominantly cytoplasmic staining (50%, including cases c>m and c). Papillary tumours all had cytoplasmic staining.

In univariate analysis for survival predominantly membranous staining (Figure 1) was associated with good prognosis, HR 8.0; 95%CI 2.0-33.2;  $p=0.004$ . When EGFR expression was handled as a continuous numerical factor (score) or divided in two classes (cut-off score 20 or 100), there was no statistically significant association with RCC survival. In multivariate analysis TNM stage was a very strong and single significant prognostic indicator, HR 37.0; 95%CI 8.2-167.5;  $p<0.001$ . The membranous immunostaining was not associated with low stages 1-2 ( $p=0.091$ ), but there was a statistically significant association with low Fuhrman grades ( $p=0.001$ , Fisher's exact test).



**Fig 1.** Survival of RCC patients with different cellular EGFR immunostaining patterns.

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#### 5.4. Expression of Bax and Bcl-2 (Paper IV)

The number of patients in this study was 138. Altogether 73 of the tumours (53%) were local (T1-2, N0, M0), 27 (20%) locally advanced (T3-4, N0, M0) and 38 (27%) disseminated (T1-4, N+/M+); 125 (91%) tumours had usual (clear cell), 6 (5%) papillary, 3 (2%) chromophobe and 2 (1%) collecting duct histology. Two of the tumours (1%) remained unclassified. In Fuhrman's nuclear grading five (4%) of the tumours were classified as grade 1, 60 (43%) as grade 2, 59 (43%) as grade 3 and 14 (10%) as grade 4. Bax index values varied from 0 to 300, 47 patients having the value 0. The median Bax index value was 50. Bcl-2 indices varied from 0 to 300, 60 patients having 0 and median being 20. MIB-1 indices ranged from 0.0 to 40.1, median 3.2. In univariate analysis according to survival statistically significant differences were reached by Bax (positive vs. negative; HR 3.04; 95%CI 1.27-7.23), Bcl-2 (positive vs. negative; HR 0.43; 95%CI 0.23-0.81), MIB-1 (continuous; HR 1.03; 95%CI 1.001-1.064), nuclear grade by Fuhrman (grade 4 vs. grades 1 plus 2; HR 8.15; 95%CI 3.13-21.20) and stage (4 vs.1; HR 60.04; 95%CI 13.99-257.68). Only stage (HR

47.96; 95%CI 10.85-212.03) and Fuhrman classification (HR 4.32; 95%CI 1.60-11.65) reached statistical significance in Cox regression multivariate analysis. When Bax and Bcl-2 values were handled as continuous parameters or divided into two classes at median the findings were similar. The same occurred using the Bax/Bcl-2 index. In cross-tabulation negative Bax values were associated with low Fuhrman grades 1 and 2 and negative Bcl-2 values with high Fuhrman grades 3 and 4 (Fisher's exact test), explaining their dropout as independent prognostic factors in multivariate analysis. Bax and Bcl-2 values had no specific associations with stage. MIB-1 was not associated with Bax or Bcl-2 (Mann-Whitney test). MIB-1 values showed no association either with stage or Fuhrman grade (Kruskall-Wallis test).

## 5.5. Genetic polymorphisms in platelet receptors (Paper V)

Alltogether 128 male patients were taking part to this study. The GPIIIaPl<sup>A2</sup> allele was more frequently (p=0.024) found among patients with metastatic RCC (43.6%) compared to local disease (24.7%). The respective frequencies were 23.6% and 17.8% for GPIb 3550T and 9.1% and 11.9% for GPIa 807T (p=NS for both). When occurrences of confounding factors were compared between metastatic and local disease groups, there were no differences in ASA or anticoagulant medication, in alcohol consumption, smoking or age at time of operation. Differences were found in NSAID (non-steroidal anti-inflammatory drug) medication (metastatic 51% vs. local 19%, p<0.001) and in the diameter of the primary tumour (metastatic 8.7cm vs. local 6.2cm, p=0.02). For further evaluation these possible confounding factors and presence/ absence of the Pl<sup>A2</sup> allele stepwise logistic regression was used to analyze differences between metastatic and local RCC disease groups. In this testing (metastatic vs. local RCC) statistical significance was reached by prevalence of the Pl<sup>A2</sup> allele, OR 2.7 (95%CI 1.1-6.5), p=0.02; diameter of primary tumour, p=0.007; NSAID medication, OR 4.5 (95%CI 1.9-11.0), p=0.001.

## 6. DISCUSSION

### 6.1. General aspects

This study was conducted in a single unit, Tampere University Hospital, in the Departments of Urology and Oncology. The patients of the study represent a typical RCC patient population in our clinics. The patients were consecutive and were followed in a prospective manner with a strict follow-up schedule. The number of patients in papers I and II was small, which limits the possibility to draw firm conclusions; only obvious trends could be evaluated. In contrast, in papers III-IV the size of the material offers possibilities for more reliable statistics and multivariate analysis.

### 6.2. Genetic aberrations and proliferation activity

Genetic aberrations were found in all RCCs investigated, but showed no association with histopathology and clinical outcome. Losses were more common than gains. A high number of losses in common-type RCCs showed no association with poor survival, but a high MIB-1 index was associated with poor survival, poor histological differentiation and presence of metastases. All grade-4 tumours progressed.

. The most frequent alteration in non-papillary RCC is a deletion of the short arm of chromosome 3 (Moch et al. 1996), as also found in 14 out of the 18 non-papillary RCCs in the present study. Other genetic alterations involve deletions of 6q, 8p, 9p, 13q, and 14q (Yang et al. 2000), again seen in our patients. Papillary RCCs are characterized by a normal chromosome 3 and they frequently have additional copies of chromosomes 7, 12 and 17, as was the case in our two patients with papillary RCC. One patient had a tumour with predominantly spindle cell morphology but also areas consistent with conventional RCC and was, therefore, classified as clear RCC. This tumour had a 3p depletion and 9 pq depletion which also fit a clear cell type, demonstrating that the analysis of chromosome aberrations may help to classify a tumour genetically into the correct histological RCC type. However, another patient had a chromophobe type of tumour with typical histopathology throughout the sections although abundant gains atypical for chromophobe RCC were found. Also the immunophenotype was consistent with chromophobe RCC: the staining for cytokeratin 7 was strongly positive and there was no vimentin expression (cytokeratin 8 was positive).

The genetic aberrations seen in our patients involved entire arms, as is typical in RCC (Mertens et al. 1997). There were very few subregional deletions or losses, and no high-level amplifications were detected, which would suggest that inactivation of tumour suppressor genes is more important in inducing renal tumours than amplification of oncogenes. However, in this prospective study the high number of losses showed no association with poor survival.

CGH provides an overview of DNA copy number changes taking place in tumours. The overall frequency of DNA gains and amplifications, the clustering of these changes to particular chromosomal subregions and the size and number of regions affected can be evaluated. CGH is not useful only for classification of tumours but also for further targeting of chromosomal areas for more sensitive and precise tools such as the polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP).

So far two studies have suggested a putative prognostic role of a deletion in a single chromosome in RCC. Moch and colleagues (Moch et al. 1996) demonstrated that a large number of deletions are associated with recurrence-free survival, and loss of 9p was the only individual locus associated with recurrence. Their study included 41 nonmetastatic T3 tumours. Jiang and colleagues (Jiang et al. 2000) found prognostic significance with deletions in 9p and 13q arms. The development of metastases is, however, the main event resulting in poor prognosis and CGH studies have shown that metastases can be genetically almost completely different from the primary tumours (Bissig et al. 1999; Junker et al. 2000). This may at least partly explain why we found no association between genetic aberrations and clinical outcome and raises concern as to whether the CGH of primary tumours can give any additional prognostic information in clinical practice.

In the present study high MIB-1 indices also associated with poor histological differentiation and presence of metastases; MIB-1 gave more prognostic information than CGH. On contrary in the study IV no association between MIB-1 indices and and Fuhrman classes was found. The clearly higher number of patients in the study IV (138 vs. 20) gives more weight to the finding of missing association. This is also in accordance with MIB-1 status as an independent prognostic factor.

There were certain limitations to this study. The number of patients was small, which makes it difficult to demonstrate any major clinicopathological correlations or draw final conclusions between the CGH findings and the clinical characteristics of renal cell cancer. However, not even any clear trends were found, which would support the conception that CGH may not offer any

additional information on the prognosis of RCC. There was also no selection in tumours here, which, however, gives more practical experience of the usefulness of CGH in routine clinical work. A further strength is that the study was prospective, which made it possible to follow up patients according to a structured protocol, and none have been lost to follow-up. In addition, the follow-up time was long enough to see the true clinical outcome of the disease.

### 6.3. Soluble immunological parameters and early prognosis of renal cell carcinoma

Our findings showed that RCC effects on the immunological cascade and removal of the primary tumour could alter the immunological parameters. sIL-2R levels, IL-6 and sICAM-1 concentrations were higher in non-local tumours than in local, suggesting higher immunological activation due to a larger tumour burden. In contrast, the IL-12 concentration was high in local tumours and increased significantly after removal of the primary growth. This might be related to some other mechanism in tumour cell killing in the early phase of the disease, or to higher stimulation of cytotoxic activity in non-local tumour patients. According to a study by Yao and colleagues IL-12 inhibits angiogenesis by inducing IFN gamma and other downstream modulators, and there is evidence that the natural killer cell cytotoxicity of endothelial cells is a potential mechanism by which IL-12 can suppress neovascularization (Yao et al. 1999); thus our patients' higher IL-12 concentrations may inhibit the angiogenesis necessary to tumour growth and occurrence of metastases and so develop smaller, local tumours.

Our results on IL-6 are in contrast to some earlier findings where IL-6 did not correlate with size of primary tumour or number of metastases (Blay et al. 1992; Dosquet et al. 1994; Stadler et al. 1992). On the other hand, the higher levels in non-local tumours in the present material are in line with studies where IL-6 has been shown to be an autocrine growth factor for RCC cells. In addition, in our previous study during IL-2 plus IFN treatment the two patients with the highest IL-6 levels had the shortest survival (Vuoristo et al. 1996). We have also shown that renal cancer cells can form a leukemia inhibitory factor (LIF) which belongs to the same cytokine family as IL-6, expressing their activity via the g130 receptor pathway. Thus in that study renal cancer cells could not be stimulated with the addition of exogenous LIF in vitro, because they had sufficient endogenous LIF for growth stimulation (Kellokumpu-Lehtinen et al. 1996). In general, IL-6 values in all patients with local disease were below the mean value in healthy controls (10 pg/ml, according to the manufacturer of the kit) and all patients except one with non-local tumours had values over 10 pg/ml.

Thus IL-6 could in the future be used in tumour staging prior to surgery and should be evaluated in planning possible subsequent adjuvant treatment.

Much attention has been focused on IL-2 in the treatment of advanced RCC after the first reports of high dose IL-2 by Rosenberg and associates (Rosenberg et al. 1985), where they achieved an objective response in one third of patients with RCC. Since then other researchers have reported poorer responses. Even the addition of lymphokine active killer cells or tumour-infiltrating lymphocytes to IL-2 therapy has not yielded better response rates (Palmer et al. 1993). We did not measure IL-2 concentrations in our patients in the present study because an earlier study has shown that in general the levels are rather low (median 11 pg/ml). Even after the administration of SC IL-2 and IFN the concentration of IL-2 was between 359 and 1130 pg/ml. Moreover, at the early phase of treatment serum levels have been lower than before treatment (Vuoristo et al. 1996). The highest values (up to 1130 pg/ml) were measured 4 hours after the injection.

Soluble IL-2 receptor is a marker of T-cell activation (Rubin and Nelson 1990). In addition it might be shed into the circulation by tumour cells, as has been shown in haematological malignancies. Treatment with IL-2 also induces IL-2 receptors into the circulation. sIL-2R may serve as a negative feedback mechanism by binding the IL-2 molecule and thus reducing its effect. In an earlier study a more pronounced increase in sIL-2R levels were measured in patients with shorter survival during IL-2 and IFN treatment (Vuoristo et al. 1996). Thus the higher sIL-2R observed in the present study might negatively correlate to IL-2 tumour response or longer stimulation of IL-2 during tumour growth in patients with non-local tumours.

In the present study the IL-12 concentration increased after removal of the primary growth in local tumours. This phenomenon might have several explanations; 1) suppression of IL-12 secretion by some factors secreted from tumour cells, 2) restoration of the immunological system after removal of the tumour burden and 3) competition of IL-12 from the same receptors as IL-6. It has been shown in laboratory studies that advanced cancer cases evince impaired cytotoxic function of both natural killer cells and T-lymphocytes. IL-12 has been shown to augment this cytotoxicity, but to have little or no proliferative effects on resting NK- or T-cells. Lissoni and colleagues (Lissoni et al. 1998) have suggested that high levels of serum IL-12 may have a favourable prognostic significance in solid tumour patients either in baseline conditions or in response to IL-2 immunotherapy (23 RCC patients).

Moreover, the high IL-6 and sICAM-1 values and low IL-12 value in the present study were associated with poor outcome in RCC. Earlier investigators have shown that low expression of the ICAM-1 molecule on the cell surface correlates with favourable clinical behaviour (Banks et al. 1993; Santarosa et al. 1995). The serum levels of sICAM-1 were higher in metastatic tumours, as also in the present study, and this was also shown to be a pretreatment predictor of overall survival (Hoffmann et al. 1999). In addition Steinbach and associates (Steinbach et al. 1995) showed high expression of ICAM-1 in their novel RCC line, which could be used in the study of cell adhesion molecules and haematogenous metastasis as well as in host defence mechanisms in human RCC. In conclusion, the measured marker (sIL-2R, IL-6, IL-12, sICAM-1) levels might have a role as prognostic factors in RCC. However, more patients should be followed up for longer periods in order to draw final conclusions as to the usefulness of these markers.

#### 6.4. Influence of the location of EGFR immunostaining on prognosis

High expression of EGFR has been associated with advanced stage, poor prognosis and high metastatic potential in many human tumours (Liebermann et al. 1985; Neal et al. 1990; Slamon et al. 1987). No association between EGFR expression and prognosis in RCC has as yet been established. There are several complicating factors; namely the histological heterogeneity of RCC and the individual properties of different assessment methods. For example, the ligand binding method, which has been held to be a sensitive method for measurement of EGFR, measures functional properties and the technique concentrates on membranous proteins only (Yoshida et al. 1997). Immunohistochemistry, on the other hand, offers a simple and economical means for cellular detection of EGFR and analysing its location in tumour cells. So far, several immunohistochemical studies have shown that positive EGFR staining in RCC is common and is associated with cell proliferation (Moch et al. 1997), but its role as a prognostic factor remains uncertain (Hofmockel et al. 1997). In an immunohistochemical study by Uhlman and co-workers (Uhlman et al. 1995) membranous EGFR expression was associated with high tumour grade, metastatic disease and poor disease-specific survival. In contrast, in our study membranotic positivity was associated with good prognosis, while cytoplasmic or negative EGFR staining was not. The explanation for this discrepancy is unclear. However, in the present study, both the intensity and location of EGFR staining in tumour cells were taken to account when evaluating its associations with prognosis, while the report by Uhlman and co-workers gives no description of staining pattern and differences in antibody and staining process may partly explain divergent



results. An adverse prognostic role of cytoplasmic EGFR staining similar to that demonstrated in our study has also been shown in squamous cell carcinoma of the lung (Piyathilake et al. 2002). The association between poor prognosis and cytoplasmic EGFR staining may derive from changes in ligand-EGFR complex internalization and activation of associated signalling pathways in the progression of RCC, a theory Piyathilake and colleagues have further strengthened by cell culture experiments (Piyathilake et al. 2002). The localization pattern of immunostaining has also been shown to be of prognostic value with other markers in cancer such as bcl-2 expression in malignant melanoma. In studies by Vlaykova and associates (Vlaykova et al. 2002) a diffuse localization of bcl-2 expression upon immunostaining was associated with better survival than negative or focal expression in malignant melanoma. Similarly, aberrant cellular location of some adhesion molecules such as alpha-catenin may result in tumour dedifferentiation and aggressive, metastatic phenotype in laryngeal carcinoma (Hirvikoski et al. 1998). In the present study, the presence of immunostaining in the membranes was associated with exceptionally good prognosis, 3-year survival 95% compared to 50% in other EGFR locations. It can be speculated that in RCCs in which the EGFR distribution in cell membranes is maintained, the growth is probably still controlled by EGF rather than by activation of new signalling pathways. Thus the distinction between two different survival groups within the EGFR-positive RCCs, in addition to being a candidate as a simple prognostic marker, opens up the challenging possibility of different molecular targets for drug development for these patient groups. The third group in our study, RCCs with totally negative EGFR staining, may include a group of cancers in which mechanisms other than EGF are responsible for cancer growth and progression.

Here prominent membranous EGFR immunostaining was associated with good prognosis in renal cell carcinoma. However, further studies will be needed to clarify the role of the different EGFR patterns in the development and progression of RCC.

## 6.5. Expression of Bax and Bcl-2

According to our results the Bax indices are positively and Bcl-2 indices negatively associated with poor prognosis in RCC. As Bax is considered to act as an accelerator and Bcl-2 as an inhibitor of apoptosis, this would lead to the conclusion that high apoptotic activity is associated with poor prognosis in RCC. This is contradictory to progression, but there are probably other strong factors

for it and apoptosis is only a compensatory phenomenon. Similar associations between Bax, Bcl-2 and apoptosis in RCC have been verified by Yang and colleagues (Yang et al. 1998). In some studies elevated Bcl-2 expression has been associated with small tumour size, low histological grade and low stage (Lipponen et al. 1995a; Oudard et al. 2002). In contrast, Vasavada and associates found an association between Bcl-2 expression and higher tumour grades (Vasavada et al. 1998). However, published results are conflicting and to our knowledge evidence of Bcl-2 and Bax being associated with prognosis in RCC is lacking. Although in our study Bcl-2 and Bax were not independent prognostic factors when stage was included in multivariate analysis, this does not rule out a possible role of Bax and Bcl-2 as independent prognostic factors within the same stage. However, such analysis would call for a much larger population. Lipponen and colleagues have shown a positive association between intense expression of Bcl-2 protein and favorable prognosis in breast carcinoma (Lipponen et al. 1995b) and a group under Törmänen found non-small cell lung carcinomas with enhanced apoptosis to carry an elevated risk for poor survival (Tormanen et al. 1995). These findings are in accord with our own. Surprisingly, however, in the latter study the extent of apoptosis did not correlate with the expression of Bcl-2. Gobe and colleagues summarize the findings on Bcl-2, Bcl-X<sub>L</sub> and Bax in individual variability in the importance of these genes in the adult human kidney. They identified a subgroup of RCCs in which a strong positive correlation prevailed between anti-apoptotic Bcl-2 and apoptosis. Bax correlated positively with apoptosis, as expected (Gobe et al. 2002). Overall, the susceptibility to apoptosis is determined by multiple competing dimerizations in which Bax and Bcl-2 constitute two functional partners (Sedlak et al. 1995). The key factor in RCC tumorigenesis is thought to be the loss of function of tumour suppressor genes. These genes also mediate their effects via apoptotic pathways: the von Hippel-Lindau gene product inhibits renal cell apoptosis via the Bcl-2 –family (Devarajan et al. 2001) and the tumour suppressor p53 is a direct transcriptional activator of the human Bax gene (Miyashita and Reed 1995). In addition to these, other regulating factors are present: epidermal growth factor, insulin-like growth factor-I and transforming growth factor (Gobe et al. 2000). The final status of apoptosis is determined by co-operation of all these regulators.

## 6.6. Fibrinogen receptors

We found in this study that the  $PI^A$  polymorphism of the  $GPIIIa$  ( $\beta_3$  integrin) gene has an association with the occurrence of metastases in RCC. In non-metastatic cases the  $PI^{A2}$  allele (heterozygotes and homozygotes combined) was found in one out of four patients, which corresponds to the level in Caucasian populations (Kekomaki et al. 1995), whereas almost half of metastatic cases carried this variant allele. This is in accordance with earlier mostly preclinical findings of a central role of fibrinogen in the development of metastases (Dvorak et al. 1983; Gunji and Gorelik 1988; Olander et al. 1985; Palumbo et al. 2000) and increased aggregability of platelets associated with the  $PI^{A2}$  allele (Feng et al. 1999; Szczeklik et al. 2000). The equal frequencies of  $PI^{A2}$  both in the normal population and in patients with local RCC give no role for this allele in the carcinogenesis of the primary RCC tumour. Bojesen and associates have recently reported that individuals homozygous for the  $PI^{A2}$  allele had an increased cancer incidence of ovarian and breast cancers and melanoma, but this was not found for RCC (Bojesen et al. 2003). This association could be due to an increased risk of metastasis among  $PI^{A2}$  homozygotes. However, these investigators found an association only among women and our study included only men. Carriers of  $\beta_3$  integrin T/T genotype ( $PI^{A1/A1}$ ) were shown in a recent study by a group under Ayala to have higher risk of breast cancer (Ayala et al. 2003), although their study is compromised by selection bias: their cases had a  $PI^{A2}$  frequency similar to that in their general population, while their controls had an exceptionally high frequency of this variant allele. However, in line with our results integrin  $\alpha_2$  ( $GPIa/IIa$ ) genotypes did not differ between patients and controls. The study was naturally limited to women. The carcinogenic potential of the  $\beta_3$  integrin is not immediately plausible biologically. However,  $\alpha_{IIb}\beta_3$  is a central receptor in tumour angiogenesis and could thus affect clinical cancer incidence. The more frequent use of NSAID by patients with metastatic disease is naturally explained by the increased need for palliation in this patient group. Of the known prognostic factors a small diameter of the primary tumour is associated with good prognosis, and also in our study the tumours were in general smaller in the local disease group.

In addition to platelets there are also fibrinogen receptors in tumour cells. The integrin  $\alpha_{IIb}\beta_3$  membrane receptor has been shown to be expressed by a variety of tumour cell lines, also by renal cell carcinoma cells (Chen et al. 1997). In experimental conditions platelet-mediated tumour cell adhesion to the extracellular matrix was mediated by platelet  $GPIIb-IIIa$  and by tumour cell alpha integrins independently of the nature of the beta subunit (Dardik et al. 1997). The histological and

molecular diversity of metastases and primary tumours could possibly be explained by ectopic expression of certain adhesive molecules in tumour cells prone to cell-platelet cross-linking and hematogenous spread. Recently integrin-linked kinase expression has been shown to correlate with invasion and metastases of gastric carcinoma (Ito et al. 2003). In addition, levels of plasma D-dimer, a fibrinogen degradation product, have been shown to be markers of lymphovascular invasion, clinical stage and lymph node involvement in operable breast cancer (Blackwell et al. 2000).

Clinical trials have also been performed on the effect of anticoagulant therapy in inhibition of metastases in various malignancies. Positive results have been observed with warfarin and dipyridamole analogue in lung carcinoma, while in colorectal cancer the results are controversial (Hejna et al. 1999) and in RCC studies they have been negative (Creagan et al. 1991; Zacharski et al. 1992). No studies have focused on interindividual variability in the effect of anticoagulant/antithrombotic treatment. An example of a more precisely targeted treatment option is abciximab, a monoclonal antibody to GPIIb/IIIa working against receptors both in platelets and in tumours (Cohen et al. 2000). The efficacy of GPIIb/IIIa blockade in long-term therapy has, however, been disappointing in trials on coronary disease patients (Chew and Bhatt 2001), even though preliminary data on cancer patients is encouraging (Amirkhosravi et al. 2003). In conclusion, presence of the  $PI^{A2}$  allele of platelet fibrinogen receptor GPIIb/IIIa is associated with metastatic disease in renal cell cancer. In future, better knowledge of adhesive receptors both on platelets and on tumour cells will afford better tools in evaluating prognosis and planning adjuvant treatments.

## 6.7. Future aspects

Newer techniques such as multi-tissue arrays are providing an enormous amount of new data on carcinogenesis and progression. The function of genes and also the world of proteomics will be better understood. The field of prognostic factors is becoming ever more complicated and diverse, and more sophisticated statistical models will be needed to handle this information in a meaningful way. On the other hand, more precise prognosis will be achieved on survival, progression, location of possible metastases and response to specific new treatments. In RCC and in general, parallel different pathways of progression will be found and concomitantly specific treatment of these with specific prognostic aspects. In future, prognostic models may be available which are based on a wide real time clinical database and where the statistical model is adjusted (real time, automatically) with inflowing data (clinical information and prognostic parameters).

## 7. CONCLUSIONS

1. The number of deleted areas in conventional-type (clear cell) carcinomas was not associated with prognosis. The total number of changed areas and the number of amplifications also remained without prognostic significance. These changes had no associations with histopathology or proliferation. The findings suggest that CGH does not necessarily offer any additional information on the prognosis of the disease in clinical practice. In contrast, proliferation was associated with histological grade, prognosis and development of metastases. Histopathological grading was associated most clearly with poor outcome of RCC. The small study population limits the value of these findings. However, CGH can be used for the classification of tumours and to locate genetic regions possibly of significance in the development of both cancer and metastases and constituting targets for further more sophisticated research.

2. Serum IL-6, sIL-2R and sICAM-1 levels prior to nephrectomy were significantly higher in non-local than in local tumours. In contrast, IL-12 levels were higher in local tumours and the levels increased significantly after removal of the primary tumour in patients with local disease. All patients with local tumours had normal IL-6 values. In addition, IL-6 and sICAM-1 levels before operation were significantly higher in patients with short (less than one year) survival. In contrast, patients with shorter survival had significantly lower IL-12. Our findings suggest that RCC induces changes in several immunological parameters. The broad distribution of these values makes their routine clinical use with single RCC patients less informative. These soluble immunological factors, IL-6, IL-12, sIL-2R and sICAM-1, might have a role as prognostic factors in RCC.

3. Tumours with prominently membranous EGFR immunostaining had a good prognosis compared to tumours with cytoplasmic or negative staining. RCCs with totally negative EGFR staining may include a group of cancers in which mechanisms other than EGF are responsible for cancer growth and progression. However, there is discrepancy between earlier studies dealing with EGFR findings and associations with prognosis. Future studies on the different EGFR expression patterns in renal cell carcinoma will clarify their role in the progression of the disease and their value as prognostic markers.

4. Both apoptotic regulators, Bax and Bcl-2 showed a statistically significant association with prognosis in RCC, but did not reach the status of independent prognostic factors. Surprisingly, our results suggested that high apoptotic activity is associated with poor prognosis. However, the regulation of the apoptotic process is complex, also other apoptosis-regulating factors are present and the final status is determined by co-operation among all of these.

5. Presence of the P1<sup>A2</sup> allele of platelet fibrinogen receptor GPIIb/IIIa is associated with an increased risk of metastatic disease in renal cell carcinoma. Future studies will provide a better knowledge of adhesive receptors both on platelets and on tumour cells and will offer better tools in evaluating prognosis and planning adjuvant treatments.

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