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NOVEL PROGNOSTIC BIOMARKERS FOR RENAL CELL CARCINOMA

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Abstract

Background and aims: Stage and grade are the most widely used prognostic parameters for renal cell carcinoma (RCC). The clinical course of this disease is not, however, always predictable by traditional prognostic factors. In the era of new molecular targeted therapies a more accurate prognostication of RCC patient survival is important for the individualization of treatment and follow-up of patients. Despite exhaustive research there are still no prognostic biomarkers for RCC in clinical practice. In order to find novel prognostic tissue markers for RCC, we examined the expression of 14 biomarkers involved in carcinogenesis and clarified their prognostic significance in RCC.

Material and methods: Out of 189 consecutive patients who underwent surgery for kidney cancer at Oulu University Hospital in the 1990s, 152 patients with histologically verified RCC were included in this study. The stage distribution was 70 (46%), 12 (8%), 51 (34%) and 19 (12%) patients with stages I-IV, respectively. The majority of the tumours (83 tumours, 55%) were nuclear grade II and 5 (3%), 40 (27%) and 22 (15%) of the tumours were grades I, III and IV, respectively. Clinical and follow-up data were obtained from patient records, the Finnish Cancer Registry and on demand from the Population Register Centre of Finland. The biomarkers studied included markers of the oxidative and neuroendocrine systems as well as proteins related to cell adhesion and migration, invasion, metastasis, inflammation and immune responses. The expression of various biomarkers was characterized via immunohistochemical tests of archival tumour material. The staining intensity was compared to clinicopathological parameters and patient RCC-specific survival.

Results: The 5-year RCC-specific survival was 77%. The expression of Toll-like receptor 9 (TLR9) was an independent marker of favourable RCC-specific survival whereas cytoplasmic myosin VI expression was found to be an independent prognostic factor of poor RCC-specific survival. Cell culture experiments showed how cyclooxygenase-2 (COX-2) expression is regulated by HuR in RCC. HuR and COX-2 immunoeexpression were also related to decreased RCC-specific survival. Immunostaining of Keap1 was associated with advanced RCC and a marker of a poorer RCC-specific prognosis. The expression of different neuroendocrine markers was evaluated but we could not establish any prognostic value for them.

Conclusions: In particular, TLR9, HuR and myosin VI can be regarded as promising novel prognostic biomarkers in RCC. Stage, however, is the most important single prognostic factor for RCC.

Keywords: biological tumour markers, cyclooxygenase-2 (COX-2), HuR, Keap1, myosin VI, prognosis, renal cell carcinoma, survival, Toll-like receptor 9 (TLR9)
Ronkainen, Hanna-Leena, Uusia munuaissyövän ennusteellisia merkkiaineita. Oulun yliopiston tutkimuskeskus; Oulun yliopist o, Lääketieteellinen tiedekunta, Kliinisen lääketieteen laitos, Kirurgia; Diagnostiikan laitos, Patologia, PL 5000, 90014 Oulun yliopisto  
Oulu

Tiivistelmä


Väitöskirjatutkimus paljasti useita uusia kudosmerkkiaineita, joiden ilmeneminen munuaissyöväpidotilassa on yhteydessä potilaan ennusteeseen. Näistä merkittävimpä ovat myosini VI, joka liittyy syöväpidotilavien metastasointiin, sekä immuunipuolustuksessa vaikuttava Tollin kalainen reseptori 9 (Toll-like receptor 9, TLR9). Molemmat merkkiaineet osoittautuivat itseään eri ennustetekijöiksi munuaissyövässä. Muita ennusteeseen vaikuttavia merkkiaineita ovat tutkimuksen mukaan oksidatiivista stressiä aistiva Keap1 sekä immunologiisiin reaktioihin liittyvä syklo-oksigenaasi 2 (COX-2) ja sen ilmenemistä säätävä HuR.

Asiasanat: biologiset kasvaimerkkiaineet, ennuste, HuR, Keap1, munuaissolu- karsinooma, syklo-oksigenaasi 2 (COX-2), TLR9, tyypin VI myosini
Acknowledgements

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Finally, I dedicate this work to my family: to my husband Jussi, who has always encouraged and supported me and to our son Niilo, for being the light and enjoyment in our lives. Jussi’s assistance with technical aspects and linguistic problems has been invaluable.

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Oulu February 2012

Hanna-Leena Ronkainen
### Abbreviations

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<th>Description</th>
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<tr>
<td>3’-UTR</td>
<td>3’ untranslated region (of mRNA)</td>
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<tr>
<td>5HT</td>
<td>5-hydroxytryptamine, serotonin</td>
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<tr>
<td>AEC</td>
<td>3-amino-9-ethylcarbazol</td>
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<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli, a tumour suppressor gene</td>
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<tr>
<td>ARE</td>
<td>antioxidant response element or AU-rich element</td>
</tr>
<tr>
<td>B7</td>
<td>a family of immune-regulatory ligands</td>
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<tr>
<td>B7-H1</td>
<td>B7 homologue 1, programmed cell death 1 ligand 1, PD-L1, CD274, an immunosuppressing protein</td>
</tr>
<tr>
<td>BHD</td>
<td>Birt-Hogg-Dubé syndrome, folliculin (FLCN) tumour suppressor gene</td>
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<tr>
<td>CAIX</td>
<td>carbonic anhydrase IX</td>
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<td>CD56</td>
<td>neural cell adhesion molecule, NCAM</td>
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<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>c-MET</td>
<td>mesenchymal-epithelial transition factor, a proto-oncogen</td>
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<tr>
<td>COX</td>
<td>cyclooxygenase, prostaglandin endoperoxide H2 synthase, PGHS</td>
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<tr>
<td>COX-1</td>
<td>cyclooxygenase-1</td>
</tr>
<tr>
<td>COX-2</td>
<td>cyclooxygenase-2</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>DAB</td>
<td>3–3’-diaminobenzidine</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>epithelial cadherin, uvomorulin, L-CAM, cell-CAM 120/80, CD324</td>
</tr>
<tr>
<td>ELAV</td>
<td>embryonic lethal abnormal vision, a family of RNA-binding proteins</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
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<tr>
<td>FH</td>
<td>fumarate hydratase, a tumour suppressor gene</td>
</tr>
<tr>
<td>FLCN</td>
<td>folliculin tumour suppressor gene</td>
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<tr>
<td>H &amp; E</td>
<td>haematoxylin and eosin</td>
</tr>
<tr>
<td>HGF</td>
<td>hepatocyte growth factor</td>
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<tr>
<td>HIF</td>
<td>hypoxia-inducible transcription factor</td>
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<tr>
<td>HR</td>
<td>hazard ratio</td>
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<tr>
<td>HuR</td>
<td>ELAVL1, HuA</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
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<tr>
<td>IGFR1</td>
<td>type I insulin-like growth factor receptor</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IL-2R</td>
<td>interleukin-2 receptor</td>
</tr>
<tr>
<td>Keap1</td>
<td>Kelch-like ECH-associated protein 1, BTB-Kelch-type substrate-adaptor protein, INrf2</td>
</tr>
<tr>
<td>Ki-67</td>
<td>proliferation marker detected by the monoclonal antibody MIB-1, MKI67</td>
</tr>
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<td>LEF</td>
<td>lymphoid enhancer-binding factor, a transcription factor</td>
</tr>
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<td>MET</td>
<td>mesenchymal to epithelial transition, mesenchymal-epithelial factor, tyrosine kinase membrane receptor</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>MVI</td>
<td>microscopic venous invasion</td>
</tr>
<tr>
<td>NCAM</td>
<td>neural cell adhesion molecule, CD56</td>
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<tr>
<td>NF-kappaB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B-cells, a transcription factor</td>
</tr>
<tr>
<td>Nfr2</td>
<td>erythroid transcription factor NF-E2, nuclear factor erythroid-2-related factor 2, NF-E2-related factor 2</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>non-stereoidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>NSE</td>
<td>neurone-specific enolase, gamma-enolase, $\gamma$-enolase</td>
</tr>
<tr>
<td>NSS</td>
<td>nephron-sparing surgery</td>
</tr>
<tr>
<td>p53</td>
<td>(tumour) protein 53, a tumour suppressor protein encoded by the TP53 gene</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PG</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PSA</td>
<td>prostate-specific antigen</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homologue, a tumour suppressor gene</td>
</tr>
<tr>
<td>PTHrP</td>
<td>parathyroid hormone-related protein</td>
</tr>
<tr>
<td>pVHL</td>
<td>von Hippel Lindau protein</td>
</tr>
<tr>
<td>RCC</td>
<td>renal cell carcinoma</td>
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List of original publications

This thesis is based on the following articles, which are referred in the text by Roman numerals. In addition, some unpublished data are presented.


*Contributed equally
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1 Introduction

Renal cell carcinoma (RCC) is the most lethal urological malignancy, accounting for 100,000 deaths worldwide annually (Parkin et al. 2005, Lam et al. 2008). Approximately 20–30% of patients are diagnosed at the metastatic stage of disease and half of the remaining patients will experience recurrence after an initially curative treatment (Motzer et al. 1996, Crispen et al. 2008, Lam et al. 2008). Predicting the clinical outcome of individual RCC patients is challenging and not always possible with classic prognostic factors, staging and grading, which are primarily used when assessing cancer prognosis (Crispen et al. 2008, Volpe & Patard 2010). During the last decade the treatment of metastatic RCC has dramatically changed, giving new hope to patients suffering from this malignancy, where survival has traditionally been regarded as poor in advanced stages. In the era of new molecular targeted therapies there is a definite need for better tools to predict the clinical course of RCC. Accurate prognostication would help in patient counselling and to plan and individualize patient treatment and follow-up. (Volpe & Patard 2010) High-risk patients could be selected for more effective treatments, more careful surveillance and clinical trials with adjuvant therapies. On the other hand, patients with indolent disease could be spared from over-treatment, psychological stress and adverse effects of follow-up, such as radiation exposure, which would also save health care costs and resources. (Eichelberg et al. 2009, Volpe & Patard 2010)

Biomarkers are objectively measured and evaluated indicators of biological or pathological processes or treatment responses. Cancer biomarkers are typically produced by the tumour or by the body in response to the tumour. Prognostic biomarkers are used to categorize patients into different risk groups and to predict the course of a disease. (Bensalah et al. 2007) The ideal prognostic biomarker provides prognostic information that is not provided by available clinicopathological indices. Other criteria for prognostic biomarkers are statistical significance, reproducibility, standardization, external validation and feasibility, such as suitability for daily clinical practices, for example evaluations based on urine and blood samples, and reasonable costs. (Crispen et al. 2008) Immunohistochemical techniques are used to determine the expression and cellular and subcellular locations of proteins of interest in tissue samples. In the tissue microarray technique (TMA), multiple samples are collected in the same block, which allows the high-throughput analysis of hundreds of specimens at the
Renal cell carcinoma is recognized as a family of cancers that originate from the renal tubular epithelium and have distinct genetic and molecular backgrounds, unique morphological features and a characteristic clinical course (Lam et al. 2008). The advances in gene technology, molecular biology and proteomics have enabled the identification of underlying molecular pathways of RCC as well as therapeutic targets for advanced disease (Pfaffenroth & Linehan 2008, Finley et al. 2011). Thousands of genes and a large number of molecular biomarkers have been screened and tested in order to find a prognostic biomarker for RCC. Despite exhaustive research, none of the markers tested so far have shown promise as an independent prognostic factor that could improve the predictive accuracy of current prognostic systems and be suitable for clinical practice. (Nogueira & Kim 2008, Volpe & Patard 2010)

Malignant transformation from normal to neoplastic cells involves acquired gain-of-function mutations in oncogenes and loss-of-function mutations in tumour suppressor genes and genome maintenance genes (Vogelstein & Kinzler 2004). Together, these provide growth advantages to tumour cells that are connected to the characteristics of cancer, such enhanced cell division (proliferation), the evasion of growth suppressors, resistance to apoptosis, induction of angiogenesis, invasion and metastasis, reprogramming of energy metabolism and the avoidance of antitumoural immune responses. Genomic instability is a fundamental underlying mechanism in cancer that provides genetic diversity, which further enhances carcinogenesis. In addition, inflammation promotes multiple hallmarks of cancer. (Hanahan & Weinberg 2011)

The present study was designed to evaluate the expression and prognostic value of novel biomarkers in RCC in order to find prognostic biomarkers for clinical use. The markers selected contribute to the basic hallmarks of cancer, such as cell proliferation, the inhibition of apoptosis, invasion and metastasis and angiogenesis. The markers included proteins involved in the oxidative system, cell adhesion and cell migration, inflammation and immune surveillance and neuroendocrine differentiation. The markers were studied using the immunohistochemical TMA technique in archival RCC tumour materials collected from patients who were operated in the 1990s at Oulu University Hospital. The immunoexpression of the biomarkers was assessed in context with various clinicopathological features of RCC tumours and compared to patient RCC-specific survival.
2 Review of the literature

2.1 Epidemiology and aetiology

2.1.1 Incidence and prevalence

Renal cell carcinoma accounts for approximately 85% of malignant kidney tumours and 2% of all malignant neoplasms (Motzer et al. 1996). The highest incidence rates occur in Europe and North America and the lowest in Asia and Africa. Among females, the incidence rates are generally about one half of those observed among males. (Lipworth et al. 2006, Chow et al. 2010) At the time of diagnosis the average age of patients is in the early sixties (Lipworth et al. 2006). The incidence of RCC is 8.9 per 100,000 persons per year, with the peak incidence occurring between 60 and 70 years of age (Campbell et al. 2007). During the last few decades the incidence and mortality of RCC have shown a continual increase. The increase in incidence can partly be explained by incidentally discovered, smaller and more often localized tumours. In recent years, however, the incidence and mortality rates of RCC have stabilized worldwide. (Chow et al. 2010) In Finland, approximately 900 new kidney cancers are diagnosed and over 300 RCC patients die of their disease each year. In 2010, there were over 6500 kidney cancer patients in Finland. (Finnish Cancer Registry 2011)

2.1.2 Risk factors

The most common and generally recognized risk factors for RCC are obesity and cigarette smoking, accounting for 30% and 20% of RCCs, respectively. Hypertension increases the risk of RCC, while antihypertensive drugs such as diuretics are not independent risk factors. Acquired renal cystic disease and end-stage renal disease are associated with an increased risk of RCC. (Lipworth et al. 2006, Chow et al. 2010) Ionizing radiation weakly increases the risk of RCC (Lipworth et al. 2006). Diets containing a large amount of fruit and vegetables, a lower caloric intake as well as physical activity and moderate alcohol consumption may have a protective effect against RCC (Chow et al. 2010).

Exposure to different occupational agents such trichloroethylene (TCE) and asbestos is an ambiguous risk factor for RCC and, generally, RCC is not regarded
as an occupational disease (Lipworth et al. 2006, Chow et al. 2010). Furthermore, there is no convincing evidence to suggest that non-steroidal anti-inflammatory drugs (NSAIDs), largely salicylates and acetaminophen, are risk factors for RCC (Lipworth et al. 2006).

A self-reported family history of RCC is associated with a 2–3-fold increase in RCC risk and it is believed that genetic factors associate with environmental factors to increase the risk of RCC (Lipworth et al. 2006, Chow et al. 2010).

2.2 Diagnosis

2.2.1 Clinical presentation

A renal mass can grow rather large in the retroperitoneum, until it becomes symptomatic and palpable. Symptoms related to RCC are due to the local growth of the tumour, haemorrhage, paraneoplastic syndromes or metastasis. (Campbell et al. 2007) The most common presentations of RCC are hematuria, abdominal pain and a palpable mass in the flank or abdomen. The classic triad of flank pain, gross hematuria and a palpable abdominal mass is found in less than 10% of cases. A varicocele, usually left-sided, resulting from the obstruction of the testicular vein, is present in 2% of male patients. (Motzer et al. 1996) Paraneoplastic syndromes result from the humoral release of various tumour-associated proteins, which are directly produced by the cancer cells or by the immune system in response to the tumour. Paraneoplastic symptoms and signs include, for example, hypertension, elevated erythrocyte sedimentation rate, anaemia, polycythemia, cachexia, weight loss, malaise, pyrexia, hypercalcaemia, neuromyopathy, amyloidosis, hypoalbuminemia or hepatic dysfunction and occur in 20% of RCCs. (Campbell et al. 2007, Lam et al. 2008) Patients can suffer from symptoms due to metastases, such as persistent cough and bone pain. The most frequent sites for RCC metastases are lung parenchyma, bone, liver and brain, but RCCs can metastasize to virtually any organ site. (Motzer et al. 1996, Ljungberg et al. 2010) The role of a physical examination is limited in the diagnosis of RCC but it might provide clues about a more advanced stage of disease, such as venous involvement and metastases (Ljungberg et al. 2010).
2.2.2 Macroscopic tumour presentation

Macroscopically, RCC usually appears as a solid round or ovoid lesion circumscribed by a pseudocapsule. Cystic degeneration and calcification are found in 10–25% and 10–20% of the tumours, respectively. One unique feature of RCC is its capability to grow into the venous system. Most sporadic RCCs are unilateral and unifocal, whereas bilateral and multicentral involvement is more common with the familial form of RCC. (Campbell et al. 2007)

2.2.3 Histopathology

Renal cell carcinomas originate from the renal tubular epithelium and have distinct genetic abnormalities and unique morphological features (Kovács et al. 1997, Lam et al. 2008). The histological diagnosis of RCC is usually based on morphological assessment under a microscope. When histological findings overlap, immunohistochemical and microRNA (miRNA) techniques can be helpful in distinguishing different subtypes of RCC. (Kovács et al. 1997, Kim & Kim 2002, Eble 2004, Youssef et al. 2011) The nuclear grading of RCC is mostly based on the Fuhrman grading system, which is a four-tiered grading system that takes into account nuclear and nucleolar size, shape and content (Fuhrman et al. 1982).

Classification of renal cell carcinomas

Clear cell carcinoma accounts for approximately 70–80% of RCCs. The most frequently recognized genetic abnormalities in clear cell RCC are mutations of the von Hippel Lindau (VHL) gene, the duplication of chromosome 5q and deletions at chromosomes 3p, 6q, 8p, 9p and 14q. The histological picture of clear cell RCC is characterized by the predominance of cells with a clear cytoplasm, although there can also be foci with an eosinophilic cytoplasm. (Kovács et al. 1997, Campbell et al. 2007)

Papillary carcinoma represents 10–15% of RCCs and it has a tendency towards multicentricity. Microscopically, basophilic and eosinophilic cells are arranged in papillary or tubular configurations. Type 1 papillary RCC consists of basophilic cells with scant cytoplasm, whereas the potentially more aggressive type 2 consists of eosinophilic cells and an abundant granular cytoplasm. (Eble et al. 2004, Campbell et al. 2007) Papillary RCCs are marked by the loss of Y
chromosomes in males and trisomy of chromosomes 3q, 7, 8p, 12, 16, 17 and 20. (Kovács et al. 1997)

Chromophobic carcinoma accounts for 3–5% of RCCs and it is mostly sporadic, although some cases are related to the Birt-Hogg-Dubé (BHD) syndrome (Eble et al. 2004, Campbell et al. 2007). Microscopically, the cells are characterized by a pale or eosinophilic granular cytoplasm. The underlying genetic abnormality is monosomy in chromosomes 1, 2, 6, 10, 13, 17 or 21. (Kovács et al. 1997)

Collecting duct or Bellini’s duct carcinoma represents less than 1% of RCCs (Eble et al. 2004, Campbell et al. 2007). It is characterized by irregular channels lined by a highly atypical epithelium, which can have a hobnail appearance (Kovács et al. 1997). Renal medullary carcinoma is considered a subtype of collecting duct carcinoma and associated with the sickle cell trait. Less than 3% of RCCs are characterized by indeterminant histological features and they are called unclassified or undifferentiated RCCs. (Campbell et al. 2007)

2.2.4 Laboratory examinations

The most commonly used laboratory parameters in RCC are C-reactive protein (CRP), haemoglobin, erythrocyte sedimentation rate, alkaline phosphatase, serum calcium, serum creatinine and glomerular filtration rate. A more precise evaluation of renal functioning, such as by an isotope renogram, is particularly important for treatment decisions when the tumour involves a solitary kidney, when the tumours are bilateral, when renal function is decreased or if there is a risk of renal impairment in the future. (Ljungberg et al. 2010)

2.2.5 Radiological evaluation

Over one half of RCCs are detected incidentally in radiological examinations (Pantuck et al. 2000). A renal mass observed by ultrasound examination (US) will be investigated further by an enhanced computed tomography (CT) scan. Preoperative staging includes chest and abdominal CT scans. When vena caval involvement is suspected, magnetic resonance imaging (MRI) or Doppler ultrasound examination may provide further information. (Heidenreich et al. 2004, Ljungberg et al. 2010) Angiography and embolization may be indicated preoperatively for large tumours in order to reduce intraoperative bleeding or as palliative management for pain and hemorrhage (Heidenreich et al. 2004). Further
examinations such as a bone scan and brain MRI or CT are performed when metastases are suspected. A needle biopsy under image-guidance is performed when a histological diagnosis is needed before oncological or minimally invasive treatment or surveillance is considered. (Ljungberg et al. 2010)

2.3 **Tumour biology in renal cell carcinoma**

The majority of RCCs are sporadic; inherited forms represent only about 2% of RCCs (Lipworth et al. 2006). However, current knowledge about the underlying genetics and molecular biology of sporadic RCCs is predominantly based on work with familial RCC (Linehan et al. 2003, Pfaffenroth & Linehan 2008).

### 2.3.1 The hypoxia-inducible pathway

Angiogenesis is an important part of the growth, invasive progression and metastatic spread of solid tumours (Diaz-Flores et al. 1994). Hypoxia and the compensatory hyperactivation of angiogenesis are important in the pathogenesis of RCC, which is typically a highly vascularized neoplasm (Kaelin 2002, Ruan et al. 2009). The most commonly inherited form of RCC is associated with von Hippel-Lindau (VHL) disease, which is an autosomal dominantly inherited disorder caused by germline mutations in the VHL tumour suppressor gene. In addition to multiple, bilateral clear cell RCCs, VHL disease is characterized by haemangioblastomas of the retina and central nervous system, phaeochromosytomas and renal, pancreatic and epididymal cysts. (Maher et al. 1991, Gnarra et al. 1994) The VHL gene resides on chromosome 3p25 (Latif et al. 1993) and its inactivation by somatic mutations or promoter hypermethylation is also a common feature in sporadic clear cell RCCs, being present in 57% and 19% cases, respectively (Gnarra et al. 1994, Herman et al. 1994).

The VHL gene encodes the VHL protein (pVHL), which shuttles between the nucleus and cytoplasm and is found in various cellular compartments such as the endoplasmic reticulum and mitochondria (reviewed in Kaelin 2002). The VHL protein is a component of a multiprotein complex called the E3 ubiquitin-ligase complex that targets hypoxia-inducible transcription factors (HIFs) for ubiquitin-mediated degradation. In normal cells and under normoxic conditions, HIFs are hydroxylated and then bound by pVHL and targeted for ubiquitin-mediated proteolysis. Under hypoxia, unhydroxylated HIFs avoid detection by pVHL and accumulate. (Maxwell et al. 1999, reviewed in Kaelin 2004) The accumulation of
HIF also takes place as a consequence of the lack of pVHL. Hypoxia-inducible transcription factors are heterodimers consisting of an α-subunit and a β-subunit. Their accumulation results in the transcription of various genes involved in adaptation to hypoxia. The target genes of HIF include, for example, vascular endothelial growth factor (VEGF), which is involved in angiogenesis, and transforming growth factor alpha (TGF-α) and platelet-derived growth factor (PDGF), which contribute to mitogenesis. In addition to angiogenesis and cell cycle control, the loss of functioning VHL has been implicated in various other central processes of carcinogenesis, including differentiation and extracellular matrix formation and turnover. (reviewed in Kaelin 2002, Kaelin 2004, Pfaffenroth & Linehan 2008)

2.3.2 The PI3K/AKT/mTOR pathway

Protein kinase B (AKT) and the mammalian target of rapamycin (mTOR) have a central role in signalling pathways that drive tumorigenesis (Shaw & Cantley 2006). The growth factors VEGF and PDGF bind to their receptor tyrosine kinases and activate phosphatidylinositol-3-kinase (PI3K) and AKT. Activated AKT regulates cell survival, growth and metabolism, as well as cell-cycle progression, and leads to the inhibition of apoptosis, the accumulation of cell cycle promoters and the activation of mTOR. (Shaw & Cantley 2006, reviewed in Banumathy & Cairns 2010) The suggested underlying mechanism of AKT activation in RCC is the negative regulation by the tumour suppressor gene called the phosphatase and tensin homologue (PTEN). However, the genetic deficiencies in the PI3K/AKT/mTOR pathway that cause RCC are still unclear. (reviewed in Banumathy & Cairns 2010)

The mTOR kinase is an intracellular serine/threonine kinase that consists of two distinct complexes: TORC1 and TORC2. The TORC1 complex regulates protein synthesis and controls various specific cell growth regulators, such as the transcription factor HIF-1α, that link mTOR signalling to diverse oncogenic processes like angiogenesis. In turn, the TORC2 complex controls cell polarity and spatial growth. The activity of mTOR is regulated by various growth factors, the availability of nutrients and AKT, which in turn is also activated by mTOR. (Shaw & Cantley 2006, reviewed in Banumathy & Cairns 2010)
2.3.3 The Wnt/beta-catenin pathway

The wingless-type (Wnt) signalling transduction pathway is a network of separate but interacting pathways that have a central role in the pathogenesis of RCC (reviewed in Karim et al. 2004, Banumathy & Cairns 2010). Several components of the Wnt pathway are associated with carcinogenesis, especially beta-catenin and the adenomatous polyposis coli (APC) tumour suppressor gene (see section 2.6.2). The signalling of Wnt positively regulates beta-catenin by inhibiting its phosphorylation, ubiquitination and degradation. The tumour suppressor gene APC in turn facilitates beta-catenin proteosomal degradation. The cytoplasmic accumulation of beta-catenin results in the translocation of beta-catenin to the nucleus, where it regulates gene expression by direct interaction with transcription factors of the TCF/LEF family (T-cell factor, lymphoid enhancer-binding factor). (Behrens et al. 1996, reviewed in Karim et al. 2004) The eventual outcome of Wnt signalling is the transcription and expression of target genes involved in the proliferation, differentiation, apoptosis and oncogenic transformation and progression. Furthermore, Wnt signalling may also activate the mTOR pathway (see section 2.3.2). (reviewed in Karim et al. 2004, Banumathy & Cairns 2010)

2.3.4 Epithelial to mesenchymal transition

In the embryonic development of the kidney, mesenchymal to epithelial transition is needed for organogenesis. The reverse phenomenon, epithelial to mesenchymal transition (EMT), is a complex process that involves various signalling receptors, transcriptional regulators and target genes and is essential before clear cell RCC can metastasize. The main target of these regulators is E-cadherin, which is crucial for maintaining an epithelial phenotype and also regulates the expression of beta-catenin, which is involved in Wnt signalling (see sections 2.3.3 and 2.6.2). (reviewed in Banumathy & Cairns 2010)

2.3.5 The HGF/MET pathway

The mesenchymal-epithelial transition factor (c-MET) is a proto-oncogene that encodes a tyrosine kinase membrane receptor (MET receptor), the ligand of which is the hepatocyte growth factor (HGF). Both the c-MET gene and the gene encoding HGF are located on chromosome 7. Ligand binding to the MET
receptor launches a signalling cascade with multiple events involving, for example, proliferation, survival, motility and the morphogenesis of many cell types. Following a gain-of-function mutation the MET receptor is constitutively activated, leading to a dysregulated tumourigenic state characterized by unregulated proliferation, transformation and increased invasive potential of the cells. (reviewed in Pfaffenroth & Linehan 2008, Banumathy & Cairns 2010, Finley et al. 2011) It has also been established that the loss of VHL promotes oncogenic signalling via HGF (Peruzzi et al. 2006). The germline mutations of c-MET are responsible for the hereditary papillary RCC (HPRCC) syndrome characterized by type 1 papillary RCC (Schmidt et al. 1997). In addition, activating mutations of c-MET are found in 5–13% of sporadic papillary RCCs, although the primary underlying genetic abnormality in sporadic papillary RCC is trisomy of chromosome 7, which is found in approximately 75% of cases (reviewed in Pfaffenroth & Linehan 2008).

### 2.3.6 Other genetic alterations

Hereditary leiomyomatosis and renal cell carcinoma (HLRCC) is characterized by germline mutations in the tumour suppressor gene fumarate hydratase (FH) and papillary type 2 RCC (Linehan et al. 2003). Thus far, no relevant evidence has been found to suggest somatic mutations of FH in sporadic RCC (reviewed in Pfaffenroth & Linehan 2008). A germline mutation in the tumour suppressor gene BHD (folliculin gene, FLCN gene) is associated with Birt-Hogg-Dubé (BHD) syndrome that is characterized by renal tumours with variable histology, including, for example, chromophobic and clear cell RCC. It has been suggested that folliculin is a downstream effector of mTOR signalling. (reviewed in Pfaffenroth & Linehan 2008) In addition, familial RCC syndromes have been shown to be associated with the chromosome 3:8 translocation (Li et al. 1993, Gnarra et al. 1994), tuberous sclerosis complex genes TSC1 and TSC2 and the succinate hydrogenase (SDH) gene (reviewed in Banumathy & Cairns 2010). Increased risk of RCC has also been reported in patients with hyperparathyroidism-jaw tumour syndrome (Haven et al. 2000) and hereditary nonpolyposis colorectal carcinoma (Baiyee & Banner 2006).
2.4 Survival and prognostic factors in renal cell carcinoma

The clinical course of RCC is variable. Small, incidentally discovered tumours can have indolent course even without treatment and in metastasized or recurrent disease survival rates have traditionally been poor. The prognosis of RCC has generally improved, which can be explained by early diagnosis and advances in imaging, staging and treatment, including both surgery and medical therapy. (Pantuck et al. 2001, Crispen et al. 2008) The most important prognostic factors for RCC are the stage and grade (Volpe & Patard 2010).

2.4.1 Clinical prognostic factors

The presence of clinical symptoms is associated with decreased survival, whereas incidentally found tumours seem to have a better prognosis, which is probably related to smaller size and lower stage of the tumours (Pantuck et al. 2000, Méjean et al. 2003, Schips et al. 2003). Weight loss of more than 10% of body weight during 6 months is related to decreased survival and cachexia is an independent marker of poor prognosis (Méjean et al. 2003, Lam et al. 2008). The performance status, as assessed by the Eastern Cooperative Oncology Group (ECOG) or Karnofsky scales, which indicate the impact of disease on the overall health of the patient, have become established and significant prognostic markers for RCC (Tsui et al. 2000, Lam et al. 2008). Younger age has been shown to be an independent marker of a more favourable prognosis (Lang et al. 2004), whereas gender does not have any prognostic potential (Schips et al. 2003).

Many laboratory parameters correlate with patient survival. In advanced RCC, high CRP caused by excessive interleukin-6 (IL-6) production, which is a multifunctional cytokine with growth factor activities, is associated with an unfavourable prognosis. An elevated erythrocyte sedimentation rate and thrombocytosis are also correlated with poor patient outcome. (Lam et al. 2008) In addition, serum calcium, haemoglobin, albumin, lactate dehydrogenase, alkaline phosphatase and neurone-specific enolase (NSE, γ-enolase) have at least some prognostic value in RCC (Takashi et al. 1989, Rasmuson et al. 1993, Méjean et al. 2003).
2.4.2 Anatomical prognostic factors: stage

The cancer stage is the most reliable prognostic factor in RCC. Generally, 5-year RCC-specific survival rates after radical nephrectomy are 75–95% for localized disease, 65–80% for locally advanced disease, 40–60% for tumours with vena cava thrombus, 10–20% for lymph node involvement and 0–5% for metastatic RCC. (Lam et al. 2008) Staging integrates the anatomical features of a tumour including tumour size, renal capsule and venous invasion and adrenal involvement and the presence of lymph node and distant metastasis which all are well-known prognostic factors and, when assessed together, provide the most reliable prognostic information in RCC (Delahunt et al. 2007, Ljungberg et al. 2010, Volpe & Patard 2010). The most commonly used staging systems for RCC are the Tumour Node Metastasis (TNM) classification by the International Union Against Cancer (UICC), presented in Figure 1 (Sobin & Wittekind 2002), and the Robson classification, which is mostly used in the United States (Robson et al. 1969). The TNM scores can be further combined in stages I-IV (Figure 1). The TNM classification was recently revised in 2009 (Sobin et al. 2009). Compared to the previous version from the year 2002 (Sobin & Wittekind 2002), tumour class T2 is now divided into subclasses T2a and T2b, including tumours that are more than 7 cm but less than 10 cm in diameter and tumours more than 10 cm in diameter, both limited to the kidney, respectively. In addition, T3a now includes tumours with a tumour thrombus extending into the renal vein only and adrenal gland invasion is now classified as T4. (Ljungberg et al. 2010)
Figure 1. TNM classification of renal cell carcinoma by the UICC (2002) From: Eble JN et. al. 2004. Reproduced with permission by IARC, Lyon.

<table>
<thead>
<tr>
<th>TNM classification</th>
<th>N</th>
<th>M</th>
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<tbody>
<tr>
<td><strong>T – Primary Tumour</strong></td>
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<tr>
<td>TX: Primary tumour cannot be assessed</td>
<td>N0</td>
<td>MX</td>
</tr>
<tr>
<td>T0: No evidence of primary tumour</td>
<td>N1</td>
<td>M0</td>
</tr>
<tr>
<td>T1: Tumour 7 cm or less in greatest dimension, limited to the kidney</td>
<td>N2</td>
<td>M1</td>
</tr>
<tr>
<td>T1a: Tumour 4 cm or less</td>
<td></td>
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<td>T1b: Tumour more than 4 cm but not more than 7 cm</td>
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<tr>
<td>T2: Tumour more than 7 cm in greatest dimension, limited to the kidney</td>
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<tr>
<td>T3: Tumour extends into major veins or directly invades adrenal gland or perinephric tissues but not beyond Gerota fascia</td>
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<td>T3a: Tumour directly invades adrenal gland or perinephric tissues but not beyond Gerota fascia</td>
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<tr>
<td>T3b: Tumour grossly extends into renal vein(s) or vena cava or its wall below diaphragm</td>
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<tr>
<td>T3c: Tumour grossly extends into vena cava or its wall above diaphragm</td>
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<tr>
<td>T4: Tumour directly invades beyond Gerota fascia</td>
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**Notes:**
- *Includes renal sinus (peripelvic) fat*
- *Includes segmental (muscle-containing) branches*

<table>
<thead>
<tr>
<th>Stage grouping</th>
<th>Stage I</th>
<th>Stage II</th>
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<tr>
<td>T1</td>
<td>N0</td>
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<td>T1, T2, T3</td>
<td>T4, N0, N1</td>
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<td>N0</td>
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<td>M0</td>
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<td>M1</td>
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1 (UICC 2002)
2 A help desk for specific questions about the TNM classification is available at http://www.uicc.org/tnm
2.4.3 Histological prognostic factors

Despite criticism concerning the validity and predictive value of the Fuhrman grading system, it is the most accurate histological grading system for RCC and an independent prognostic factor for clear cell RCCs at least (Lang et al. 2004, Delahunt et al. 2007, Volpe & Patard 2010). The 5-year RCC-specific survival is 89%, 65% and 46% for grades I, II and III-IV, respectively (Tsui et al. 2000).

Generally, the prognosis for papillary and chromophobic RCCs is better than that of clear cell RCC, and the survival rate of collecting duct RCC is poor. After the stratification of tumour stage, however, the prognostic information regarding the histological subtype is lost. (Ljungberg et al. 1999, Delahunt et al. 2007, Ljungberg et al. 2010, Volpe & Patard 2010) Among papillary RCCs, type 1 tumours have a better prognosis than type 2, which are independent markers of a poor prognosis (Delahunt et al. 2001). Out of the various histological features of RCC, microscopic venous invasion (MVI), sarcomatoid differentiation, necrosis and invasion of the collection system are associated with reduced survival rates, whereas cystic composition is related to a more benign clinical course (Lang et al. 2004, Delahunt et al. 2007, Ljungberg et al. 2010).

2.4.4 Molecular prognostic markers

Although numerous biomarkers have been recognized as being associated with patient outcome, no prognostic biomarker has yet been found that would be suitable for routine clinical practice in RCC (Nogueira & Kim 2008, Volpe & Patard 2010).

Markers of the hypoxia-inducible pathway

The prognostic impact of markers of the hypoxia-inducible pathway is controversial, indicating that the signalling network may be more complicated than previously thought. Somatic VHL alterations are associated with a favourable prognosis (Yao et al. 2002). In clear cell RCC, cytoplasmic HIF-1α expression correlates with a more favourable prognosis, whereas nuclear immunoexpression of HIF-1α is related to poor prognosis in metastatic disease ( Lidgren et al. 2005, Klatte et al. 2007). It has been suggested that HIF-2α might be more important in the tumorigenesis of clear cell RCC than HIF-1α since with dysfunctional VHL the transcription of hypoxia-regulated genes such as VEGF
has been shown to be regulated by HIF-2α (Nogueira & Kim 2008). However, there are no reports of the prognostic value of HIF-2α immunoeexpression as yet. The overexpression of downstream targets of VHL and HIF-1α are associated with both more and less favourable survival as carbonic anhydrase IX (CAIX) was shown to be a marker of better prognosis whereas VEGF, on the other hand, is related to worse survival rates (Bui et al. 2003, Bui et al. 2004, Jacobsen et al. 2004).

**Markers of the mammalian target of rapamycin pathway**

A loss of the tumour suppressor gene phosphatase and tensin homologue (PTEN) activity and the following activation of the mTOR pathway are related to decreased survival in RCC (Pantuck et al. 2007).

**Markers of cell proliferation, cell cycle regulation and apoptosis**

Cell proliferation, regulation of the cell cycle and programmed cell death (apoptosis) are key factors in the initiation and progression of malignant tumours (Linden et al. 1992, Hofstädter et al. 1995). However, it has been suggested that in RCC mitotic activity is too low to provide survival information in individual cases, but it could be associated with survival rates in larger populations. Proliferation markers such as proliferating cell nuclear antigen (PCNA, cyclin), Ki-67, argyrophilic nucleolar organizer regions (AgNOR) and spermine (SAT1) have been reported to be correlated with prognosis. (Delahunt et al. 1993, Bui et al. 2004, Kallio et al. 2004, Pantuck et al. 2007, Nogueira & Kim 2008) Cell cycle regulators p53 and p27 (cyclin-dependent kinase inhibitor 1B, CDKN1B, Kip1) are independent predictors of poor survival in clear cell RCC (Migita et al. 2002, Cho et al. 2005, Shvarts et al. 2005, Nogueira & Kim 2008). The prognostic role of apoptosis-related genes and proteins has not been thoroughly elucidated and mixed observations have been reported regarding their prognostic value in RCC (Lam et al. 2008). The B-cell lymphoma 2 (Bcl-2) family of apoptosis-regulating proteins, including Bcl-2 and the Bcl-2-associated factor X (Bax), have been correlated with patient survival (Kallio et al. 2004). In addition, the expression of p21 (cyclin-dependent kinase inhibitor 1, CDK-interacting protein, WAF1, cip1), survivin and DIABLO (second mitochondria-derived activator of caspaces, SMAC) have been associated with patient outcome in RCC (Mizutani et al. 2005, Parker et al. 2006, Weiss et al. 2007).
Markers of cell adhesion

A loss of cellular adhesion between neoplastic cells and between neoplastic cells and the extracellular matrix is one of the key processes in the development of metastatic disease (Freemont & Hoyland 1996, van Kilsdonk et al. 2010). Cell adhesion molecules have been studied as potential prognostic biomarkers for RCC with variable results. The down-regulation of E-cadherin and cadherin-6 is correlated with a poor prognosis in RCC but the prognostic significance of N-cadherin seems to be quite the opposite (see section 2.6.2) (Katagiri et al. 1995, Shimazui et al. 2006). The prognostic value of the cell-surface glycoprotein CD44 seems to be debatable (Méjean et al. 2003). Expression of the epithelial cell adhesion molecule (EpCAM) and the vascular cell adhesion molecule-1 (VCAM-1) have been related to a more favourable prognosis in RCC, whereas the L1 cell adhesion molecule (L1CAM) and cellular adhesion molecule EphA2 have been shown to be associated with advanced disease and decreased survival, respectively (Herrem et al. 2005, Nogueira & Kim 2008, Eichelberg et al. 2009).

Markers of degradation of the extracellular matrix

Matrix metalloproteinases (MMPs) degrade components of the extracellular matrix and basement membrane, allowing tumour to grow, invade and metastasize. Increased expression of MMPs has been demonstrated to be associated with impaired prognosis in RCC. (Kallakury et al. 2001, Nogueira & Kim 2008) Also, the expression of urokinase-type plasminogen activator (uPA), its receptor uPAR and plasminogen activator inhibitor type 1 (PAI-1), which are involved in regulating the degradation of the extracellular matrix, have been related to the outcome of RCC patients (Nogueira & Kim 2008).

Markers of immune protection

It has been established that renal cell carcinoma induces the apoptosis of T-cells and thus promotes tumorigenesis by suppressing host antitumour immunity (Rayman et al. 2004). The B7 family is a group of co-regulatory ligands that regulate T-cell-mediated immunity: B7-H1 and B7-H4 suppress immune responses and their increased expression correlates with an unfavourable patient outcome (Nogueira & Kim 2008). A cytokine receptor CXCR3 regulates tumour-mediated immunity, angiogenesis and metastatic spread, and it is an independent
marker of a more favourable prognosis in RCC (Klatte et al. 2008). On the other hand, the overexpression of another chemokine receptor, CXCR4, is related to poor RCC-specific survival (Eichelberg et al. 2009). The soluble immunological factors interleukin-6 (IL-6), interleukin-12 (IL-12) and the interleukin-2 receptor (IL-2R) have also been correlated with the prognosis of RCC patients (Kallio et al. 2001).

**Other prognostic biomarkers**

Of the numerous tissue markers studied, for example insulin-like growth factor II mRNA-binding protein IMP3 and vimentin are associated with an unfavourable prognosis, whereas fascin has only been correlated with negative clinicopathological features of tumours (Thompson et al. 2006, Eichelberg et al. 2009).

Some of the markers studied, such as CAIX, VEGF, amyloid A, insulin-like growth factor 1 (IGF-1) and NSE, as well as some immunological factors, have been demonstrated to predict patient survival when analysed in patient blood or serum samples (Takashi et al. 1989, Rasmuson et al. 1993, Kallio et al. 2001, Eichelberg et al. 2009). The presence of circulating tumour cells, endothelial cells and tumour enzymes also has some prognostic value in RCC (Bluemke et al. 2009, Namdarian et al. 2010, Nisman et al. 2010).

The prognostic value of the quantification of tumour vasculature (microvascular density) is controversial (Lam et al. 2008). The cytogenetic profile of the tumour is correlated with the prognosis. In addition, many signature genes have been recognized that, when used as a gene pattern, predict the clinical outcome of patients (Nogueira & Kim 2008, Klatte et al. 2009a).

**2.4.5 Prognostic factors in metastasized in renal cell carcinoma**

In advanced RCC, classic anatomical and histological features of the primary tumour have traditionally had limited predictive value (Volpe & Patard 2010). The prognostic factors that have been identified in metastasized disease are performance status, number and locations of metastatic sites, time to appearance of metastases, prior nephrectomy and curative surgical resection of metastases (Méjean et al. 2003). Bone metastases have been traditionally regarded as a marker of shorter survival. However, it has been shown that the number of metastatic sites is a more important prognostic marker than the location. (Lam et
al. 2008) Some laboratory findings such as low haemoglobin and elevated lactate dehydrogenase, corrected serum calcium and inflammatory markers have been correlated with patient survival (Volpe & Patard 2010).

2.4.6 Prognostic systems and nomograms

In order to improve predictive accuracy and categorize patients into different risk groups, several algorithms called nomograms have been developed by combining previously known prognostic factors. The most frequently used prognostic models in localized RCC are the University of California Los Angeles Integrated Staging System (UISS), which includes both clinical and pathological parameters, and the Mayo Clinic’s Stage, Size, Grade and Necrosis (SSIGN) score. The accuracy of both of these nomograms has been shown to be approximately 80%, which is superior to TNM staging alone. (Zisman et al. 2001, Frank et al. 2002, Volpe & Patard 2010) The Kattan nomogram was designed to estimate the probability of 5-year recurrence-free survival after nephrectomy and after its update in 2005 its accuracy is about 80% as well (Kattan et al. 2001, Volpe & Patard 2010). In a novel prognostic model by Karakiewicz et al. both the clinical and pathological variables combined within the algorithm were shown to provide a predictive accuracy of over 80% (Karakiewicz et al. 2007).

The most widely used prognostic tools for advanced RCC are the Memorial Sloan-Kettering Cancer Center (MSKCC) and the French group of immunotherapy instruments, which combine clinical parameters including performance status, serum laboratory examinations, time from diagnosis to initiation of systemic therapy and also the number and location of metastatic sites in order to stratify patients into different risk categories. Lately, new prognostic models have been developed and old ones updated in order to predict patient outcomes under molecular targeted therapy. (Motzer et al. 1999, Volpe & Patard 2010)

The addition of set of molecular markers to the nomogram of classic variables could improve the predictive accuracy considerably (Kim et al. 2004, Klatte et al. 2009b). Prognostic models that combine various independent prognostic molecular markers have been developed, such as the biomarker panel BioScore that includes B7-H1, survivin and Ki-67 (Parker et al. 2009). The molecular signature, which consists of Ki-67, p53, VEGF and VEGF receptors (VEGFR), is more accurate than clinical or pathological prognostic factors alone in predicting patient outcome in RCC (Klatte et al. 2009b).
2.5 Treatment

2.5.1 Treatment of localized renal cell carcinoma

Historically, the gold standard curative therapy for patients with localized RCC has been radical nephrectomy (Robson et al. 1969). Nowadays, partial nephrectomy (nephron-sparing surgery, NSS) is a recommended standard treatment for local renal tumours up to a diameter of 7 cm and an option for larger, local tumours whenever technically feasible (Uzzo & Novick 2001, Ljungberg et al. 2010, Van Poppel et al. 2011). The oncological efficacy of NSS has been confirmed in large, prospective randomized studies (Van Poppel et al. 2011), and there is evidence to show that NSS decreases the risk of renal failure and relative adverse effects on general health, including cardiovascular events, compared to radical nephrectomy (Huang et al. 2009).

With its adherence to the principles of open radical surgery, laparoscopic renal surgery is considered a standard of care for selected RCC patients and it is associated with a lower rate of morbidity than open surgery (Link et al. 2005, Ljungberg et al. 2010). Laparoscopic nephrectomy is a recommended standard care for local tumours that are not suitable for NSS and it provides equivalent oncologic outcomes to open surgery (Patard et al. 2004, Becker et al. 2006, Ljungberg et al. 2010). In experienced hands and with carefully selected patients, laparoscopic NSS is an alternative to open partial nephrectomy. Robotic-assisted partial nephrectomy is under evaluation. (Ljungberg et al. 2010)

In surgical treatment of RCC the official role of lymphadenectomy is restricted to staging purposes, principally at the hilar region. In selected cases with lymph node metastases restricted to the retroperitoneal area, extended lymphadenectomy may improve RCC patient survival rates. When the adrenal gland is normal in preoperative imaging, routine adrenalectomy is only recommended for large upper pole tumours or tumours with a diameter greater than 7 cm. (Ljungberg et al. 2010)

Minimally-invasive techniques, for example, percutaneous radiofrequency (RF) ablation (Lui et al. 2003), cryoablation (Gill et al. 2005), microwave ablation (Johnson & Nakada 2001), laser ablation (Hinshaw & Lee 2004) and high-intensity focused ultrasound (HIFU) (Janzen et al. 2002) are alternatives for surgical treatment in selected RCC patients with decreased performance status or multiple tumours. In addition, small renal tumours may be actively followed and treated if they progress. (Ljungberg et al. 2010)
2.5.2 Treatment of metastasized renal cell carcinoma

Resistance against radiotherapy, cytotoxic drugs and hormones is a typical feature of RCC (DiBiase et al. 1997, Finley et al. 2011). Immunotherapy with interferon alpha (IFN-α) or interleukin-2 (IL-2) can produce complete and durable responses, although response rates have generally been shown to be 6–15% for IFN-α and 7–27% for IL-2, providing only a modest survival benefit in advanced RCC. Nowadays, the official role of immunotherapy has been restricted to adjuvant use with bevacizumab. (Ljungberg et al. 2010, Finley et al. 2011)

Molecular targeted therapies are designed to block the critical signalling pathways underlying the pathogenesis of RCC. They are categorized into three groups: tyrosine kinase and multikinase inhibitors, VEGF antibodies and mTOR inhibitors. (Hutson 2011) Targeting agents are a recommended systemic treatment for metastasized RCC (Ljungberg et al. 2010). The efficacy of molecular targeted therapies has been proven in clinical trials where they have improved both progression-free and overall survival. Durable remissions are, however, rare because these drugs do not eradicate the disease. (Finley et al. 2011, Hutson 2011) The advantages of molecular targeted therapies compared to immunotherapy are better efficacy and tolerability, as well as oral administration (Hutson 2011).

Cytoreductive nephrectomy and resection of metastases improve patient survival and are recommended for patients with a good performance status (van der Poel et al. 1999, Flanigan et al. 2004, de Reijke et al. 2009, Ljungberg et al. 2010). In the era of molecular targeted therapies the role and timing of palliative surgery have, however, been blurred, and they should be re-evaluated in clinical trials (Polcari et al. 2009).

Palliative radiotherapy is indicated for symptomatic brain and bone metastases that do not respond to systemic treatment. The embolization of bone and paravertebral metastases can also relieve pain caused by metastases. (Ljungberg et al. 2010)

2.6 Reasoning for studied biomarkers

2.6.1 Oxidative stress in carcinogenesis and markers of the oxidative system

Oxidative stress is a state where the amount of reactive oxygen species (ROS) exceeds the antioxidant capability of the target cell (Chance et al. 1979, Klaunig
et al. 1998). In addition to mitochondrial oxidative phosphorylation and some other endogenous sources of ROS, ionizing radiation, ozone, various drugs, hormones and other xenobiotic chemicals alter the oxidative status in cells (reviewed in Klaunig et al. 1998, Oberley 2002). At low levels, ROS have physiological functions such as the regulation of signal transduction pathways, transcription factors and mitochondrial enzyme activity. A moderate increase in ROS activity promotes cell proliferation and differentiation, but excessive oxidative stress is harmful for cells. (reviewed in Oberley 2002, Trachootham et al. 2009)

Oxidative stress promotes carcinogenesis via several mechanisms that cover cancer initiation, promotion and progression. Reactive oxygen species cause structural alterations in DNA, prevent DNA damage repair and activate chemical carcinogens. (reviewed in Klaunig et al. 1998) In addition, ROS activate proto-oncogenes, inactivate tumour suppressor genes and modify cytoplasmic and nuclear signal transduction. By activating transcription factors, ROS are involved in gene expression regulation. (reviewed in Toyokuni et al. 1995, Wiseman & Halliwell 1996, Klaunig et al. 1998) Oxidative stress-mediated signalling events have been shown to contribute to various functions involved in carcinogenesis, such as cell proliferation and survival, apoptosis, energy metabolism, cell morphology, cell-cell adhesion, cell motility and metastasis, angiogenesis and the maintenance of tumour stemness (reviewed in Liou & Storz 2010). Finally, oxidative stress induces chemoresistance against anticancer drugs by activating specific antioxidant systems (reviewed in Toyokuni et al. 1995).

Persistent oxidative stress is present in cancer cells, including RCC (Szatrowski & Nathan 1991, Okamoto et al. 1994, Toyokuni et al. 1995). Simultaneously with increased ROS production there may also be an increase or decrease in antioxidants and ROS-scavenging enzymes, which makes regulation of the redox balance quite complex in cancer cells (reviewed in Trachootham et al. 2009). High levels of oxidative stress have been shown to be associated with aggressive behaviour and poor prognosis in cancer (reviewed in Kumar et al. 2008, Trachootham et al. 2009). In RCC, oxidative DNA damage has been shown to be related to poor differentiation (Soini et al. 2006b).

Nfr2 and Keap1

The erythroid transcription factor NF-E2 (nuclear factor erythroid-2-related factor 2, NF-E2-related factor 2, Nfr2) is a nuclear transcription factor that regulates
genes encoding antioxidants, xenobiotic detoxifying enzymes and drug efflux pumps in response to oxidative, electrophilic and xenobiotic stresses (Itoh et al. 1997). It is negatively regulated by Kelch-like ECH-associated protein 1 (BTB-Kelch-type substrate adaptor protein, Keap1, Nrf2) (Itoh et al. 1999). The Keap1 protein is a cysteine-rich cytoplasmic protein that binds to actin. It is localized in the nuclear periphery at the cytoskeleton and in adhesion complexes. (Itoh et al. 2004) The primary task of Keap1 is to sense xenobiotic and oxidative stresses and to modulate Nfr2 stability (Motohashi & Yamamoto 2004). Under normal conditions, the Neh2 domain of Nfr2 is bound to the double glycine repeat (DGR) domain of Keap1, which results in constant ubiquitination and proteosomal degradation of Nfr2 and further suppression of Nfr2 activity. In addition, Keap1 regulates Nfr2 by altering its subcellular location. (McMahon et al. 2003, Itoh et al. 2004) Oxidative stress antagonizes the interaction between Nfr2 and Keap1 by modifying cysteine residues of Keap1, resulting in the dissociation of Nfr2 from Keap1 and the accumulation of Nfr2 (McMahon et al. 2003, Yamamoto et al. 2008). When the Keap1-Nfr2 complex disrupts, Nfr2 is able to translocate into the nucleus where it binds to the antioxidant response element (ARE) and activates the transcription of various detoxification and antioxidant proteins. Then, Nfr2 activation is switched off and Nfr2 is exported back to the cytoplasm where it binds to Keap1 and is degraded. (Kaspar et al. 2009) The Nfr2-Keap1 system is regarded as being one of the most important cellular defence mechanisms against oxidative and xenobiotic stresses (Motohashi & Yamamoto 2004).

It is assumed that either too much or too little Nfr2 activity can result in physiological impairments (Liu et al. 2005). The biological consequences of Nfr2 activation are ambiguous in carcinogenesis. On the one hand, Nfr2 activity protects DNA against oxidative and xenobiotic damage by eliminating ROS and detoxifying carcinogens and, thus, it may suppress cancer initiation. It may also prevent metastasis by affecting the redox balance. (Satoh et al. 2010, Taguchi et al. 2011) On the other hand, Nfr2 activation can promote tumourigenicity and metastasis via several ways, such as by facilitating proliferation, anchorage-independent growth and cell survival, as well as by inhibiting apoptosis (Singh et al. 2008, Satoh et al. 2010, Shibata et al. 2010, Taguchi et al. 2011). Moreover, Nfr2 mutation activates the mTOR pathway (Shibata et al. 2010). Through the up-regulation of antioxidant enzymes and drug efflux pathways, Nfr2 activation induces chemoresistance (Singh et al. 2008).

Mutations in the Keap1 or Nfr2 genes result in impairments in Keap-mediated Nfr2 regulation and increased Nfr2 activity. Gain-of-function mutations
of the Nfr2 gene have mainly been reported in squamous cell carcinomas such as head and neck, lung, oesophageal and skin carcinomas and result in inactivated Keap1-mediated degradation of Nfr2 and the nuclear accumulation of Nfr2 (Shibata et al. 2008b, Kim et al. 2010). Loss-of-function mutations in the Keap1 gene have been described in several adenocarcinomas, including breast, gallbladder and lung cancers (Singh et al. 2006, Nioi & Nguyen 2007, Shibata et al. 2008a). In lung cancer, Keap1 mutations are associated with decreased Keap1 immunoeexpression (Ohta et al. 2008). In head and neck squamous cell carcinoma, increased Nfr2 expression has been reported in 92% of tumours at the same time as increased expression of the Nfr2-downstream target thioredoxin and the relative of overexpression Keap1 (Stacy et al. 2006).

Mutations of the Nfr2 and Keap1 genes have been found to be associated with poor survival in both lung and head and neck carcinomas (Shibata et al. 2010, Takahashi et al. 2010). Decreased Keap1 expression and the resulting nuclear accumulation of Nfr2 has been shown to stimulate tumour growth in lung adenocarcinoma (Ohta et al. 2008) and to be correlated with worse overall survival rates in non-small cell lung cancer (Solis et al. 2010).

**Thioredoxin**

Thioredoxin (Trx, adult T-cell leukaemia-derived factor, ADF) is a thiol-containing antioxidant enzyme, the expression of which is regulated by Nfr2 (Laurent et al. 1964, Powis et al. 2000, Stacy et al. 2006). Thioredoxin is predominantly a cytosolic protein but it has also been detected in the nucleus in malignant cells. The expression of Trx is cell-cycle dependent. Hypoxia and oxidative stress are able to induce Trx expression as well as facilitate its translocation into the nucleus. The antioxidant function of Trx is primary based on its ability to donate electrons for oxidized thioredoxin peroxidase, which then removes oxidant species. The catalytic site (-Trp-Cys-Gly-Pro-Cys-Lys-) of Trx is reversibly oxidized to the cysteine disulphide Trx-S2. (reviewed in Powis et al. 2000, Powis & Montfort 2001) In addition, Trx acts directly by removing hydrogen peroxide (Spector et al. 1988).

Thioredoxin may promote tumorigenesis via several mechanisms. Trx stimulates tumour cell growth by sensitizing tumour cells to growth factors produced by the tumour itself (Gasdaska et al. 1995). It also regulates transcription factors involved in tumorigenesis, such as nuclear factor-kappaB (NF-kappaB), activator protein 1 (AP-1) and HIF-1α, as well as the tumour
suppressor gene p53 (reviewed in Powis & Montfort 2001), and it inhibits apoptosis and thus offers both survival and growth advantages for cancer cells (Baker et al. 1997). The association between Trx expression and cell proliferation is contradictory. In some cancers, Trx expression is positively associated with cell proliferation activity (Grogan et al. 2000, Turunen et al. 2004), but the opposite situation has also been reported (Rubartelli et al. 1995). In addition, Trx facilitates chemoresistance (Singh et al. 2008).

Secretion of Trx has been shown in neoplastic cells (Rubartelli et al. 1992). Increased expression of Trx has been observed in several malignomas, for example in cervix, colorectal, gastric and non-small cell lung carcinomas (Fujii et al. 1991, Gasdaska et al. 1994, Berggren et al. 1996, Grogan et al. 2000). Thioredoxin overexpression is associated with features of more aggressive tumour growth, such as increased proliferation and decreased apoptotic activity (reviewed in Powis & Montfort 2001). In colorectal carcinoma, Trx is an independent marker of shortened survival rates (Raffel et al. 2003). In RCC, the immunoexpression of Trx is associated with older age and a lower apoptotic index (Soini et al. 2006b).

2.6.2 Cell adhesion, migration, invasion and metastasis and related proteins

The ability to invade other tissues and spread to distant organs is characteristic of cancer cells (Hanahan & Weinberg 2011). Tumour metastasis is the most important cause of mortality in cancer. It is a complex, multistep process that begins when tumour cells lose their cell-cell attachments, dissociate from the primary tumour and invade the extracellular matrix through the epithelial basement membrane. After entering the blood stream or lymphatic vessels, tumour cells disseminate via the circulation to distant organs where they form secondary lesions. The key processes in metastasis are changes in cellular adhesion, the production of proteolytic enzymes, which degrade the extracellular matrix, and the secretion of various cytokines, which promote invasion and angiogenesis. (reviewed in Meyer & Hart 1998)

Cell adhesion molecules are transmembrane glycoproteins that mediate interactions between cells and between cells and the tissue matrix. Changes in cell adhesion allow tumour cells to migrate, invade and metastasize. In addition, cell adhesion molecules are involved in cell signalling, proliferation and tumour growth. (reviewed in Freemont & Hoyland 1996, van Kilsdonk et al. 2010) Cell
adhesion molecules are divided into four major groups; one of these is the large cadherin family which mediates calcium-dependent intercellular adhesion. They interact with the cytoskeleton through cytoplasmic regulatory proteins called catenins. (Takeichi et al. 1988, reviewed in Makrilia et al. 2009)

**E-cadherin**

Epithelial cadherin (E-cadherin, uvomorulin, L-CAM, cell-CAM 120/80, CD324) (Sommers et al. 1991, van Kilsdonk et al. 2010) mediates homotypic and homophilic cell-cell adhesion in the epithelium and is essential for epithelial integrity and polarity. It consists of an extracellular domain, a transmembrane segment and a cytoplasmic domain. (reviewed in Freemont & Hoyland 1996, Chetty & Serra 2008, Makrilia et al. 2009) The cytoplasmic domain is complexed with either beta- or gamma-catenin (plakoglobin), which then binds directly to alpha-catenin and thus connects the E-cadherin-catenin complex to the actin cytoskeleton. (Aberle et al. 1996)

The loss of E-cadherin promotes tumorigenesis via several routes. The decreased expression of E-cadherin disrupts cell adhesion, allowing tumour cells to detach, and it facilitates conversion to a more migratory phenotype and invasion and metastasis (reviewed in van Kilsdonk et al. 2010). The loss of E-cadherin is a crucial step in the epithelial to mesenchymal transition (EMT), which is related to a tumour’s ability to metastasize (see section 2.3.4) (reviewed in Banumathy & Cairns 2010). In addition, the loss of E-cadherin liberates beta-catenin, which translocates to the nucleus and activates the transcription of genes responsible for cell proliferation, differentiation and migration (see section 2.3.3) (reviewed in Banumathy & Cairns 2010, van Kilsdonk et al. 2010). Several genetic and epigenetic mechanisms have been found that inactivate E-cadherin-mediated cell adhesion and promote tumour invasiveness and progression. The mechanisms that result in impaired E-cadherin functioning are reported to be DNA hypermethylation around the promoter region of the E-cadherin gene, mutations in E-cadherin and catenin genes, the transcriptional inactivation of E-cadherin, tyrosine phosphorylation of the E-cadherin/beta-catenin complex and the phosphorylation of beta-catenin. In addition, several proteases such as some matrix metalloproteases are capable of cleaving E-cadherin; the soluble E-cadherin fragments released inhibit E-cadherin functioning in a paracrine way. A cell membrane glycoprotein called dysadherin also down-regulates E-cadherin. (reviewed in Makrilia et al. 2009)
The disruption of E-cadherin-mediated cell-cell adhesion followed by a more invasive and aggressive phenotype has been established in many cancers of epithelial origin, such as squamous cell carcinomas of the head and neck and oesophagus and carcinomas of the bladder, breast, cervix, colon, endometrium, lung, pancreas, prostate and thyroid. In addition, decreased E-cadherin expression has been shown in precancerous conditions such as Barrett’s oesophagus and in the progression of dysplasia to adenocarcinoma. (Ilyas & Tomlinson 1997; reviewed in Chetty & Serra 2008, Makrilia et al. 2009) E-cadherin negativity is characteristic of invasive lobular breast carcinoma and diffuse-type gastric cancer (Gamallo et al. 1993, Becker et al. 1999).

In general, the loss of E-cadherin is associated with decreased differentiation, invasiveness and metastatic potential and higher mortality rates in cancer (Ilyas & Tomlinson 1997, reviewed in Makrilia et al. 2009). The relationship between decreased E-cadherin immunoexpression and unfavourable clinicopathological tumour features was previously established, for example in breast, colon and oesophageal carcinomas (Sommers et al. 1991, Doki et al. 1993, Dorudi et al. 1993). For example in bladder, prostate and oesophageal carcinomas decreased E-cadherin immunoexpression is a marker of poor patient prognosis (Giroldi et al. 1994, Tamura et al. 1996). However, the prognostic significance of the loss of E-cadherin is not independent of tumour stage and grade (Ilyas & Tomlinson 1997).

Inactivation of the VHL-HIF pathway is a critical step in the development of clear cell RCC (Gnarra et al. 1994, Kaelin 2004) and a direct association between VHL status and E-cadherin expression has been established in clear cell carcinoma (Esteban et al. 2006, Krishnamachary et al. 2006, Evans et al. 2007). It is believed that the loss of E-cadherin is an early pathogenic event in clear cell RCC (Esteban et al. 2006, Russell & Ohh 2007). The loss of VHL and the subsequent accumulation of HIF-1 reduce E-cadherin expression via the action of various transcriptional repressors (Krishnamachary et al. 2006, Evans et al. 2007). Hypermethylation of the E-cadherin gene was reported in 11% of primary RCCs (Dulaimi et al. 2004). The decreased expression of E-cadherin was established in several studies on RCC (Terpe et al. 1993, Katagiri et al. 1995, Shimazui et al. 1997, Kuroiwa et al. 2001) and the loss of E-cadherin immunoexpression was associated with advanced stage and decreased survival (Katagiri et al. 1995).
Beta-catenin

Beta-catenin (β-catenin) is a member of the regulatory cytoplasmic protein family called catenins and is a vertebrate homologue of the Armadillo protein in *Drosophila*. It is directly associated with the cytoplasmic domain of E-cadherin and forms a structural link between E-cadherin and the actin cytoskeleton via alpha-catenin. In addition to its essential role in cell adhesion as a component of the E-cadherin complex, beta-catenin is involved in intracellular signal transduction. (reviewed in Gumbiner 1995) It is believed that beta-catenin could be a connection between cell adhesion and mitogenic signalling (reviewed in Karim *et al.* 2004).

Beta-catenin is a part of the Wnt pathway (see section 2.3.3) and the accumulation of beta-catenin seems to play a central role in oncogenesis. Beta-catenin promotes the transcription of target genes and is involved in the regulation of cell proliferation, differentiation and migration, as well as the inhibition of apoptosis. (Behrens *et al.* 1996, reviewed in Karim *et al.* 2004) On the other hand, a lack of beta-catenin disrupts E-cadherin-mediated cell adhesion, which has also been implicated in carcinogenesis (reviewed in Makrilia *et al.* 2009). It is believed that the overexpression of beta-catenin is important in tumour initiation, whereas the loss of beta-catenin and the resulting loss of function of the E-cadherin-beta-catenin complex and impaired cellular adhesion are involved in tumour invasion. The cytoplasmic level of beta-catenin is regulated by Wnt and APC, where Wnt increases the expression of beta-catenin and, in turn, APC decreases it. (Ilyas & Tomlinson 1997) In addition, as E-cadherin binds beta-catenin it recruits it to the cell membrane and prevents its nuclear translocation (Orsulic *et al.* 1999).

Beta-catenin gene mutations and/or accumulation have been found in several malignancies such as colorectal, gastric, hepatocellular, prostate, oesophageal, and anaplastic thyroid carcinomas, as well as lung adenocarcinoma and malignant melanoma (reviewed in Karim *et al.* 2004). On the other hand, decreased levels of beta-catenin expression have also been reported in cancers such as colon, gastric and oesophageal carcinomas (reviewed in Takayama *et al.* 1996, Ilyas & Tomlinson 1997). The prognostic significance of beta-catenin is not similar in different cancers. For example, in colon and liver cancers increased levels of cytoplasmic and nuclear beta-catenin are associated with invasion and poor prognosis, whereas in melanoma they are related to a favourable outcome (Arozarena *et al.* 2011).
The involvement of the Wnt/beta-catenin pathway has been established in the pathogenesis of RCC and beta-catenin is regarded as one of the key molecules in the oncogenesis of RCC. In animal studies, the overexpression of beta-catenin was shown to induce renal tumours. (reviewed in Banumathy & Cairns 2010) Mutations in the beta-catenin gene are, however, rare in RCC and are only reported in clear cell variants. Instead, the accumulation of beta-catenin is a more frequent event which suggests that there are other mechanisms activating the Wnt signalling pathway in RCC. (Kim et al. 2000) For example, hypermethylation of the APC gene promoter, splicing isoforms of the transcriptional factor TCF-4 and the loss of various Wnt antagonists may regulate beta-catenin expression and Wnt signalling (Battagli et al. 2003, Shiina et al. 2003, Awakura et al. 2008, Hirata et al. 2009, Kawakami et al. 2009, Kojima et al. 2009). There are no APC tumour suppressor gene mutations in RCC (Kim et al. 2000). It has been found that VHL down-regulates beta-catenin by stabilizing a protein named Jade-1 (Chitalia et al. 2008). In addition, VHL represses the HGF-driven oncogenic signalling of beta-catenin (see section 2.3.5) (Peruzzi et al. 2006).


Myosin VI

Myosins are a large family of molecular motor proteins that hydrolyse adenosine-5’-triphosphate (ATP) and convert chemical energy into a mechanical force in order to move along actin filaments. Myosin VI is one of the so-called unconventional myosins and was first identified in Drosophila melanogaster. It moves in the reverse direction compared to other myosins, i.e. towards the pointed (-) end of actin. (Kellerman & Miller 1992, Wells et al. 1999) The movement of myosin VI is directed away from the plasma membrane into the cell and away from the surfaces of internal organelles such as the Golgi complex (Buss et al. 2001, Buss et al. 2004). Intracellularly, myosin VI is located in clathrin-coated endocytic vesicles, membrane ruffles and the Golgi complex (Buss et al. 2001, Roberts et al. 2004), but nuclear myosin VI expression has also been described (Vreugde et al. 2006). Myosin VI forms a complex with E-cadherin and beta-catenin and protects them from degradation. Similarly, E-
cadherin and beta-catenin preserve myosin VI expression. (Küssel-Andermann et al. 2000, Geisbrecht & Montell 2002)

Myosin VI has multiple roles that are connected to oncogenesis (Knudsen 2006, Sweeney & Houdusse 2007). By anchoring to the actin cytoskeleton, myosin VI maintains cell shape and rigidity (Millo et al. 2004). Myosin VI strengthens cellular adhesion as it provides tension between the E-cadherin-beta-catenin cell adhesion complex and the cytoskeleton. In addition, myosin VI fixes epithelial cells to the basement membrane and thus maintains the polarity of epithelial cell layers. (Küssel-Andermann et al. 2000, Geisbrecht & Montell 2002, Millo et al. 2004) By connecting to membrane proteins such as E-cadherin, myosin is able to produce the protrusion force needed in cell migration and is thus involved in cell motility (Geisbrecht & Montell 2002). Myosin VI has several transport roles, such as endocytosis and secretion (Buss et al. 2001, Warner et al. 2003); it carries cargo from the periphery towards the centre of the cell via clathrin-mediated endocytosis (Buss et al. 2001). Myosin VI-mediated membrane transport has a crucial role in cell division and the inhibition of myosin VI expression results in a defect in cytokinesis (Arden et al. 2007). In addition, myosin VI modulates gene expression and receptor clustering (Dunn et al. 2006, Vreugde et al. 2006, Sweeney & Houdusse 2007), and it may form a link between endocytosis and signalling pathways (Buss et al. 2001).

The overexpression of myosin VI has been demonstrated in human cancers such as ovarian and prostate carcinomas (Yoshida et al. 2004, Dunn et al. 2006, Loikkanen et al. 2009). Myosin VI expression is related to malignant properties such as cancer cell migration and dissemination, as well as to the aggressive clinical behaviour of tumours (Geisbrecht & Montell 2002, Yoshida et al. 2004, Dunn et al. 2006). In the normal kidney, myosin VI is highly expressed at the base of the brush border in proximal tubule cells (Biemesderfer et al. 2002).

2.6.3 Inflammation-related cancer and the immunologic nature of renal cell carcinoma

The immune system consists of innate and acquired (adaptive) immunity that protect the host from various foreign substances and damaging agents such as microbes and cancer cells (reviewed in Reddy & Reddy 2010). The first line of defence against infections is based on innate immunity, whereas the initiation of acquired immunity takes several days. Innate immunity is composed of phagocytic cells such as macrophages, neutrophils and dendritic cells, which
digest pathogens in a non-specific manner and present pathogen-derived antigens to T-lymphocytes. Acquired immunity consists of T- and B-lymphocytes, which are capable of recognizing specific peptide antigens through antigen receptors located on their surfaces. Acquired immunity is characterized by specificity and memory. However, innate immunity is also capable of recognizing pathogen-associated molecular patterns through Toll-like receptors (TLRs) on cell surfaces. (reviewed in Akira & Hemmi 2003, Wagner 2004) The B-cells are involved in the humoral immune response, which is based on secreted antibodies. Antigen-specific cytotoxic T-cells are capable of inducing apoptosis and account for cell-mediated immunity along with macrophages and natural killer (NK) cells. (reviewed in Reddy & Reddy 2010) In addition, cells of the immune system, like helper T-cells, can secrete various immunomodulating agents such as interferons (IFNs), interleukins (ILs) and tumour necrosis factor alpha (TNF-α), which may be involved in several processes in the body (reviewed in Akira et al. 2001).

The relationship between inflammation and cancer development has been recognized for years, but the exact mechanisms by which inflammation results in carcinogenesis are not fully known. It is known that chronic inflammation causes a repeated cycle of genomic damage and healing with accelerated cell proliferation that promotes both malignant transformation and clonal expansion of transformed cells. (reviewed in Farrow & Evers 2002) The peritumoural inflammation observed could also indicate a relationship between renal carcinogenesis and inflammation (Tuna et al. 2004).

Malignant tumours repress immunosurveillance as they produce immunosuppressive mediators by themselves or they cause surrounding inflammatory cells to release them (Huang et al. 1998). In RCC, the apoptosis of T-cells is induced, which suppresses host antitumour immunity and thus promotes tumorigenesis (Uzzo et al. 1999, Rayman et al. 2004). On the other hand, inflammatory activation is associated with a more favourable clinical outcome in some cancers such as melanoma (Hernberg et al. 1997). Bacterial DNA purified from Bacillus Calmette-Guerin (BCG) stimulates NK cells and IFN production, which have antitumour activity in cancer (Tokunaga et al. 1984).

Renal cell carcinoma is generally considered as one of the immunogenic malignancies. Immunotherapy can produce significant responses in advanced RCC (Vogelzang et al. 1992) and tumour vaccines have been shown to have some benefits as well (Finley et al. 2011). Rare cases of the spontaneous regression of RCC metastases are presumably caused by immunological mechanisms (Lokich 1997). Renal cell carcinomas attract several kinds of immune cells (Geiger et al. 1992).
2009) and various tumour-associated antigens have been detected that arouse tumour-specific T-cell-defined immune responses (Neumann et al. 1998). Immunological parameters such as the amount of peripheral blood and intratumoural neutrophils and other immune cells, as well as serum cytokine levels, have been shown to be related to the outcome of RCC patients (Kallio et al. 2001, Donskov & von der Maase 2006).

Cyclooxygenase-2

Cyclooxygenase (COX, prostaglandin endoperoxidase H2 synthase, PGHS) is a key enzyme in the synthesis of prostaglandins (PGs) from arachidonic acid (DeWitt & Smith 1988). The activity of COX can be inhibited by non-steroidal anti-inflammatory drugs (NSAIDs) (Vane 1971). There are two isoforms of COX, COX-1 and COX-2, which share a similar structure and catalytic activity but differ in expression pattern and substrate and inhibitor selectivity (reviewed in Smith et al. 1996, Vane et al. 1998). Cyclooxygenase-1 is permanently expressed in almost all cell types; it catalyses the synthesis of protective PGs involved in shielding gastric and intestinal mucosa and in maintaining normal renal function in compromised kidneys. In platelets, the inhibition of COX-1 results in the loss of aggregation. (reviewed in Vane et al. 1998)

Cyclooxygenase-2 expression is not observed in most mammalian tissues under normal conditions. However, various stimuli including inflammatory factors such as bacterial lipopolysaccharides and several cytokines, hormones, growth factors, oncogenes and tumour promoters induce COX-2 expression within hours. (Sheng et al. 1998; reviewed in Smith et al. 1996, Dubois et al. 1998, Vane et al. 1998) The induction of COX-2 can be decreased by anti-inflammatory cytokines, corticosteroids and NSAIDs (reviewed in Vane et al. 1998). Cyclooxygenase-2 participates in numerous important physiological and pathophysiological conditions such as inflammation, cell growth control, nerve transmission (especially pain and fever), neural development and adaptation, reproduction, bone metabolism and wound healing (reviewed in Dubois et al. 1998, Vane et al. 1998). The side effects of COX-2 inhibition include cardiovascular toxicity such as stroke, myocardial infarction and other thromboembolic events (reviewed in Menter et al. 2010).

The relationship between COX-2 and carcinogenesis was originally observed in epidemiological studies where aspirin use was associated with a reduced risk in colorectal carcinoma (Thun et al. 1991). Up-regulation of COX-2 gene expression
was later established in colorectal carcinoma specimens (Eberhart et al. 1994). There are several mechanisms via which COX-2 can promote carcinogenesis. Indeed, COX-2 has also been shown to induce tumorigenic transformation in transgenic mice (Liu et al. 2001). It catalyzes the conversion of pro-carcinogens to proximate carcinogens (Eling et al. 1990) and enhances cell migration and adhesion to the extracellular matrix, thus promoting invasion and metastasis (Tsujii & DuBois 1995, Tsujii et al. 1997, Cao & Prescott 2002). Cyclooxygenase-2 expression favors cancer cell growth by stimulating proliferation (Sheng et al. 1998) and inducing tumour neoangiogenesis (Tsujii et al. 1998, Masferrer et al. 2000). Cyclooxygenase-2 overexpression also inhibits apoptosis (Tsujii & DuBois 1995). It is assumed that COX-2 has an important role in inflammation-induced carcinogenesis (Farrow & Evers 2002) and, in addition, that it suppresses antitumour immunity (Huang et al. 1998, Williams et al. 1999).

The up-regulation of COX-2 has been reported in several malignomas, including breast, colorectal, gastric and also renal cell carcinoma (Eberhart et al. 1994, Subbaramaiah et al. 1996, Ristimäki et al. 1997, Miyata et al. 2003). It is associated with a poor clinical outcome in various cancers such as breast, colorectal and oesophageal carcinomas (Sheehan et al. 1999, Buskens et al. 2002, Ristimäki et al. 2002). In RCC, COX-2 is involved in angiogenesis as well as invasiveness of the tumour (Chen et al. 2004a, Chen et al. 2004b).

**HuR**

The HuR protein (ELAVL1, HuA) is a member of the RNA binding protein family ELAV (embryonic lethal abnormal vision), which is involved in diverse physiological processes (Ma et al. 1996, reviewed in Hinman & Lou 2008). Gene expression is regulated at the DNA and protein level and, in addition, it can be altered via the modulation of messenger RNA (mRNA) stability and translation (reviewed in Brennan & Steitz 2001). The HuR protein stabilizes mRNA by binding to the adenylate and uridylate residues in the 3’ untranslated region (3’-UTR), which are called AU-rich elements (AREs) (Ma et al. 1996, Fan & Steitz 1998). Furthermore, HuR is predominantly a nuclear protein that binds to ARE-containing mRNAs in the nucleus and escorts them into the cytoplasm where it protects them from degradation (Fan & Steitz 1998). Through its target mRNAs, HuR has been linked to various physiological functions including cell growth, differentiation and proliferation, as well as oncogenesis (Peng et al. 1998, López de Silanes et al. 2003, reviewed in Brennan & Steitz 2001).
The HuR protein regulates COX-2 expression by stabilizing COX-2 mRNA (Sengupta et al. 2003), which has been found in several types of carcinomas including breast, colon, gastric and ovarian carcinomas (Dixon et al. 2001, Erkinheimo et al. 2003, Denkert et al. 2004b, Mrena et al. 2005). In addition to COX-2, HuR has other target mRNAs that are involved in carcinogenesis and, for example, inhibit apoptosis and promote cell proliferation, invasion, metastasis and angiogenesis (Levy et al. 1998, Wang et al. 2000, reviewed in López de Silanes et al. 2005). The HuR protein may also induce the expression of immunosuppressive cytokines, which might help the tumour to avoid detection via immune surveillance (Nabors et al. 2001, reviewed in López de Silanes et al. 2005).

In normal tissue, the expression pattern of HuR is weak or moderate and primarily nuclear, whereas up-regulated and cytoplasmic HuR expression is detected in virtually all cancer tissues, including RCC (López de Silanes et al. 2003). Mutations of the HuR gene have never been detected in cancer but there are other mechanisms that increase HuR expression, such low levels of 5-adenosine monophosphate-activated protein kinase (AMPK) activity (Wang et al. 2002, reviewed in López de Silanes et al. 2005). The association between cytoplasmic HuR expression and adverse clinicopathological features of malignant tumours such as higher grades and advanced stages has been reported in several carcinomas such as breast, colon and ovarian carcinomas (Erkinheimo et al. 2003 Denkert et al. 2004b, Heinonen et al. 2005, Denkert et al. 2006). The immunoexpression of HuR predicts a poor outcome, for example in breast, gastric and ovarian carcinomas (Erkinheimo et al. 2003, Heinonen et al. 2005, Mrena et al. 2005). Moreover, the up-regulation of HuR has been found to accelerate tumour growth in colon carcinoma animal models (López de Silanes et al. 2005).

Toll-like receptor 9

The Toll-like receptors (TLRs) are a family of recognition proteins that detect both microbial and host-derived molecular patterns and introduce them to the immune system (reviewed in Akira & Hemmi 2003, Wagner 2004). At least 13 different mammalian TLRs, each responding to a different ligand, have been identified (Akira & Hemmi 2003, Shi et al. 2011). Based on amino acid sequence similarities, the TLR family members are divided into different subfamilies. The members of the TLR9 subfamily (TLR7, TLR8 and TLR9) recognize viral and bacterial RNA and DNA. (reviewed in Wagner 2004) In addition, host RNA,
DNA and RNA- or DNA-associated proteins can stimulate TLRs and result in the development of systemic autoimmune disease (Lamphier et al. 2006, Marshak-Rothstein & Rifkin 2007). The subcellular expression sites of the different TLRs vary. Some of them are expressed and bind their ligands on the cell surface (Nishiya & DeFranco 2004), while the TLR9 subfamily resides in the intracellular vesicles (Leifer et al. 2004). Ligand binding to TLRs launches signalling pathways, resulting in an immune reaction (reviewed in Akira & Hemmi 2003).

The TLR9 protein is most abundantly expressed in B-cells and plasmacytoid dendritic cells of the immune system in humans (reviewed in Wagner 2004). In addition to the immune system, the expression of TLR9 has been shown, for example, in normal epithelial cells of the gastric mucosa (Schmausser et al. 2004), uterus (Schaefer et al. 2004) and respiratory epithelium (Platz et al. 2004), as well as in keratinocytes (Mempel et al. 2003). In the normal kidney, TLR9 expression has been detected in renal tubules and interstitial tissue. The glomerular expression of TLR9 has been reported in autoimmune renal disease, such as in lupus nephritis. (Papadimitraki et al. 2009)

The expression of TLR9 has also been observed in several cancers such as breast, gastric, lung, ovarian and prostate carcinomas (Droemann et al. 2005, Schmausser et al. 2005, Merrell et al. 2006, Ilvesaro et al. 2007, Berger et al. 2010). An association between TLR9 expression and poor differentiation of the tumour has been described in breast, ovarian and prostate carcinomas, and also in glioma (Jukkola-Vuorinen et al. 2008, Berger et al. 2010, Väisänen et al. 2010, Wang et al. 2010). Stimulation of TLR9 increases the invasiveness of tumours in several malignomas such as in breast, lung and prostate carcinomas and in glioma (Merrell et al. 2006, Ilvesaro et al. 2007, Ren et al. 2007, Wang et al. 2010). In breast carcinoma and glioma, TLR9 expression is correlated with an unfavourable clinical outcome (Jukkola-Vuorinen et al. 2008, Wang et al. 2010). The molecular mechanism via which TLR9 expression influences tumour behaviour is not fully understood, but there is evidence linking TLRs to cell migration, invasion and metastasis (Anderson et al. 1985, Kelleher et al. 2006, Tomchuck et al. 2008). It has been suggested that TLR9 expression might be a part of the epithelial-to-mesenchymal transition (EMT) and that TLR9 is involved in metastasis rather than participating in oncogenesis itself (Jukkola-Vuorinen et al. 2008).
2.6.4 Neuroendocrine activity in cancer

In addition to specific neuroendocrine cells and neuroendocrine tumours, neuroendocrine differentiation, characterized by the expression of scattered clusters of differentiated neuroendocrine cells, the presence of secretory granules and the production of peptide hormones or the presence of immunoreactivity for certain neuroendocrine markers, can be detected in multiple organs and malignomas throughout the body (reviewed in Edgren et al. 1996, Abrahamsson 1999, Erickson & Lloyd 2004). Rare neuroendocrine tumours of the kidney include carcinoid, large cell neuroendocrine carcinoma, primitive neuroectodermal tumour, neuroblastoma, phaeochromocytoma, small cell carcinoma and mucinous tubular and spindle cell carcinoma of the kidney (Eble et al. 2004, Kuroda et al. 2006, Lane et al. 2007). Neuroendocrine activity is also expressed in normal renal cells (Edgren et al. 1996).

Neuroendocrine differentiation has been observed in several types of cancer such as breast, colorectal, lung and prostate carcinomas, as well as in RCC (Abrahamsson et al. 1987, Gazdar et al. 1988, Nesland et al. 1988, Westlin et al. 1995). In prostate carcinoma, neuroendocrine differentiation is a common feature that occurs in 30–100% of tumours, and it is possibly a part of the oncogenic process (Abrahamsson et al. 1987, Abrahamsson 1999, Bostwick et al. 2002). As the clinical behaviour of specific neuroendocrine tumours varies from aggressive to indolent (Viallet & Ihde 1991, Dahabreh et al. 2009), the evidence of the prognostic influence of neuroendocrine differentiation in cancer is contradictory, too. However, it has been found that neuroendocrine products may stimulate tumour growth and cell proliferation in an autocrine-paracrine fashion (Abrahamsson 1999).

Serotonin

Serotonin (5-hydroxytryptamine, 5HT) is a monoamine neurotransmitter derived from the amino acid tryptophan. It mediates diverse physiological actions in the human body by binding to multiple receptor subtypes. (Zifa & Fillion 1992, reviewed in Abrahamsson 1999) Serotonin is implicated in psychiatric and neurological disorders, but it has also growth factor activity and is involved in differentiation, cell proliferation and gene expression. In tumorigenesis, serotonin has been connected to oncogenes, malignant transformation and tumour growth. (Julius et al. 1989; reviewed in Vicaut et al. 2000, Siddiqui et al. 2005)
tumour growth promoting effect of serotonin has been established in several types of cancer such as lung, pancreatic and prostate carcinoma. On the other hand, serotonin may inhibit tumour growth through its vasoconstrictive effect on tumour vasculature. (reviewed in Vicaut et al. 2000) So far, there is no clear evidence showing that serotonin expression is a prognostic factor in cancer. For example, in prostate carcinoma, both increased and decreased immunoreactivity have been associated with advanced and aggressive disease (Bostwick et al. 2002, Dizeyi et al. 2004).

CD56

Cell adhesion molecules play an important role in tumorigenesis and the development of metastatic disease (see section 2.6.2) (reviewed in Freemont & Hoyland 1996, Meyer & Hart 1998). The neural cell adhesion molecule (NCAM, CD56) is a member of the immunoglobulin superfamily of cell adhesion molecules (Jin et al. 1991). It mediates both cell-cell and cell-substrate interactions via homophilic and heterophilic mechanisms (reviewed in Abbate et al. 1999, Daniel et al. 2001) and is a rather sensitive marker of neuroendocrine differentiation. It is present in the central nervous system and adrenal gland and in most neuroendocrine cells and tumours, whereas most non-endocrine cells and tumours do not express CD56. (Jin et al. 1991, Lahr et al. 1993) The expression of CD56 has been detected in neuroblastomas, brain tumours, embryonal rhabdomyosarcomas and various other sarcomas, neuroendocrine tumours and subsets of carcinomas such as lung and ovarian carcinomas, and in RCC (Garin-Chesa et al. 1991, Terpe et al. 1993). The polysialated isoforms of CD56 are involved in tumour invasion and metastasis (Daniel et al. 2000, Daniel et al. 2001).

Neurone-specific enolase

Enolases are cytoplasmic glycolytic enzymes consisting of three immunologically distinct subunits α, β and γ, where subunit γ is called neurone-specific enolase (NSE, gamma-enolase) (Fletcher et al. 1976, Schmechel et al. 1978, Ariyoshi et al. 1983). Neurone-specific enolase is a broad-spectrum, non-specific neuroendocrine marker expressed in addition to neurons and neuroendocrine cells in various non-endocrine cells such as myoepithelial cells and lymphocytes (reviewed in Erickson & Lloyd 2004). In the normal kidney, NSE is expressed in
macula densa and in epithelial cells of loops of Henle and collecting ducts, but not in proximal tubules. Both the up-regulation of NSE immunoexpression and elevated serum NSE levels have been shown in RCC. (Haimoto et al. 1986) The immunoexpression of NSE has also been observed in other cancers of non-neuroendocrine origin, such as in breast, colorectal, lung and oesophageal carcinomas (Ariyoshi et al. 1983, Vinores et al. 1984). The activity of glycolytic enzymes such as NSE presumably reflects enhanced anaerobic glycolysis of the malignant tumour. Serum NSE is regarded as a useful tumour marker for indicating the extent of disease (Ariyoshi et al. 1983, Haimoto et al. 1986). In RCC, increased serum levels of NSE are associated with advanced disease and decreased survival (Takashi et al. 1989, Rasmuson et al. 1993). In contrast, in breast carcinoma, the immunoexpression of NSE is related to improved survival (Makretsov et al. 2003).

**Chromogranin A**

Chromogranins are a class of secretory proteins that reside in the secretory granules of endocrine, neuroendocrine and neuronal cells (reviewed in Montero-Hadjadje et al. 2008). The best characterized member of the granin family, chromogranin A, was originally identified as a major soluble protein in adrenal medullary chromaffin granules (Blaschko et al. 1967). Chromogranin A is important in secretory granule formation and is thus needed in the packaging, processing and release of a variety of hormones, neurotransmitters and peptides in response to physiological and pharmacological stimuli. It is a precursor to several bioactive peptides that have diverse autocrine, paracrine and endocrine activities. Chromogranin A and its peptides play multiple roles in the endocrine, cardiovascular and nervous systems and they are implicated in cardiovascular and neurodegenerative diseases. In addition, chromogranin A has apoptosis modulating activity. (reviewed in Taupenot et al. 2003, Montero-Hadjadje et al. 2008)

Chromogranin A immunoexpression has been demonstrated in several non-endocrine tumours, including breast, colorectal, gastric, lung and prostate carcinomas (Abrahamsson et al. 1989, Pagani et al. 1990, Skov et al. 1991, Park et al. 1992). Increased serum levels of chromogranin A have been reported in patients with colorectal, prostate and renal cell carcinoma (Kadmon et al. 1991, Syversen et al. 1995, Rasmuson et al. 1999). Chromogranin A is regarded as a potential tumour marker for several types of neoplasms (reviewed in Taupenot et
al. 2003). Although the prognostic significance of chromogranin A expression remains unclear in cancer, a tendency towards shorter survival has been reported in colorectal carcinoma (Syversen et al. 1995, reviewed in Taupenot et al. 2003).

**Synaptophysin**

Neurons, as well as neuroendocrine and endocrine cells, contain small presynaptic secretory vesicles that store and release low molecular weight components such as classic neurotransmitters. The expression of a protein called synaptophysin at the cytoplasmic site of the presynaptic vesicle membrane is characteristic of these small neurosecretory vesicles. (reviewed in Wiedenmann & Huttner 1989) Synaptophysin is a calcium-binding transmembrane glycoprotein involved in forming the transmembrane channels needed in the accumulation and release of vesicular contents (reviewed in Wiedenmann & Huttner 1989, Erickson & Lloyd 2004). It is a marker of nerve terminals and a broad-spectrum marker of neuroendocrine differentiation (reviewed in Erickson & Lloyd 2004). In addition to neurons and many endocrine and neuroendocrine cells, the immunoeexpression of synaptophysin has been reported in various cancers, for example in breast, endometrial and lung carcinomas, with no significant association with patient survival (Kayser et al. 1988, Makretsov et al. 2003, Taraif et al. 2009; reviewed in Wiedenmann & Huttner 1989, Erickson & Lloyd 2004).
3 Aims of the study

The purpose of this thesis was to screen renal cell carcinoma tumours with different immunohistochemical biomarkers to find novel prognostic tissue markers for RCC. Specifically, the aims of the study were:

1. to investigate the expression of markers of the oxidative system, including Keap1, Nfr2 and Trx, in RCC tumours and their correlation with clinicopathological parameters such as TNM classification and nuclear grade and survival in RCC;

2. to evaluate the expression of myosin VI and its prognostic significance in RCC and to discover how myosin VI expression relates to the expression of E-cadherin and beta-catenin and clinicopathological features in RCC;

3. to study the expression and prognostic role of the mRNA stability factor HuR in RCC and to investigate the presence of HuR-regulated COX-2 expression in RCC;

4. to explore the expression of TLR9 in RCC and to determine whether or not it is correlated with patient RCC-specific survival as well as with TNM stage and nuclear grade of RCC tumours;

5. to discover the expression of neuroendocrine markers in RCC and their influence on RCC-specific patient survival.
4 Materials and methods

4.1 Study cohort

The retrospective study population was based on a cohort of 189 patients who underwent renal surgery for renal tumours at the Department of Surgery, Oulu University Hospital, Finland, between 1990 and 1999, and who were identified from the registry of the Department of Pathology, Oulu University Hospital. On the basis of histologically verified RCC and the availability of representative histological materials and follow-up details, 152 patients were included in the study.

4.2 Clinical data and follow-up

All relevant clinical data and follow-up details were collected from patient records and evaluated along with information from the Finnish Cancer Registry and the Population Register Centre of Finland. All information used was re-evaluated. The presence of chronic diseases, including hypertension and other cardiovascular diseases, the use of antihypertensive medication and smoking and RCC-related symptoms were recorded. Because of the retrospective nature of the study, some information, for example alcohol use, which was not documented, was not available. Performance status could not be retrospectively assessed based on the documentation. The preoperative laboratory findings and radiological evaluation, as well as information regarding surgical treatment and peri- and postoperative complications, were carefully recorded. Adjuvant oncologic treatment was also recorded. Follow-up details were meticulously collected from each patient as required from separate hospitals and health care centres all over Finland.

The exact stage was recorded according to the TNM classification of RCCs 2002 (Sobin & Wittekind 2002) (Figure 1), which was the version in clinical use in 2006 when the study began. In order to simplify the statistical analyses the TNM stages were further grouped into stages I-IV (Figure 1). The lymph node status was determined via pathological evaluations of resected lymph nodes and was classified N0 when no lymph node metastases were histologically verified. In a case of histologically verified lymph node metastasis, the tumour was staged N1 when there was only one positive regional lymph node or N2 when there were
more than one regional lymph node metastases, even if the number of lymph nodes analysed was not documented or if fewer than eight lymph nodes were analysed which is the minimum number required for TNM classification.

4.3 Tumour samples

All of the tumour samples were specimens from nephrectomy or partial resection. The tumour samples were routinely fixed in 10% buffered formalin and embedded in paraffin. The histological diagnosis was confirmed by reviewing the original haematoxylin and eosin (H & E)-stained sections by two histopathologists (Pasi Hirvikoski, M.D., Ph.D. and Saila Kauppila M.D., Ph.D.). The tumours were re-classified and graded according to the current WHO classification (Eble et al. 2004). Microscopic venous invasion (MVI), tumour necrosis, sarcomatoid features and cystic structure were also assessed.

4.4 Multi-tissue blocks

The most representative tumour region of each RCC tumour was defined and marked in the H & E-stained sections. The regions marked were punched from the original blocks and re-cast in multitissue blocks. Sections of 3 μm thick were cut from these paraffin-embedded blocks and mounted onto pre-coated slides (tissue microarray technique, TMA) (Merseburger et al. 2006).

4.5 Immunohistochemistry

Tissue sections were deparaffinized in xylene, rehydrated in a descending ethanol series and washed in phosphate buffered saline (PBS). In order to enhance immunoreactivity the tissue sections were boiled in citrate buffer (pH 6.0) or in Tris-EDTA buffer (pH 9.0), after which they were cooled for 20 min in a buffer. Endogenous peroxidase activity was blocked by incubation in methanol-containing hydrogen peroxidase. The sections were incubated with specific antibodies and the antibody reaction was visualized using commercial kits. The details of the antibodies, specific pretreatments and visualization methods used are listed in Table 1. All steps in the procedure were carried out at room temperature. The sections were washed with PBS in between each step in the staining procedure.
Table 1. Details of the immunohistochemical procedure.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Antibody; Manufacturer</th>
<th>Dilution</th>
<th>Pretreatment</th>
<th>Visualization; Chromogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nfr2</td>
<td>Rabbit polyclonal antibody, Nfr2 C-20: sc-722; Santa Cruz Biotechnology, Inc.</td>
<td>1/100</td>
<td>Citrate(^1)</td>
<td>Histostain Plus, Invitrogen Life Technologies; AEC(^4)</td>
</tr>
<tr>
<td>Keap1</td>
<td>Goat polyclonal IgG antibody, Keap-T E-20 SC-15246; Santa Cruz Biotechnology, Inc.</td>
<td>1/100</td>
<td>Citrate(^1)</td>
<td>Histostain Plus, Invitrogen Life Technologies; AEC(^4)</td>
</tr>
<tr>
<td>Trx</td>
<td>Goat anti-human IgG antibody, popuI No 705; American Diagnostica, Inc.</td>
<td>1/200</td>
<td>Citrate(^1)</td>
<td>Histostain Plus, Invitrogen Life Technologies; AEC(^4)</td>
</tr>
<tr>
<td>Myosin VI</td>
<td>Monoclonal anti-myosin VI; Sigma</td>
<td>1/250</td>
<td>Citrate(^1), TBS(^3)</td>
<td>EnVision, DakoCytomation; DAB(^5)</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Mouse monoclonal anti-E-cadherin; Zymed Laboratories</td>
<td>1/300</td>
<td>Citrate(^1), TBS(^3)</td>
<td>EnVision, DakoCytomation; DAB(^5)</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Monoclonal anti-beta-catenin; BD Biosciences</td>
<td>1/200</td>
<td>Citrate(^1), TBS(^3)</td>
<td>EnVision, DakoCytomation; DAB(^5)</td>
</tr>
<tr>
<td>HuR</td>
<td>Mouse monoclonal antibody HuR, 19F12, sc-56709; Santa Cruz Biotechnology, Inc.</td>
<td>1/1500</td>
<td>Tris-EDTA(^5)</td>
<td>EnVision, DakoCytomation; DAB(^5)</td>
</tr>
<tr>
<td>COX-2</td>
<td>Mouse monoclonal antibody COX-2; Cayman Chemical</td>
<td>1/200</td>
<td>Citrate(^1)</td>
<td>EnVision, DakoCytomation; DAB(^5)</td>
</tr>
<tr>
<td>TLR9</td>
<td>Mouse monoclonal anti-human TLR9/CD289, Img-305A, clone 26C593.2; Ingenex</td>
<td>1/200</td>
<td>Citrate(^1)</td>
<td>EnVision, DakoCytomation; DAB(^5)</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Mouse monoclonal anti-human serotonin; DakoCytomation</td>
<td>1/200</td>
<td>Citrate(^1)</td>
<td>UltraVision, Thermo Fisher Scientific; DAB(^5)</td>
</tr>
<tr>
<td>CD56</td>
<td>Mouse lyophilized monoclonal antibody for CD56; Novocastra Laboratories Ltd.</td>
<td>1/200</td>
<td>Citrate(^1)</td>
<td>UltraVision, Thermo Fisher Scientific; DAB(^5)</td>
</tr>
<tr>
<td>Marker</td>
<td>Antibody; Manufacturer</td>
<td>Dilution</td>
<td>Pretreatment</td>
<td>Visualization; Chromogen</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------------------------</td>
<td>----------</td>
<td>--------------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>NSE</td>
<td>Mouse monoclonal anti-NSE; Zymed Laboratories</td>
<td>1/1000</td>
<td>None</td>
<td>UltraVision, Thermo Fisher Scientific; DAB³</td>
</tr>
<tr>
<td>Chromogranin A</td>
<td>Rabbit polyclonal anti-chromogranin A; Zymed Laboratories</td>
<td>1/500</td>
<td>Tris-EDTA²</td>
<td>EnVision, DakoCytomation; DAB³</td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>Mouse monoclonal anti-synaptophysin; DakoCytomation</td>
<td>1/50</td>
<td>Tris-EDTA²</td>
<td>EnVision, DakoCytomation; DAB³</td>
</tr>
</tbody>
</table>

1 Boiled in citrate buffer (pH 6.0) in a microwave oven for 2 min at 800 W + 10 min at 300 W
2 Boiled in Tris-EDTA buffer (pH 9.0) in a microwave oven for 2 min at 800 W + 15 min at 300 W
3 Washed with Tris-buffered saline (TBS) (pH 7.5)
³3–3’-diaminobenzidine

### 4.6 Evaluation of immunohistochemical staining

In addition to internal controls, additional positive controls were used when appropriate. Sections that were stained without primary antibodies served as negative controls. Immunoreactivity was simultaneously assessed by two to four interpreters who had no preliminary knowledge of any clinical data and a consensus was reached.

#### 4.6.1 Nfr2 (I)

The immunostaining of Nfr2 was observed in both the nucleus and the cytoplasm. Immunoreactivity was classified into four categories representing subgroups of negative, weakly positive, moderately positive and strongly positive staining reactions. The limits for these categories were 0%, < 20% positive, 20–40% positive and > 40% positive for nuclear Nfr2 and 0%, < 20% positive, 20–50% positive and > 50% positive for cytoplasmic Nfr2.

#### 4.6.2 Keap1 (I)

Immunoreactivity for Keap1 was detected in the cytoplasm. Immunostaining for Keap1 was primarily scored into four subgroups based on the intensity of staining: negative (0), weakly positive (< 10%), moderately positive (10–40%) and
strongly positive (> 40%). In a further statistical analysis the positive subgroups were combined and compared to the negative subgroup.

4.6.3 Thioredoxin (I)

Immunostaining for nuclear and cytoplasmic Trx was classified into four groups according to the staining reaction: negative (0), weakly positive (< 25%), moderately positive (25–75%) and strongly positive (> 75%). Statistical analyses were performed between positive and negative cases for cytoplasmic Trx.

4.6.4 Myosin VI (II)

Immunoreactivity for myosin VI was observed in the nucleus and cytoplasm. Nuclear myosin VI immunoreactivity was classified as positive when at least one stained nucleus was detected and negative when no stained nuclei were detected. Immunostaining for cytoplasmic myosin VI was initially scored as negative, weakly positive, moderately positive and strongly positive to represent the intensity of staining. In a further statistical analysis the negative and weakly positive subgroups were combined and re-classified as negative and compared to the subgroups of moderately and strongly positive, which were combined and re-classified as positive.

4.6.5 E-cadherin (II)

Immunoreactivity for E-cadherin was detected in the nucleus and membranes. Immunostaining for nuclear E-cadherin was classified as positive when staining was detected and negative if no staining was detected. Immunostaining for membranous E-cadherin was primarily categorized as negative, weakly positive, moderately positive and strongly positive based on the intensity of the staining reaction. In the final analyses, immunostaining for membranous E-cadherin was re-classified as negative (negative and weakly positive combined) or positive (moderate and strongly positive combined).
4.6.6 Beta-catenin (II)

Immunoreactivity for beta-catenin was observed in the nucleus and cytoplasm and classified as positive when staining was detected or negative when no staining was detected.

4.6.7 HuR (III)

Immunoreactivity for HuR was detected in the nucleus and cytoplasm. Nuclear immunoreactivity was scored as either negative or positive. Cytoplasmic HuR immunoreactivity was initially scored depending on the cytoplasmic intensity of staining: negative (0), weakly positive (1) or strongly positive (2). For further statistical analyses the negative samples (score 0) were compared to the positive samples (scores 1 and 2).

4.6.8 Cyclooxygenase-2 (III)

Cytoplasmic COX-2 immunoreactivity was assessed by grading the intensity of staining from 0 to 3 (absent, weakly diffuse, moderately granular, strong granular or diffuse, respectively). In the statistical analysis, the COX-2 grades were divided into two subgroups, i.e. negative (scores 0–1) and positive (scores 2–3).

4.6.9 Toll-like receptor 9 (IV)

Immunoreactivity of TLR9 was detected in both the nucleus and cytoplasm. Nuclear immunostaining was assessed as negative or positive. Cytoplasmic TLR9 immunoreactivity was initially scored according to four cytoplasmic staining intensities: negative (0), weakly positive (1), moderately positive (2) or strongly positive (3). For further statistical analyses, the negative samples (score 0) were compared with the positive ones (scores 1 to 3).

4.6.10 Neuroendocrine markers (V)

Cytoplasmic immunostaining for serotonin, CD56, NSE, chromogranin A and synaptophysin was classified as positive when any staining was detected or negative when no staining was detected.
4.7 Cell culture and RNA interference (III)

Human RCC cells (769-P, ATCC CRL-1933) were cultured in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 10% foetal calf serum (FCS) (Sigma-Aldrich), 2 mmol/l L-glutamine and antibiotics (Sigma-Aldrich) and maintained at 37 °C and 5% carbon dioxide (CO₂) in air. The small interfering RNA (siRNA) duplexes were synthesized by Dharmacon, Inc. and the sequences for HuR were: sense 5′-AAC AUG ACC GAU GAG UUA dTdT-3′ and antisense 5′-UAA CUC AUC CUG GGU CAU GUU dTdT-3′. The ON-TARGETplus Non-targeting Pool (D-001810-10, Dharmacon) was used as a control for the siRNA experiments. The day before transfection, 769-P cells were trypsinized and diluted at 1:20 with optiMEM 1-medium (Life Technologies, Inc.) supplemented with 10% FCS without antibiotics and transferred to 24-well plates at 1 ml per well with a final split ratio of 1:4. Transient transfection of the siRNAs was carried out using Lipofectamine reagent (Invitrogen Life Technologies), following the manufacturer’s instructions. The final siRNA concentrations were 80 nmol/l.

4.8 Immunoblotting (III)

Proteins from the cells were isolated 78 h after transfection using a radioimmunoprecipitation assay (RIPA) buffer containing 25 mM Tris-hydrochloride (Tris-HCl) (pH=7.6), 150 mM sodium chloride (NaCl), 1% nonyl phenoxypolyethoxylethanol-40 (NP-40), 1% sodium deoxycholate and 0.1% sodium dodecyl sulphate (SDS). After boiling the samples in a reducing SDS sample buffer for 5 min, 20 µg of protein was loaded per lane and the samples were separated by 10% SDS-PAGE. Proteins from the SDS gel were transferred to a polyvinylidene fluoride (PVDF) membrane for HuR (sc-56709, Santa Cruz Biotechnology), COX-2 (sc-1745, Santa Cruz Biotechnology) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (SM7116P, Acris Antibodies GmbH) detection. Binding of target proteins on the membranes was revealed with goat anti-mouse or donkey anti-goat horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology). The protein bands were visualized by chemiluminescence.
4.9 Statistical analyses

The software package SPSS for Windows 15 was used for the statistical analysis. Associations between factors, including clinicopathological variables and the immunostaining pattern, were assessed using the chi-square test ($\chi^2$ test) or Fisher’s exact test in the case of low expected frequencies. Survival analyses were performed for the whole study population and also separately for each histological subtype. Survival rates were calculated using the Kaplan-Meier method and statistical significance between the groups was analysed using the log-rank test. The hazard ratio (HR) was assessed using a Cox regression model and univariate analysis. The RCC-specific survival was calculated from the date of diagnosis to death due to RCC or to the last day of follow-up. Deaths due to intercurrent causes were not included in the analysis. Multivariate survival analysis was performed using the Cox proportional hazards model with the covariates age, stage, grade and immunoreactivity for each biomarker tested. All of the p-values were two-sided.

4.10 Ethics

The research plan was approved by the Ethics committee of the Northern Ostrobothnia Hospital District (EETTMK:16/2005). Approval for the study was sought from the Finnish Ministry of Social Affairs and Health (STM/1117/2005) in order to obtain data from the Finnish Cancer Registry. The use of pathological archive material was licenced by the National Authority for Medicolegal Affairs (at the present time the National Supervisory Authority for Welfare and Health) (Permission No. 1441/32/300/05).
5 Results

5.1 Characteristics, treatment and follow-up of the patients

The mean and median age of the patients were 62 (standard deviation (SD) 11) and 63 (range 29–86) years, respectively. There was a slight predominance of females in the study cohort with 77 (51%) females and 75 (49%) males. Elevated blood pressure, the use of antihypertensive medication or a documented hypertension diagnosis was observed in 73 (48%) of the patients. There were 32 (21%) smokers in the study cohort but the smoking anamnesis was missing in 18 (12%) cases.

Approximately one half (77 tumours, 51%) of the tumours had been found incidentally. A preoperative abdominal US had been performed for each patient and, in addition, an abdominal CT was performed for 125 patients (82%). Preoperative chest radiography (X-ray and/or CT) was performed for 135 patients (89%). In the case of suspected metastases or vena caval involvement, additional preoperative studies such as bone scintigraphy (14 patients, 9%), skeletal radiography (17 patients, 11%), MRI (11 patients, 7%) or cavography (3 patients, 2%) were performed. Preoperative embolization was successfully performed in 37 (24%) of the tumours. Out of the patients, 6 (4%) had histologically verified lymph node metastases and 18 (12%) had distant metastases at the time of primary surgical treatment. The most common organ sites for metastases were lung, bone and liver, representing 7 (39%), 6 (33%) and 3 (17%) of metastases at the time of diagnosis, respectively. In addition, there were solitary metastases in the adrenal gland, brain, uterus, oment and subcutis. Three of the patients (2%) had metastases in more than one organ site. In the study cohort, 70 (46%), 12 (8%), 51 (34%) and 19 (12%) patients had stages I to IV, respectively. The majority of patients underwent radical nephrectomy (145 patients, 95%) and 7 (5%) partial nephrectomy. The tumour characteristics are presented in Table 2.

The median and mean times of follow-up were 90 (range 0–209, SD 63.6) months. The follow-up was complete in all cases. During the follow-up period, 44 (29%) patients died of RCC, 40 (26%) died of other causes and 68 (45%) were still alive. Thirty-two patients suffered from RCC-metastases during the follow-up period. Metastases were diagnosed in 8 patients within one year after the primary operation and in 6, 8, 5 and 5 patients after 1–3 years, 3–5 years, 5–10 years and after more than 10 years of follow-up, respectively.
Table 2. Tumour characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Side</strong></td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>76/152 (50%)</td>
</tr>
<tr>
<td>Left</td>
<td>75/152 (49%)</td>
</tr>
<tr>
<td>Bilateral</td>
<td>1/152 (1%)</td>
</tr>
<tr>
<td><strong>Tumour class</strong></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>75/152 (49%)</td>
</tr>
<tr>
<td>T2</td>
<td>12/152 (8%)</td>
</tr>
<tr>
<td>T3</td>
<td>59/152 (39%)</td>
</tr>
<tr>
<td>T4</td>
<td>6/152 (4%)</td>
</tr>
<tr>
<td><strong>Nodal status</strong></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>146/152 (96%)</td>
</tr>
<tr>
<td>N1</td>
<td>4/152 (3%)</td>
</tr>
<tr>
<td>N2</td>
<td>2/152 (1%)</td>
</tr>
<tr>
<td><strong>Distant metastases</strong></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>134/152 (88%)</td>
</tr>
<tr>
<td>M1</td>
<td>18/152 (12%)</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>70/152 (46%)</td>
</tr>
<tr>
<td>II</td>
<td>12/152 (8%)</td>
</tr>
<tr>
<td>III</td>
<td>51/152 (34%)</td>
</tr>
<tr>
<td>IV</td>
<td>19/152 (12%)</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
</tr>
<tr>
<td>Clear cell</td>
<td>134/152 (88%)</td>
</tr>
<tr>
<td>Papillary</td>
<td>11/152 (7%)</td>
</tr>
<tr>
<td>Chromophobic</td>
<td>5/152 (3%)</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>2/152 (1%)</td>
</tr>
<tr>
<td><strong>Nuclear grade</strong></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5/150 (3%)</td>
</tr>
<tr>
<td>II</td>
<td>83/150 (55%)</td>
</tr>
<tr>
<td>III</td>
<td>40/150 (27%)</td>
</tr>
<tr>
<td>IV</td>
<td>22/150 (15%)</td>
</tr>
</tbody>
</table>

5.2 Survival analysis of the clinicopathological characteristics

The 5- and 10-year RCC-specific survival and HR for deaths due to RCC, according to the major clinicopathological parameters are shown in Table 3.
The RCC-specific survival rate did not differ between males and females, age groups or symptomatic and asymptomatic tumours in univariate or multivariate analysis. Of the various preoperative symptoms reported, hypogastric pain (14 patients, 9%) had a prognostic value in RCC-specific survival, with \( p=0.02 \). Preoperative anaemia, hypersedimentation and increased CRP were markers of a poorer RCC-specific prognosis, with \( p=0.002 \), \( p=0.004 \) and \( p<0.001 \), respectively, in univariate but not in multivariate survival analysis. None of the patients with normal CRP levels died from RCC during the follow-up period. Other laboratory findings such as increased liver enzyme levels, polycythaemia and hypercalcaemia had no prognostic significance in this material (data not shown). Time to metastasis was a statistically significant prognostic factor in RCC-specific survival in both the univariate \( (p<0.001) \) and multivariate analysis \( (p=0.004) \) adjusted for age, stage and grade.

The TNM classification, stage, tumour size and several histological parameters including nuclear grade, sarcomatoid features, microscopic venous invasion (MVI) and necrosis were statistically significant prognostic factors in the univariate analysis. Although papillary and chromophobic RCCs had a slightly longer RCC-specific mean survival (155 (95% CI 124–185) and 169 (95% CI 105–234) months, respectively) than clear cell RCCs (150 (95% CI 135–165) months), the difference was not statistically significant \( (p=0.7) \). Cystic structure was not a prognostic factor in our material. In the multivariate analysis adjusted for age, stage and grade, the only independent prognostic factors of the above-mentioned clinicopathological parameters were TNM staging, stage and tumour size (see Table 3).
Table 3. The 5- and 10-year RCC-specific survival (Kaplan-Meier method) and HR for deaths due to RCC (Cox univariate analysis) according to the clinicopathological parameters measured.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>5-year survival</th>
<th>10-year survival</th>
<th>HR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>77%</td>
<td>72%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>73%</td>
<td>71%</td>
<td>1.30</td>
<td>0.72–2.35</td>
<td>0.4</td>
</tr>
<tr>
<td>Female</td>
<td>81%</td>
<td>74%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Under 65</td>
<td>81%</td>
<td>75%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>65 or over</td>
<td>72%</td>
<td>69%</td>
<td>1.47</td>
<td>0.81–2.66</td>
<td>0.2</td>
</tr>
<tr>
<td>Under 46</td>
<td>80%</td>
<td>80%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>46 or over</td>
<td>77%</td>
<td>72%</td>
<td>1.23</td>
<td>0.38–4.00</td>
<td>0.7</td>
</tr>
<tr>
<td>Presentation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asymptomous</td>
<td>78%</td>
<td>75%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptomous</td>
<td>76%</td>
<td>69%</td>
<td>1.41</td>
<td>0.78–2.56</td>
<td>0.3</td>
</tr>
<tr>
<td>T class</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>92%</td>
<td>88%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1a</td>
<td>98%</td>
<td>93%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1b</td>
<td>86%</td>
<td>83%</td>
<td>3.16</td>
<td>0.82–12.24</td>
<td>0.1</td>
</tr>
<tr>
<td>T2</td>
<td>75%</td>
<td>67%</td>
<td>6.96</td>
<td>1.66–29.17</td>
<td>0.008</td>
</tr>
<tr>
<td>T3</td>
<td>64%</td>
<td>59%</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>T3a</td>
<td>68%</td>
<td>65%</td>
<td>6.03</td>
<td>1.72–21.20</td>
<td>0.005</td>
</tr>
<tr>
<td>T3b</td>
<td>67%</td>
<td>61%</td>
<td>7.27</td>
<td>1.88–28.16</td>
<td>0.004</td>
</tr>
<tr>
<td>T3c</td>
<td>25%</td>
<td>0%</td>
<td>32.95</td>
<td>7.27–149.27</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T4</td>
<td>17%</td>
<td>17%</td>
<td>26.74</td>
<td>6.32–113.06</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Nodal status</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>79%</td>
<td>74%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N1</td>
<td>50%</td>
<td>50%</td>
<td>3.70</td>
<td>0.89–15.41</td>
<td>0.07</td>
</tr>
<tr>
<td>N2</td>
<td>0%</td>
<td>0%</td>
<td>42.64</td>
<td>8.45–215.08</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>86%</td>
<td>81%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>11%</td>
<td>11%</td>
<td>13.75</td>
<td>6.91–27.37</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>96%</td>
<td>91%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>75%</td>
<td>67%</td>
<td>5.12</td>
<td>1.62–16.17</td>
<td>0.005</td>
</tr>
<tr>
<td>III</td>
<td>75%</td>
<td>69%</td>
<td>3.80</td>
<td>1.56–9.24</td>
<td>0.003</td>
</tr>
<tr>
<td>IV</td>
<td>16%</td>
<td>16%</td>
<td>24.09</td>
<td>9.61–60.41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Parameter</td>
<td>5-year survival</td>
<td>10-year survival</td>
<td>HR</td>
<td>95% CI</td>
<td>p-value</td>
</tr>
<tr>
<td>----------------------</td>
<td>-----------------</td>
<td>------------------</td>
<td>--------</td>
<td>-------------</td>
<td>---------</td>
</tr>
<tr>
<td><strong>Tumour size (cm)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Under 5</td>
<td>92%</td>
<td>86%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>From 5 to 9</td>
<td>74%</td>
<td>70%</td>
<td>2.65</td>
<td>1.15–6.14</td>
<td>0.02</td>
</tr>
<tr>
<td>10 or over</td>
<td>50%</td>
<td>45%</td>
<td>6.70</td>
<td>2.62–17.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Nuclear grade</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>100%</td>
<td>100%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>88%</td>
<td>82%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>68%</td>
<td>68%</td>
<td>1.76</td>
<td>0.86–3.63</td>
<td>0.1</td>
</tr>
<tr>
<td>IV</td>
<td>50%</td>
<td>41%</td>
<td>3.92</td>
<td>1.90–8.09</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>MVI</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>80%</td>
<td>75%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>47%</td>
<td>47%</td>
<td>3.89</td>
<td>1.86–8.16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Sarcomatoid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>79%</td>
<td>76%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>50%</td>
<td>33%</td>
<td>3.79</td>
<td>1.75–8.23</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Necrosis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>88%</td>
<td>84%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>60%</td>
<td>55%</td>
<td>3.62</td>
<td>1.96–6.71</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clear cell</td>
<td>76%</td>
<td>71%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papillary</td>
<td>91%</td>
<td>91%</td>
<td>0.60</td>
<td>0.14–2.48</td>
<td>0.5</td>
</tr>
<tr>
<td>Chromophobic</td>
<td>80%</td>
<td>80%</td>
<td>0.58</td>
<td>0.08–4.23</td>
<td>0.6</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>50%</td>
<td>50%</td>
<td>2.31</td>
<td>0.32–16.84</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>Cystic structure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>No</td>
<td>72%</td>
<td>69%</td>
<td>1.73</td>
<td>0.59–5.01</td>
<td>0.3</td>
</tr>
<tr>
<td>Yes</td>
<td>89%</td>
<td>83%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MVI: microscopic venous invasion
1 (ref.) hazard ratio 1.0, the reference group

5.3 Staining results and their association with the clinico-pathological parameters measured and disease-specific survival

The proportion of positive immunostaining and associations between tumour markers and the clinico-pathological characteristics measured, including stage, TNM classification, histology and nuclear grade, at the time of diagnosis are presented in Table 4. The 5- and 10-year RCC-specific survival rates (Kaplan-
Meier analysis) and HR for deaths due to RCC (Cox univariate analysis), according to the expression of tumour markers, are presented in Table 5.

Table 4. Proportion of positively stained specimens and the relationship between the tumour markers and the clinicopathological characteristics.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Positive/all</th>
<th>Stage</th>
<th>T</th>
<th>N</th>
<th>M</th>
<th>Histology</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear Nfr2</td>
<td>113/139 (81%)</td>
<td>0.007</td>
<td>0.005</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.02</td>
</tr>
<tr>
<td>Cytoplasmic Nfr2</td>
<td>135/139 (97%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Keap1</td>
<td>79/144 (55%)</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>NS</td>
<td>0.002</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Nuclear Trx</td>
<td>111/143 (78%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.02</td>
</tr>
<tr>
<td>Cytoplasmic Trx</td>
<td>123/143 (86%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Nuclear myosin VI</td>
<td>51/145 (35%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic myosin VI</td>
<td>104/145 (72%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.04</td>
</tr>
<tr>
<td>Nuclear E-cadherin</td>
<td>59/148 (40%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Membranous E-cadherin</td>
<td>14/148 (9%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.001</td>
<td>NS</td>
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</tr>
<tr>
<td>Nuclear β-catenin</td>
<td>65/147 (44%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>0.003</td>
</tr>
<tr>
<td>Cytoplasmic β-catenin</td>
<td>13/147 (9%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Nuclear HuR</td>
<td>127/145 (88%)</td>
<td>NS</td>
<td>NS</td>
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<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic HuR</td>
<td>37/145 (25%)</td>
<td>0.004</td>
<td>0.04</td>
<td>NS</td>
<td>0.009</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Cytoplasmic COX-2</td>
<td>22/147 (15%)</td>
<td>0.04</td>
<td>0.03</td>
<td>0.04</td>
<td>0.02</td>
<td>0.007</td>
<td>0.04</td>
</tr>
<tr>
<td>Cytoplasmic TLR9</td>
<td>112/138 (81%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Serotonin</td>
<td>12/145 (8%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CD56</td>
<td>26/144 (18%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td></td>
</tr>
<tr>
<td>NSE</td>
<td>69/145 (48%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.01</td>
<td>NS</td>
</tr>
<tr>
<td>Synaptophysin</td>
<td>2/148 (1%)</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Chromogranin A</td>
<td>0/148 (0%)</td>
<td></td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

NS = no significant correlation; numeric value is p-value for correlation

Table 5. The 5- and 10-year RCC-specific survival rates (Kaplan-Meier method) and HR for deaths due to RCC (Cox univariate analysis) according to tumour marker expression.

<table>
<thead>
<tr>
<th>Marker</th>
<th>5-year survival</th>
<th>10-year survival</th>
<th>HR</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear Nfr2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>73%</td>
<td>65%</td>
<td>1.09</td>
<td>0.36–3.27</td>
<td>0.9</td>
</tr>
<tr>
<td>Weakly positive</td>
<td>85%</td>
<td>80%</td>
<td>0.69</td>
<td>0.25–1.92</td>
<td>0.5</td>
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<tr>
<td>Moderately positive</td>
<td>68%</td>
<td>66%</td>
<td>1.17</td>
<td>0.42–3.29</td>
<td>0.8</td>
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<td>Strongly positive</td>
<td>64%</td>
<td>64%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoplasmic Nfr2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>100%</td>
<td>75%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weakly positive</td>
<td>80%</td>
<td>78%</td>
<td>1.08</td>
<td>0.14–8.35</td>
<td>0.9</td>
</tr>
<tr>
<td>Moderately positive</td>
<td>77%</td>
<td>73%</td>
<td>1.47</td>
<td>0.19–11.08</td>
<td>0.7</td>
</tr>
<tr>
<td>Marker</td>
<td>5-year survival</td>
<td>10-year survival</td>
<td>HR</td>
<td>95% CI</td>
<td>p-value</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------</td>
<td>------------------</td>
<td>-------</td>
<td>-----------------</td>
<td>---------</td>
</tr>
<tr>
<td>Strongly positive</td>
<td>67%</td>
<td>60%</td>
<td>1.75</td>
<td>0.23–13.44</td>
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<td>Cytoplasmic Keap1</td>
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<td>88%</td>
<td>80%</td>
<td>1 (ref.)</td>
<td></td>
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<tr>
<td>Positive</td>
<td>66%</td>
<td>63%</td>
<td>2.09</td>
<td>1.11–3.94</td>
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<td>Nuclear Trx</td>
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<td></td>
</tr>
<tr>
<td>Negative</td>
<td>78%</td>
<td>69%</td>
<td>1.49</td>
<td>0.62–3.60</td>
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<tr>
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<td>76%</td>
<td>69%</td>
<td>1.53</td>
<td>0.62–3.78</td>
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<tr>
<td>Moderately positive</td>
<td>72%</td>
<td>70%</td>
<td>1.33</td>
<td>0.57–3.10</td>
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<td>78%</td>
<td>75%</td>
<td>1 (ref.)</td>
<td></td>
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<td>Cytoplasmic Trx</td>
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<td></td>
</tr>
<tr>
<td>Negative</td>
<td>70%</td>
<td>55%</td>
<td>1.87</td>
<td>0.90–3.88</td>
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<td>76%</td>
<td>73%</td>
<td>1 (ref.)</td>
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<td>Nuclear myosin VI</td>
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<td></td>
</tr>
<tr>
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<td>78%</td>
<td>72%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>75%</td>
<td>71%</td>
<td>1.04</td>
<td>0.56–1.95</td>
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<td></td>
</tr>
<tr>
<td>Negative</td>
<td>83%</td>
<td>78%</td>
<td>1 (ref.)</td>
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<tr>
<td>Positive</td>
<td>74%</td>
<td>69%</td>
<td>1.40</td>
<td>0.69–2.85</td>
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<td>Nuclear E-cadherin</td>
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</tr>
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<td>Negative</td>
<td>74%</td>
<td>70%</td>
<td>1.31</td>
<td>0.7–2.43</td>
<td>0.4</td>
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<tr>
<td>Positive</td>
<td>80%</td>
<td>75%</td>
<td>1 (ref.)</td>
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<td>Membranous E-cadherin</td>
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<td>Negative</td>
<td>78%</td>
<td>73%</td>
<td>1 (ref.)</td>
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<td></td>
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<tr>
<td>Positive</td>
<td>57%</td>
<td>57%</td>
<td>1.59</td>
<td>0.67–3.77</td>
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<td>Nuclear β-catenin</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>Negative</td>
<td>72%</td>
<td>68%</td>
<td>1.34</td>
<td>0.73–2.48</td>
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<td>Positive</td>
<td>82%</td>
<td>75%</td>
<td>1 (ref.)</td>
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<td>Cytoplasmic β-catenin</td>
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<td></td>
</tr>
<tr>
<td>Negative</td>
<td>78%</td>
<td>72%</td>
<td>1 (ref.)</td>
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<tr>
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<td>62%</td>
<td>1.41</td>
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</tr>
<tr>
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<td>94%</td>
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<tr>
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<td>72%</td>
<td>1.83</td>
<td>0.65–5.16</td>
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<tr>
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<td>83%</td>
<td>78%</td>
<td>1 (ref.)</td>
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<td>62%</td>
<td>60%</td>
<td>2.18</td>
<td>1.16–4.09</td>
<td>0.02</td>
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73
<table>
<thead>
<tr>
<th>Marker</th>
<th>5-year survival</th>
<th>10-year survival</th>
<th>HR</th>
<th>95% CI</th>
<th>p-value</th>
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<td>COX-2</td>
<td></td>
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</tr>
<tr>
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<td>81%</td>
<td>76%</td>
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<td>55%</td>
<td>50%</td>
<td>2.29</td>
<td>1.15–4.54</td>
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<td>Cytoplasmic TLR9</td>
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<td>62%</td>
<td>58%</td>
<td>2.40</td>
<td>1.24–4.63</td>
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<td>75%</td>
<td>1 (ref.)</td>
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<td>Serotonin</td>
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<tr>
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<td>75%</td>
<td>70%</td>
<td>2.08</td>
<td>0.50–8.60</td>
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</tr>
<tr>
<td>Positive</td>
<td>83%</td>
<td>83%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD56</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>80%</td>
<td>75%</td>
<td>1 (ref.)</td>
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</tr>
<tr>
<td>Positive</td>
<td>65%</td>
<td>62%</td>
<td>1.37</td>
<td>0.67–2.79</td>
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<tr>
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<td>80%</td>
<td>72%</td>
<td>1 (ref.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>73%</td>
<td>71%</td>
<td>1.09</td>
<td>0.60–1.99</td>
<td>0.8</td>
</tr>
</tbody>
</table>

1 (ref.) hazard ratio 1.0, the reference group

**5.3.1 Markers of the oxidative system (I)**

**Nfr2**

Immunoreactivity for Nfr2 was investigated in 139 RCC specimens. Nuclear Nfr2 immunoreactivity was observed in 113 (81%) RCC tumours, of which 61 (44%) were weakly positive, 38 (27%) were moderately positive and 14 (10%) were strongly positive. Cytoplasmic Nfr2 was positive in 135 (97%) cases, of which 50 (36%) were weakly positive, 52 (37%) were moderately positive and 33 (24%) were strongly positive.

Nuclear Nfr2 immunoreactivity was associated with stage (p=0.007), tumour (T) class (p=0.005) and grade (p=0.02), and cytoplasmic Nfr2 with age (p=0.007). Nfr2 immunoreactivity did not have any prognostic influence on RCC-specific survival either in uni- or multivariate analysis.

**Keap1**

The staining pattern of Keap1 was cytoplasmic. Immunoreactivity of Keap1 was evaluated in 144 RCC tumours, of which 79 (55%) were immunopositive. Of the positive cases, 44 (31%), 24 (17%) and 11 (8%) tumours were weakly,
moderately and strongly positive, respectively. In the statistical analysis the immunopositive tumours were grouped together and compared with the immunonegative ones.

The immunoreactivity of Keap1 was associated with several factors indicating the advanced pattern of RCC, including advanced stage (p<0.001), a higher T class (p=0.002) and presence of distant metastases (p=0.002). The higher nuclear grade RCC tumours were more frequently immunopositive for Keap1, although the difference was not statistically significant (p=0.07).

In the univariate analysis, Keap1 immunoreactivity correlated with reduced RCC-specific survival, with p=0.02. The mean RCC-specific survival was 167 (95% confidence interval (CI) 148–186) months for Keap1-immunonegative tumours and 133 (95% CI 113–154) months for Keap1-immunopositive tumours. When Keap1 immunoreactivity was subjected to multivariate analysis adjusted for age, stage and grade, the only independent prognostic factor was stage.

**Thioredoxin**

There were 143 RCC samples available for Trx immunohistochemistry. Nuclear Trx immunoreactivity was detected in 111 (78%) tumours, of which 29 (20%) were weakly positive, 46 (32%) were moderately positive and 36 (25%) were strongly positive. Cytoplasmic Trx immunostaining occurred in 123 (86%) cases: 14 (10%) were weakly positive, 38 (27%) were moderately positive and 71 (50%) were strongly positive. Statistical analyses were performed between immunopositive and immunonegative tumours for cytoplasmic Trx.

The immunoreactivity of Trx was more frequently observed in well-differentiated tumours than in poorly differentiated ones. Nuclear and cytoplasmic Trx immunoreactivity was associated with nuclear grade, with p=0.02 and p=0.05, respectively.

The tumours with cytoplasmic immunoreactivity of Trx had a tendency toward a better RCC-specific prognosis as the mean RCC-specific survival was 115 (95% CI 78–153) months for tumours with no cytoplasmic expression of Trx and 153 (95% CI 138–169) months for tumours with cytoplasmic expression of Trx. However, the difference was not statistically significant (p=0.09). Nuclear Trx was not associated with RCC-specific survival. In the multivariate analysis adjusted for age, stage and grade, Trx immunoreactivity was not found to be a prognostic factor.
Associations between oxidative markers

Cytoplasmic Nfr2 was associated with nuclear Nfr2 (p<0.001) and cytoplasmic Keap1 (p=0.009). Nuclear Nfr2 was correlated with Trx immunoreactivity in both the nucleus and the cytoplasm (p=0.01 and p=0.03, respectively). In addition, nuclear and cytoplasmic Trx were associated with each other (p<0.001).

5.3.2 Myosin VI, E-cadherin and beta-catenin (II)

Myosin VI

Immunoreactivity for myosin VI was assessed in 145 RCC samples. Nuclear immunostaining was observed in 51 (35%) tumours. Cytoplasmic myosin VI was primarily detected in 28 (19%), 50 (35%) and 54 (37%) tumours representing subgroups of weak, moderate and strong immunopositivity, respectively, whereas 13 tumours (9%) were immunonegative for myosin VI. In a further analysis the strongly and moderately positive tumours were combined and regarded as positive (104 tumours, 72%) and compared to the negative ones (41 weakly positive and negative tumours, 28%).

Nuclear myosin VI was not associated with any of the clinicopathological parameters tested. Cytoplasmic myosin VI was associated with a lower Fuhrman grade, with p=0.04.

The mean RCC-specific survival was 162 (95% CI 137–187) months for cytoplasmic myosin VI-negative tumours and 146 (95% CI 128–163) months for positive tumours (p=0.3). The survival difference was observed also in low grade tumours (data not shown). In the multivariate analysis adjusted for age, stage and grade, cytoplasmic myosin VI immunoeexpression was found to be an independent prognostic factor along with stage. The HR for cytoplasmic myosin VI-expressing tumours was 2.3 (95% CI 1.1–5.0, p=0.03) when compared to the immunonegative tumours. In the multivariate analysis for clear cell carcinomas, cytoplasmic myosin VI expression was almost a statistically significant (p=0.05) prognostic factor of poorer prognosis (HR 2.1, 95% CI 1.0–4.5). Nuclear myosin VI expression was not correlated with RCC-specific survival.
**E-cadherin**

There were 148 RCC tumours specimens available to evaluate E-cadherin immunoreactivity. Immunostaining for nuclear E-cadherin was observed in 59 (40%) tumours. Membranous E-cadherin was detected as follows: weak intensity in 27 (18%), moderate intensity in 8 (5%) and strong intensity in 6 (4%) of the tumours. Moderately and strongly positive tumours (14, 9%) were combined and re-classified as positive and the negative and weakly positive tumours were combined and re-classified as negative (134, 91%).

No relationships were found between nuclear E-cadherin expression and clinicopathological features. Immunoreactivity for membranous E-cadherin was associated with histology (p<0.001) and was more frequently detected in chromophobic and undifferentiated carcinomas than in clear cell tumours. None of the papillary carcinomas showed membranous E-cadherin immunopositivity.

Immunostaining for E-cadherin in either the nucleus or membranes was not a prognostic factor in RCC-specific survival.

**Beta-catenin**

Of 147 RCC tumours, 65 (44%) were immunopositive for nuclear beta-catenin. Cytoplasmic beta-catenin was observed in 13 out of 147 tumours (9%).

Nuclear beta-catenin immunoreactivity was associated with a lower Fuhrman grade (p=0.003). Cytoplasmic beta-catenin was not associated with any of the clinicopathological parameters tested.

The uni- and multivariate analyses of the whole study population did not find beta-catenin immunoexpression to be a prognostic factor. However, when the survival analysis was performed separately for the subgroup of clear cell carcinomas, cytoplasmic beta-catenin expression was almost a statistically significant independent marker of decreased survival in the multivariate analysis adjusted for age, stage and grade, with an HR of 3.0 for positive tumours compared to negative ones (95% CI 1.0–9.0, p=0.05).

**Associations between myosin VI, E-cadherin and beta-catenin**

Nuclear myosin VI was associated with cytoplasmic myosin VI (p=0.004) and nuclear beta-catenin (p=0.009). E-cadherin and beta-catenin were associated in the nucleus (p<0.001) and in the membranes and cytoplasm (p=0.02).
5.3.3 *HuR and cyclooxygenase-2 (III)*

**HuR**

There were 145 RCC tumours available to evaluate HuR expression. Nuclear HuR immunopositivity was detected in 127 tumours (88%). Six (4%) of the tumours were strongly positive, 31 (21%) were weakly positive and 108 (75%) were negative for cytoplasmic HuR immunostaining. For further statistical analysis, the subgroups of weakly and strongly positive cases were combined to form a group of 37 positive tumours (25%), which was compared to the group of negative cases.

Cytoplasmic HuR was associated with advanced stage (p=0.004), a higher T class (p=0.04) and distant metastases (p=0.009). Tumours with cytoplasmic HuR expression showed a tendency toward a higher Fuhrman grade (p=0.06). A significant association was found between the histological subtype of RCC and cytoplasmic HuR expression (p=0.01). Of the clear cell and chromophobic carcinomas, 100 (78%) and 4 (80%) did not express HuR in the cytoplasm, respectively, whereas 5 (56%) papillary carcinomas showed immunoreactivity for cytoplasmic HuR. Both of the unclassified tumours expressed HuR. Cytoplasmic HuR immunoreactivity was more common in male than in female patients (p=0.04). Nuclear HuR immunoreactivity was not associated with the clinicopathological parameters studied. The immunoreexpression of cytoplasmic and nuclear HuR was associated (p=0.007).

The mean RCC-specific survival was longer for patients whose tumours did not express cytoplasmic HuR (p=0.01). The mean RCC-specific survival times for HuR-expressing and non-expressing tumours were 118 (95% CI 89–146) and 164 (95% CI 148–179) months, respectively. The lack of HuR expression was also a prognostic factor of a more favourable RCC-specific survival in the subgroup of clear cell RCCs (p=0.006). The mean RCC-specific survival was 108 (95% CI 76–140) and 162 (95% CI 146–178) months for cytoplasmic HuR immunopositive and immunonegative clear cell RCCs, respectively. The HR for HuR-expressing clear cell tumours was 2.45 (95% CI 1.27–4.75, p=0.008) compared to clear cell carcinomas with no cytoplasmic HuR expression. In the multivariate analysis adjusted for age, stage and grade, cytoplasmic HuR expression was not found to be a prognostic factor. Nuclear HuR immunoreactivity was not a prognostic factor in RCC-specific survival.
Cyclooxygenase-2

Out of 147 RCC tumours, cytoplasmic COX-2 immunostaining was weakly positive in 22 (15%), moderately positive in 10 (7%) and strongly positive in 12 (8%) tumours. The negative and weakly positive cases and the moderately and strongly positive cases were re-classified as negative (125 tumours, 85%) and positive (22 tumours, 15%), respectively. No COX-2 immunostaining was detected in the nucleus.

Cytoplasmic COX-2 expression was associated with advanced stage (p=0.04), a higher T class (p=0.03), lymph node metastases (p=0.04), distant metastases (p=0.02) and a higher nuclear grade (p=0.04). A relationship was found between COX-2 expression and the histological type of RCC (p=0.007). Similar to cytoplasmic HuR, COX-2 expression was more frequently detected in papillary (3 tumours, 33%) and unclassified (2 tumours, 100%) tumours than in clear cell (16 tumours, 12%) and chromophobic (one tumour, 20%) carcinomas.

Lack of COX-2 expression was a marker of better prognosis (p=0.02). The mean RCC-specific survival times were 158 (95% CI 144–173) and 107 (95% CI 70–144) months for COX-2 immunonegative and immunopositive tumours, respectively. Cytoplasmic COX-2 expression was a prognostic factor also in the subgroup of tumours with clear cell histology. The mean RCC-specific survival was 96 (95% CI 54–138) months for COX-2 immunopositive and 156 (95% CI 140–172) months for immunonegative clear cell RCCs (p=0.01). The HR for COX-2-expressing clear cell carcinomas was 2.50 (95% CI 1.19–5.26, p=0.02). In the analysis of five chromophob ic carcinomas, cytoplasmic COX-2 immunopositivity was associated with unfavourable patient survival, with p=0.05. In the Cox regression analysis for age, stage, grade and COX-2 expression, the only statistically significant factor in RCC-specific survival was stage (p<0.001).

The relationship between HuR and cyclooxygenase-2

Cytoplasmic HuR was associated with cytoplasmic COX-2 (p<0.001). Both HuR and COX-2 expression was present in 16 (11%) tumours whereas 102 (70%) of the tumours were immunonegative for both of these markers. In addition, cytoplasmic COX-2 expression and membranous E-cadherin were inversely associated (p=0.002).
**Cell culture experiments**

In order to test the hypothesis that HuR regulates COX-2 expression in RCC cells, human RCC cells were treated with HuR siRNA molecules. This treatment suppressed HuR expression, as detected by immunoblotting. Furthermore, a suppression of COX-2 protein expression after the HuR siRNA treatment was observed. Control siRNA showed no effect on HuR or COX-2 expression. These results indicated that HuR can regulate COX-2 expression in RCC cells.

**5.3.4 Toll-like receptor 9 (IV)**

Of the 138 RCC tumours available for the TLR9 immunoreactivity evaluation, 60 (44%) showed some nuclear immunopositivity. Twenty-one (15%) of the tumours were strongly positive, 39 (28%) were moderately positive and 52 (38%) were weakly positive for cytoplasmic TLR9 immunostaining. For further analyses, the weakly, moderately and strongly positive cases were combined and re-classified as TLR9-positive samples (112 tumours, 81%).

No correlation was found between the clinicopathological parameters studied and TLR9 immunoreactivity. The immunoreactivity of TLR9 was observed in 100 (82%), 6 (67%), 4 (80%) and 2 (100%) tumours representing the histological subtypes of clear cell RCC, papillary RCC, chromophobic RCC and unclassified RCC, respectively. The expression of TLR9 and tumour necrosis was not associated (data not shown).

In univariate analysis, RCC-specific survival was significantly longer for patients whose tumours expressed cytoplasmic TLR9 compared to patients with TLR9-immunonegative tumours (p=0.007). The mean RCC-specific survival time was 112 (95% CI 76–147) months for tumours that did not express TLR9 in the cytoplasm and 160 (95% CI 144–175) months for TLR9-positive tumours. The expression of TLR9 was also a marker of more favourable RCC-specific survival when the histological subtypes were analysed separately. The mean RCC-specific survival period for cytoplasmic TLR9-expressing clear cell RCCs was 158 months (95% CI 142–175 months), whereas for immunonegative tumours it was 113 months (95% CI 74–152 months, p=0.02). The expression of TLR9 also indicated a more favourable RCC-specific survival (p=0.05) in the analysis of nine papillary RCCs. In the Cox regression analysis for cytoplasmic TLR9 expression, including age, stage and nuclear grade, TLR9 expression was an independent prognostic factor in RCC-specific survival along with stage and
grade. The HR was 3.52 (95% CI 1.71–7.26, p=0.001) for tumours that did not express TLR9 compared to TLR9-positive tumours. The expression of TLR9 was also an independent marker of a more favourable RCC-specific prognosis in the subgroup of clear cell RCCs. The HR was 3.59 (95% CI 1.65–7.80, p=0.001) for TLR9-negative tumours compared to immunopositive clear cell RCCs. Nuclear TLR9 was not a prognostic factor in RCC-specific survival.

5.3.5 Markers of the neuroendocrine system (V)

Out of 145 RCC tumours, 12 (8%) were immunopositive for serotonin. There were 144 specimens available for the immunohistochemical evaluation of CD56, of which 26 (18%) were immunopositive. Regarding immunopositivity for NSE, 69 cases out of 145 (48%) were detected. Synaptophysin immunopositivity was found in 2 out of 148 tumours (1%). The immunoexpression of chromogranin A was not observed in any of the 148 tumours (0%). The expression of serotonin and CD56 (p<0.001), serotonin and synaptophysin (p=0.006) and CD56 and synaptophysin (p=0.03) were associated.

The immunoexpression of NSE was more common in clear cell RCCs than in the other subtypes (p=0.01). Other NE markers (serotonin, CD56 and synaptophysin) were not found to be associated with histology or with the other clinicopathological parameters, although the correlation between CD56 expression and a higher T class was almost significant (p=0.05).

In survival analysis, none of the neuroendocrine markers tested was found to be a significant prognostic factor in RCC-specific survival, although serotonin-immunopositive and CD56- and NSE-immunonegative tumours had a tendency toward longer RCC-specific survival. The mean RCC-specific survival times for serotonin-positive and negative tumours were 176 (95% CI 138–214) months and 148 (95% CI 133–163) months, respectively (p=0.3). The mean RCC-specific survival was 156 (95% CI 140–172) months for CD56-immunonegative tumours and 153 (95% CI 143–171) months for NSE-immunonegative tumours, whereas it was 139 (95% CI 106–172) months and 149 (95% CI 127–171) months for tumours that expressed CD56 and NSE, respectively (p=0.4 for CD56 and p=0.8 for NSE). Both patients with synaptophysin-immunopositive tumours were alive at the end of follow-up. When the stainings (serotonin, CD56 and NSE) were separately included in the Cox multivariate analysis with stage, Fuhrman grade and age, the only independent prognostic factor for RCC-specific survival was stage (p<0.001).
5.3.6 The prognostic accuracy of tested biomarkers

The most important prognostic factors of the tested biomarkers were cytoplasmic TLR9, myosin VI and HuR, which improved the prognostic accuracy of the multivariate survival analysis especially when used in combination. The most important single prognostic factor was stage.
6 Discussion

In the search for novel prognostic markers for RCC we evaluated the expression of 14 molecular markers by immunohistochemistry and examined their prognostic significance. In addition, we explored the prognostic impact of widely used clinicopathological parameters in our patient material.

6.1 Material and methodology

As our study population consisted of 77 females and 75 males, the gender distribution differed from the normal male predominance (Chow et al. 2010), although the predominance of females seemed to be coincidental. The commonly recognized risk factors for RCC, i.e., smoking, hypertension and obesity (data not shown) (Lipworth et al. 2006) were frequently found in our study population. The majority of the tumours were operated on by radical nephrectomy, which was the main type of operation performed for renal tumours in the 1990s. The clinical practice at our hospital in the 1990s favoured preoperative embolization and it was successfully performed for 24% of the patients. One half of the tumours were asymptomatic and incidentally found by radiological examination, which is in line with the results of previous studies (Pantuck et al. 2000). The distribution of histological subtypes corresponds quite well with data presented in the literature, although there was a slight predominance of clear cell carcinomas (88%) whereas there were slightly fewer papillary and chromophobic carcinomas than previously established (Kovács et al. 1997). The distribution of different nuclear grades was not equal as there were only five grade I tumours whereas grade II tumours were overrepresented. This minor discrepancy may be explained by the size of study population. The stage distribution deviated a little from that generally reported as almost one half of the tumours were stage I and only 12% of the patients had metastatic disease (Motzer et al. 1996). However, each stage was represented and thus this series can be considered unselected. During the follow-up period 29% patients died of RCC, which is slightly less than the traditionally reported figure of more than 40% (Landis et al. 1999, Bui et al. 2001).

There were many variables in each study that may have biased our results. It is possible that some of the positive results were so-called false positives (alpha error, type I error) due to coincidence, for example. On the other hand, as some of the markers did not show any prognostic potential, there is a possibility of beta
errors (type II error), which may have been caused by the patient cohort being too small, for example.

6.2 Patient survival and clinicopathological features as prognostic factors

The RCC-specific survival of whole study population was in line with the results of previous reports (Tsui et al. 2000). Minor discrepancies in RCC-specific survival could be explained by the size of the study population and slightly different stage distributions. The prognostic significance of classic prognostic factors is well described in the literature (Méjean et al. 2003, Volpe & Patard 2010) and the results from our study are in agreement with these previous findings. In multivariate analysis the nuclear grade lost its prognostic significance, which was also recognized in many previous studies (Delahunt et al. 2007).

The value of tumour-related anatomical parameters, including TNM stage, stage grouping and tumour size, as well as time to appearance of metastasis, as independent prognostic factors was confirmed in our study. Interestingly, large localized tumours (T2, stage II) had a poorer prognosis in terms of shorter mean survival and higher HR for death from RCC (under long-term surveillance) than locally advanced RCCs (T3, stage III). This observation supports the results of a previous study by Murphy and co-workers (Murphy et al. 2005), as well as traditional clinical observations. However, the low number of cases in the subgroups of pT2 and stage II reduces the reliability of this finding. The TNM classification is a dynamic prognostic tool; its significance in predicting the survival of RCC patients has been confirmed in multiple trials (Volpe & Patard 2010). Despite the revisions made over the years there is an ongoing debate regarding further finetuning of the TNM staging concerning, for example, the significance of tumour size, especially in locally advanced disease, sinus fat and collecting system invasion, as well as infiltration into the vena cava wall. It might be also considered whether or not there is a need to combine novel variables such as performance status, symptoms or molecular profile in the TNM classification system. (Lam et al. 2008, Delahunt 2009, Moch et al. 2009) For our material, however, the TNM classification 2002 accurately predicted 5- and 10-year RCC-specific survival.

The prognostic significance of several histological factors but not the histological subtype or cystic structure was also confirmed in this study. The low number of cases in the subgroups of papillary and chromophobic carcinomas and
the lack of an evaluation of the possible cystic structure in almost one half of the cases may be the reasons why no statistically significant survival difference was found in our study according to these variables. The undifferentiated carcinomas showed a poor survival rate, as could be expected.

Although it is generally thought that incidentally found asymptomatic tumours have a better prognosis than symptomatic ones, not all studies, including ours, support this (Mevorach et al. 1992, Jayson & Sanders 1998). Several earlier studies indicated that the more favourable survival rates among asymptomatic tumours is related to the smaller size and lower stage of these tumours (Pantuck et al. 2000, Méjean et al. 2003, Schips et al. 2003). In our material the incidentally found and asymptomatic tumours did not differ in stage, T class or tumour size, which presumably explains the similar survival rates observed. However, the incidentally found tumours appeared to have a lower nuclear grade compared to the symptomatic tumours (p=0.005), which has also been previously reported (Pantuck et al. 2000).

6.3 Markers of the oxidative system (I)

Oxidative stress is known to promote carcinogenesis (reviewed in Klaunig et al. 1998) and oxidative stress and DNA damage have also been found in RCC (Okamoto et al. 1994, Soini et al. 2006b). Thus, we decided to investigate some of the markers involved in protection against oxidative stress in order to discover whether or not they have any prognostic significance in RCC. The markers selected were Nfr2 and Keap1, which work as a pair to sense and defend against ROS (Motohashi & Yamamoto 2004), and Trx, which is an antioxidant enzyme regulated by Nfr2 (Stacy et al. 2006).

6.3.1 Keap1 and Nfr2

The immunoexpression pattern of Nfr2 and Keap1 that was detected corresponded to the pattern generally reported (McMahon et al. 2003, Itoh et al. 2004). The expression of Nfr2 was frequent in RCC whereas just over half of the tumours were immunopositive for Keap1. The associations between these markers reflected the earlier observations that Keap1 regulates the expression of Nfr2 by binding it in the cytoplasm (Itoh et al. 2004) and that accumulated Nfr2 translocates from the cytoplasm into the nucleus (Kaspar et al. 2009).
The relationship between Keap1 expression and advanced stage largely explains the poorer prognosis of Keap1-immunopositive tumours. In addition, the higher grade RCC tumours were more frequently Keap1 positive. This is in contrast to the situation in lung cancer, where reduced Keap1 expression and the resulting nuclear accumulation of Nfr2 have been shown to stimulate cell growth and to be related to poor survival rates (Ohta et al. 2008, Solis et al. 2010). It is evident that the biological consequences of the Keap1-Nfr2 system have not been thoroughly determined in cancer. By protecting DNA against oxidative and xenobiotic damage and by modulating the redox balance, the activity of Nfr2 can suppress cancer development (Satoh et al. 2010, Taguchi et al. 2011). Thus, it is reasonable to suggest that the overexpression of Keap1 and the consequent down-regulation of nuclear Nfr2 activity could promote carcinogenesis by repressing defence mechanisms such as antioxidants against oxidative stress. Although Keap1 and Nfr2 were associated in the cytoplasm, we could not, however, find any significant association between Keap1 immunoexpression and nuclear Nfr2. The expression of Nfr2 was not found to be a prognostic factor for RCC-specific survival either. It is possible that the immunohistochemical quantification of nuclear Nfr2 did not correspond to functionally active Nfr2. The modulation of Nfr2 activity could be more complex than the ROS-induced modification of cysteine residues of Keap1 alone. For example, the phosphorylation of serine of Nfr2 can result in the release of Nfr2 from Keap1 and be another regulatory mechanism for Nfr2 activity. (Apopa et al. 2008, Yamamoto et al. 2008, Kaspar et al. 2009) Nuclear Nrf2 expression is, however, an independent prognostic marker in lung cancer (Merikallio et al. 2011). Consequently, the distribution of Nrf2 in the nucleus may be cancer-type specific and relate to exposure to specific carcinogens or specific treatments. Moreover, although the accumulation of Nfr2 and the consequently induced expression of cytoprotective genes have been regarded as the main mechanisms of action of the Keap1-Nfr2 system, additional pathways might also influence survival and contribute to cancer prognosis. Indeed, there is evidence to show that Keap1 can, for example, regulate apoptosis (Wakabayashi et al. 2010, Niture & Jaiswal 2011). As the prognostic significance of Keap1 was not explained by Nfr2, these as yet unknown mechanisms might explain the influence on survival that was observed in our study by Keap1.
6.3.2 Thioredoxin

The immunoexpression of Trx observed in our study was in line with that previously reported for RCC (Soini et al. 2006b). Furthermore, nuclear immunostaining for Trx has been previously reported in other cancers including breast, cervical and gastric carcinomas (Fujii et al. 1991, Grogan et al. 2000, Turunen et al. 2004). Thioredoxin is an Nfr2-regulated gene product and Nfr2 activation has been shown to result in the up-regulation of Trx (Stacy et al. 2006, Singh et al. 2008), which was confirmed by the association between nuclear Nfr2 and Trx expression in our study.

Thioredoxin immunoreactivity was more frequently observed in well-differentiated tumours than in poorly differentiated ones, and Trx-expressing tumours were associated with longer survival. These observations are in agreement with those of earlier studies that established the relationships between various antioxidants and lower grade, more local stage and better survival in RCC (Soini et al. 2006a, Soini et al. 2006b). It has been suggested that oxidative stress and the formation of ROS could result in the induction of antioxidant enzymes which, in turn, could protect cancer cells from further oxidative damage and restrict additional genomic instability (Soini et al. 2006a, Soini et al. 2006b).

6.4 Myosin VI, E-cadherin and beta-catenin (II)

Invasion and metastasis are the hallmarks of cancer (reviewed in Hanahan & Weinberg 2011). A cell adhesion molecule E-cadherin and a cytoplasmic regulatory protein beta-catenin are both involved in these processes, as well as in the pathogenesis of RCC (reviewed in Banumathy & Cairns 2010, van Kilsdonk et al. 2010). The previous observation that unconventional myosin VI is associated with the E-cadherin-beta-catenin complex (Küssel-Andermann et al. 2000, Geisbrecht & Montell 2002) and has multiple roles that may contribute to invasion and metastasis (Dunn et al. 2006, Knudsen 2006) inspired us to study the immunoexpression of myosin VI in RCC tumours in an attempt to discover whether or not myosin VI expression has any prognostic significance in RCC.

6.4.1 Myosin VI

The staining pattern of myosin VI was equal to patterns previously reported (Vreugde et al. 2006, Sweeney & Houdusse 2007). In ovarian and prostate
carcinomas myosin VI expression was associated with a higher grade (Yoshida et al. 2004, Dunn et al. 2006) and thus our observation of a relationship between cytoplasmic myosin VI immunoreactivity and greater differentiation is controversial.

When confounding factors were eliminated in the survival analysis, myosin VI expression was found to be an independent marker of poorer prognosis in RCC. Moreover, myosin VI also predicted survival in the subgroup of low grade tumours which is an interesting observation from a clinical point of view. Similar observations of myosin VI as a marker of unfavourable cancer prognosis have been reported in ovarian and prostate carcinomas and in leukaemia, where myosin VI overexpression is related to adverse clinical features of tumour and cancer progression. In detail, the knock-down of myosin VI inhibits cancer cell spreading and colony formation. (Yoshida et al. 2004, Dunn et al. 2006, Jbireal et al. 2010)

Myosin VI is involved in cancer cell migration, which contributes to tumour invasion in ovarian and prostate carcinomas and in leukaemia (Geisbrecht & Montell 2002, Yoshida et al. 2004, Dunn et al. 2006, Buss & Kendrick-Jones 2008, Jbireal et al. 2010). The exact mechanism via which it enhances cell migration is not fully understood. Myosin can produce a protrusive force by binding to adhesion complexes such as E-cadherin-beta-catenin in the membrane and, by pushing the actin filaments outwards, it can move the cell forward (Buss & Kendrick-Jones 2008). Myosin VI might also be involved in the internalization and recycling of key mediators of cell migration (Yoshida et al. 2004) and increased myosin VI expression could facilitate tumour invasion by enhancing cancer cells to respond to growth factor stimulation (Knudsen 2006). During tumour progression changes occur in cell polarity, proteins at cell-cell junctions become reorganized and, furthermore, myosin VI translocates from cell-cell junctions to membrane ruffles, which could be related to both invasion and metastasis (Knudsen 2006). A recent study on prostate cancer found that the overexpression of myosin VI enhances the secretion of VEGF, which could also be related to cancer progression (Puri et al. 2010). These mechanisms could explain the prognostic impact of myosin expression observed in our study. Vascular endothelial growth factor (VEGF) plays a central role in the pathogenesis of RCC (reviewed in Pfaffenroth & Linehan 2008) and thus the relationship between myosin VI and VEGF (Puri et al. 2010) is intriguing and warrants further investigation.
6.4.2 E-cadherin

The membranous immunoexpression of E-cadherin detected in the present study was slightly less frequent than commonly reported for RCC (Terpe et al. 1993, Katagiri et al. 1995, Shimazui et al. 1997). Altered E-cadherin expression is, however, a less frequent phenomenon in RCC compared to other epithelial carcinomas, presumably due to the origin of RCC. The majority of RCCs originate from proximal tubular epithelial cells, which normally do not express E-cadherin at all. Instead, E-cadherin is present in the distal tubules and collecting ducts of the normal kidney. (Katagiri et al. 1995) Our study replicated the previous observations of the association between histology and E-cadherin expression in RCC (Gervais et al. 2007).

Some previous studies associated the down-regulation of E-cadherin with advanced stage (Katagiri et al. 1995, Shimazui et al. 1997) and an unfavourable clinical outcome in RCC (Katagiri et al. 1995). These results were not replicated in our study. However, the prognostic role of E-cadherin was not proven in every previous study (Shimazui et al. 1997).

In addition to membranous expression, nuclear E-cadherin immunostaining has been reported in some cancers (Gervais et al. 2007, Chetty & Serra 2008). The exact mechanism by which E-cadherin translocates to the nucleus is unclear, but it is assumed that it is related to some cooperative molecules such as beta-catenin (Chetty & Serra 2008). In a previous study by Gervais and co-workers, nuclear E-cadherin expression was correlated with a low Fuhrman grade, VHL immunoreactivity and better prognosis, and it was only observed in clear cell variants (Gervais et al. 2007). Although nuclear E-cadherin expression was also detected in our study, these observations could not be statistically confirmed, which was presumably related to methodological differences and a different study population.

6.4.3 Beta-catenin

The normal immunoexpression pattern of beta-catenin is membranous and/or cytoplasmic (Kim et al. 2000). Beta-catenin gene mutations, which seem to be uncommon in the pathogenesis of RCC (Kim et al. 2000), could result in nuclear and/or cytoplasmic accumulation of beta-catenin (Bilim et al. 2000). Previous reports of beta-catenin immunoexpression in RCC are variable, as both increased and decreased beta-catenin immunoreactivity have been observed. For example,
in the study of Shimazui and co-workers, 24% of RCCs showed abnormal, i.e. absent, or heterogeneous immunoreactivity for beta-catenin (Shimazui et al. 1997). In other studies, decreased membranous or cytoplasmic immunoreactivity was observed in 16% and 42% of RCC tumours, respectively (Bilim et al. 2000, Aaltomaa et al. 2004). Some studies established a connection between the normal immunostaining pattern or cytoplasmic accumulation of beta-catenin and clear cell carcinoma (Shimazui et al. 1997 Kim et al. 2000, Guo et al. 2001). The comparison of individual studies seems to be quite infeasible due to fundamental differences in immunohistochemical procedures and evaluations. However, it seems that cytoplasmic beta-catenin expression was less usual and nuclear immunoreactivity more frequent in our study population than reported previously (Aaltomaa et al. 2004). Previous reports of the association between beta-catenin and tumour differentiation are controversial: on the one hand an association between preserved beta-catenin expression and better differentiation was reported (Bilim et al. 2000), whereas on the other hand the down-regulation of membranous beta-catenin was reportedly associated with low grade clear cell carcinomas (Guo et al. 2001). We observed an association between nuclear beta-catenin expression and better differentiation, whereas cytoplasmic expression was not correlated with nuclear grade.

The prognostic influence of beta-catenin differs between various cancers. Beta-catenin expression has been connected to invasion and reduced survival in colon and liver cancers, for example, whereas it has been found to be related to a more favourable prognosis in malignant melanoma and transitional cell carcinoma (Shimazui et al. 1996, Arozarena et al. 2011). Studies concerning the prognostic value of beta-catenin in RCC have mainly reported that decreased beta-catenin expression is related to advanced stage, tumour recurrence and decreased patient survival (Shimazui et al. 1997, Bilim et al. 2000, Kim et al. 2000, Zhu et al. 2000, Kuroiwa et al. 2001, Aaltomaa et al. 2004). However, it is very difficult to compare these previous studies as they have mixed and differing immunostaining patterns and the cut-off values for decreased, normal and increased levels of immunoreexpression were unique for each study, among other methodological differences. Our study found that cytoplasmic beta-catenin expression was related to poorer RCC-specific survival in clear cell carcinoma. It is evident that beta-catenin plays convoluted roles in the pathogenesis of RCC and thus the impact of beta-catenin immunoreexpression might vary in this malignancy.
6.4.4 Associations between myosin VI, E-cadherin and beta-catenin

We could not prove an association between myosin VI and E-cadherin-beta-catenin in the cytoplasm, unlike previously described (Küssel-Andermann et al. 2000, Geisbrecht & Montell 2002). However, there was an association between nuclear myosin VI and beta-catenin. As myosin VI is a cytoplasmic protein (Sweeney & Houdusse 2007), its role in the nucleus remains obscure, but it did not appear to have any prognostic significance in RCC. Nuclear beta-catenin expression was previously reported in various malignancies such as colon, gastric and endometrial carcinomas (Iwamoto et al. 2000, Ougolkov et al. 2000, Saegusa et al. 2001), as well as in RCC (Aaltomaa et al. 2004). We suggest that myosin VI and beta-catenin could somehow co-operate or interact. The role of beta-catenin as a regulator of transcription factors has been clearly described (Behrens et al. 1996, Karim et al. 2004). In fact, myosin VI has also been linked to the regulation of gene expression and transcriptional modulation (Dunn et al. 2006, Vreugde et al. 2006).

The positive relationship between E-cadherin and beta-catenin expression has previously been shown in gastrointestinal carcinomas (Han et al. 2000, Gervais et al. 2007, Serra et al. 2007) and it was also confirmed in our study. Moreover, E-cadherin binding has been shown to prevent beta-catenin nuclear localization (Orsulic et al. 1999). Indeed, we also found that nuclear beta-catenin was more frequently absent in clear cell RCC with membranous E-cadherin expression (data not shown). It is assumed that nuclear E-cadherin is a cleavage product of the membranous full-length E-cadherin and a direct association was previously found between membranous and nuclear E-cadherin expression in RCC (Gervais et al. 2007). This association was also confirmed in our study.

6.5 HuR and cyclooxygenase-2 (III)

Inflammation is regarded as one of the risk factors for cancer development and progression (reviewed in Farrow & Evers 2002). Up-regulation of the inflammation-associated enzyme COX-2 has been shown to be correlated with adverse clinicopathological features and more aggressive tumour behaviour in various malignomas including RCC (Sheehan et al. 1999, Miyata et al. 2003). In this study, we decided to find out whether or not COX-2 expression is regulated by the mRNA stability protein HuR in RCC and whether or not HuR expression has some prognostic value in RCC.
6.5.1 HuR

The expression of HuR has been found to be greater and more frequently cytoplasmic in RCC than in the normal kidney (López de Silanes et al. 2003). The nuclear immunoexpression of HuR observed in the present study is in line with the results of a previous study by Danilin et al. (Danilin et al. 2010). However, cytoplasmic HuR reactivity seemed to be less frequent in our study population than in earlier studies (López de Silanes et al. 2003, Danilin et al. 2010). This discrepancy could be explained, at least partly, by methodological aspects since we used paraffin-embedded, formalin-fixed specimens compared to the freshly harvested tumour samples used in the study by Danilin and co-workers, and by the low number of cases in the study by López de Silanes et al.

We detected a significant association between HuR and COX-2 expression at the protein level and showed the regulation of COX-2 expression by HuR in RCC cell lines, which were previously shown in several other malignomas (Dixon et al. 2001, Erkinheimo et al. 2003, Denkert et al. 2004b, Mrena et al. 2005, Denkert et al. 2006, Lim et al. 2007). The similar relationship observed in the present study between cytoplasmic HuR expression and clinicopathological parameters of advanced stage and decreased cancer survival was previously demonstrated in other carcinomas (Erkinheimo et al. 2003, Denkert et al. 2004a, Denkert et al. 2004b, Heinonen et al. 2005, Mrena et al. 2005, Denkert et al. 2006, Lim et al. 2007).

Although the ability to induce COX-2 expression is regarded as the explanation for the prognostic significance of HuR expression in many cancers (Erkinheimo et al. 2003, Mrena et al. 2005), HuR also regulates the stability and/or translation of numerous other mRNAs involved in malignant transformation and cancer promotion (López de Silanes et al. 2005, Hinman & Lou 2008,). The HuR protein enhances angiogenesis as it increases HIF-1α and VEGF expression, which was previously shown in colon carcinoma cells, for example (Levy et al. 1998, Dixon et al. 2001, Sheflin et al. 2004). The overexpression of HuR was shown to be related to increased cell proliferation by the stabilization of mRNAs of epidermal growth factor (EGF) (Sheflin et al. 2004), granulocyte macrophage colony-stimulating factor (GM-CSF) (López de Silanes et al. 2005) and proto-oncogenic and proliferative factors such as c-myc, c-fos, cyclin A and cyclin B1 (Peng et al. 1998, Wang et al. 2000, Wang et al. 2001). Furthermore, HuR inhibits apoptosis via the antiapoptotic protein prothymosin α (ProTα) (López de Silanes et al. 2005). Other HuR-regulated
target proteins have been reported in studies on colon carcinoma, for example beta-catenin (López de Silanes et al. 2003), as well as some invasion and metastasis related proteins including matrix metalloproteinase 9 (MMP-9), urokinase plasminogen activator (uPA) and the metastasis-associated protein (López de Silanes et al. 2005). In addition, there is evidence to show that HuR induces the expression of immunosuppressive cytokines, which may help the tumour to avoid immune surveillance (López de Silanes et al. 2005). Many immunomodulating factors such as TGF-β, TNF-α and interleukins IL-6 and IL-8 have been found to be the target mRNAs of HuR (Dixon et al. 2001, Nabors et al. 2001).

Evidence has shown that HuR has a central role in the pathogenesis of RCC (Danilin et al. 2010), where HuR was found to be overexpressed in all of the samples of conventional RCC tested, regardless of the VHL status (Danilin et al. 2010). Renal cell carcinoma cell culture specimens showed that the down-regulation of HuR reduces proliferation (Danilin et al. 2009) and facilitates apoptosis (Danilin et al. 2010). Moreover, HuR knock-down was found to inhibit RCC tumour growth in both cell cultures and an in vivo animal model (Danilin et al. 2010). In conventional RCC, HuR expression has shown to be associated for example with VEGF and HIF-2α expression as well as with the constitutive activation of the PI3K/AKT pathway (Danilin et al. 2010).

The relationship between HuR and VHL has been shown in several studies on RCC. The stabilization of tumour suppressor p53 mRNA by HuR is pVHL-dependent and could partly explain the tumour suppressive functions of pVHL that have been observed in RCC (Galbán et al. 2003). On the other hand, pVHL reduces HuR expression via sequestration, resulting in decreased expression of type I insulin-like growth factor receptor (IGF1R) in clear cell RCC (Yuen et al. 2007). In turn, IGF1R is one of the key mediators of tumour cell survival and its expression is connected to a worse prognosis in clear cell RCC (Parker et al. 2002). In addition, the parathyroid hormone-related protein (PTHrP), which is responsible for humoral hypercalcaemia associated with RCC, has growth factor-like activities and is an independent prognostic factor of patient outcome in RCC (Iwamura et al. 1999), where it was shown to be regulated by HuR in a VHL-dependent manner (Danilin et al. 2009).

Although nuclear HuR expression has been shown to correlate with clinicopathological features, including stage and grade, and patient prognosis in gynaecological carcinomas (Lim et al. 2007, Yi et al. 2009), these observations were not replicated in our RCC material.
Based on the results of our study, HuR regulates COX-2 expression in RCC, which presumably explains the prognostic influence of HuR in this disease. However, HuR can also be connected to the pathogenesis of RCC via several other routes, as described above. The HuR protein has been suggested as a potential target in cancer therapy due to its presumed central role in carcinogenesis (López de Silanes et al. 2005, Lim et al. 2007). The antitumoural activity of miRNAs repressing HuR expression has been shown in breast carcinoma in vitro and in cervical carcinoma in vivo (Guo et al. 2009, Abdelmohsen et al. 2010). The therapeutic role of HuR inhibitors or antagonists should also be evaluated in RCC.

6.5.2 Cyclooxygenase-2

The expression of COX-2 is a common feature in RCC and is reported in over one half of RCC tumours (Miyata et al. 2003, Hashimoto et al. 2004, Tuna et al. 2004, Mungan et al. 2006). However, in our study population COX-2 expression was found less frequently than in previous studies. Part of this discrepancy could be explained by the cut-off values used, for example we classified weakly positive tumours as negative tumours, and partly by differences in the immunohistochemical procedures and study population. Our results were, however, in line with those of a previous Finnish study where COX-2 immunostaining was reported in 26% of primary RCC tumours (Kankuri-Tammilehto et al. 2010). The cytoplasmic expression pattern observed in our study was similar to previously reported patterns (Miyata et al. 2003, Tuna et al. 2004). The expression of COX-2 was less frequent in clear cell carcinomas than in other histological subtypes. Previously, Hashimoto et al. found that COX-2 expression is more common in the granular cell subtype than in clear cell variants (Hashimoto et al. 2004). As the classification “granular” is no longer recommended, it was not documented in our study protocol.

In our material, the expression of COX-2 was associated with clinicopathological variables related to advanced disease and reduced RCC-specific survival. The relationships observed between COX-2 immunoreactivity and various clinicopathological features such as TNM stage, tumour size and nuclear grade were also observed in several previous studies on RCC (Miyata et al. 2003, Hashimoto et al. 2004, Tuna et al. 2004, Rini et al. 2006), although not in every study (Yoshimura et al. 2004, Cho et al. 2005, Kankuri-Tammilehto et al. 2010). In light of the results from previous studies, the prognostic potential of
COX-2 seems to be contradictory in RCC. Our material appeared to show that COX-2 immunoreactivity is not an independent prognostic factor for RCC-specific survival, as found in a previous study by Miyata et al. (Miyata et al. 2003). Several other studies were not able to prove a prognostic significance of COX-2 expression (Tuna et al. 2004, Cho et al. 2005), although an association was reported between COX-2 expression and more favourable overall survival in patients with metastatic RCC treated with immunotherapy (Kankuri-Tammilehto et al. 2010). However, a comparison of these earlier studies is unreliable because of the different study populations and study protocols used, as well as variable immunohistochemical procedures and cut-off values. To the best of our knowledge, our study population was the largest so far for this type of immunohistochemical study and our follow-up time was considerably longer than in most previous studies.

We observed an inverse relationship between COX-2 and membranous E-cadherin expression, which could reflect the previous observation that the down-regulation of COX-2 alters cell adhesion molecules such as E-cadherin and inhibits invasion in RCC (Chen et al. 2004a). Thus, this finding of an association between COX-2 and E-cadherin expression warrants further investigation. It has been hypothesized that beta-catenin could activate COX-2 expression through transcription factor LEF-1 (Prescott & White 1996). In colorectal carcinoma, APC mutations have been shown to be correlated with both the up-regulation of COX-2 and increased nuclear beta-catenin expression (Dimberg et al. 2001). In our study, however, no association was found between COX-2 and beta-catenin expression.

### 6.6 Toll-like receptor 9 (IV)

The idea of studying TLR9 expression and its relationship to patient survival arose from previous observations in other types of cancer where TLR9 expression has been shown to be related to higher grades and the invasiveness of cancer (Ilvesaro et al. 2007, Jukkola-Vuorinen et al. 2008, Väisänen et al. 2010).

The cytoplasmic expression pattern of TLR9 observed in the present study corresponds to expression patterns reported in other cancers (Jukkola-Vuorinen et al. 2008, Väisänen et al. 2010). The nuclear TLR9 expression observed in the present study was not reported previously and might represent unspecific staining.

The expression of TLR9 was related to a more favourable clinical outcome in RCC. Furthermore, it was not associated with the clinicopathological parameters
and, based on the results of our study, TLR9 expression is indeed an independent prognostic factor for RCC-specific survival. Although there were a few previous studies on the prognostic value of TLR9 expression in cancer, these studies mainly connected TLR9 with adverse clininopathological features of cancer such as poor differentiation (Jukkola-Vuorinen et al. 2008, Berger et al. 2010, Väisänen et al. 2010, Wang et al. 2010). Moreover, the mechanism by which TLR9 stimulation promotes cancer cell invasion through the up-regulation of MMP activity in brain, breast, lung and prostate cancers in vitro has also been determined (Merrell et al. 2006, Ilvesaro et al. 2007, Ren et al. 2007). In addition, the stimulation of TLR9 has been shown to promote cell proliferation, inhibit apoptosis and regulate several genes and transcription factors related to tumorigenesis and cancer progression in hepatocellular carcinoma (Tanaka et al. 2010). In glioma, TLR9 expression is associated with poor patient outcome (Wang et al. 2010).

It is evident that the function of TLR9 expression differs in RCC from that of other cancers as the lack of TLR9 confers aggressive behaviour to renal carcinoma cells. However, some parallel observations have been reported in other malignomas. In recurrent breast carcinoma, TLR9 expression in fibroblast-like cells has been shown to be correlated with a low probability of metastasis (González-Reyes et al. 2010) and, in neuroblastoma, TLR9 expression is inversely associated with disease stage (Brignole et al. 2010). Although TLR9 expression has been connected with higher nuclear grade and although TLR9 stimulation has been shown to induce cancer invasion in prostate carcinoma, its activation by immunomodulatory nucleotides was reported to induce apoptosis in vitro and to cause the regression of tumour growth in an in vivo xenograft model of prostate cancer (Rayburn et al. 2006). Thus, the contribution of either high or low TLR9 expression to the pathophysiology of cancer may be highly tumour specific.

The immunogenic nature of RCC is generally recognized. As cytokines such as IFN-α and IL-2 can produce complete and durable responses in metastatic RCC (Vogelzang et al. 1992, Finley et al. 2011), one might hypothesize that the immune response generated by ligand binding to TLR9 is the mechanism by which TLR9 expression results in more favourable survival in RCC. Upon recognition of the CpG-DNA ligand, TLR9 recruits specific intracellular adaptor proteins to initiate signalling pathways that activate transcription factors such as NF-kappaB. The eventual outcome is an immune reaction characterized by the stimulation of B-cell proliferation, the activation of macrophages and dendritic
cells and the production of inflammatory mediators such as interferon, interleukins and other inflammatory cytokines. (Akira & Hemmi 2003, Wagner 2004, Kawai & Akira 2008) We suggested that in the absence of TLR9 expression such immune responses are not evoked in RCC cells and that cancer cells are less susceptible to immunosurveillance and can progress further. Further studies are needed to clarify the mechanism and the role of TLR9 expression in RCC. The underlying yet hypothetical mechanisms of TLR9 activation should be examined in the future.

Hypoxia and the compensatory hyperactivation of angiogenesis are important features in the pathogenesis of RCC tumours (reviewed in Banumathy & Cairns 2010). Hypoxia has been shown to increase the expression of various TLRs including TLR9 (Kuhlicke et al. 2007, Liu et al. 2009). Moreover, HIF-1 has been shown to coordinate the induction of TLRs (Kuhlicke et al. 2007). Although in our study population TLR9 expression and tumour necrosis were not found to be associated, it would be interesting to discover whether or not hypoxia and HIF-1 regulate TLR9 expression in RCC cancer cells.

The prognostic role of TLR9 evokes expectations of the therapeutic potential of TLR9 agonists in RCC. Synthetic TLR9 agonists are being examined as anti-tumour immunomodulants against various cancers and they seem to have potential as anticancer treatments in preclinical and early clinical trials (Krieg 2008). However, a synthetic TLR9 agonist only showed modest antitumour activity in advanced RCC (Thompson et al. 2009). It would be reasonable to study whether or not there is difference in the response rates between TLR9-expressing and non-expressing tumours.

6.7 Markers of neuroendocrine activity (V)

Neuroendocrine cells are involved in the regulation of cell growth and differentiation and neuroendocrine products may stimulate cell proliferation and tumour growth (Abrahamsson 1999, Bostwick et al. 2002, Erickson & Lloyd 2004). In some cancers, neuroendocrine differentiation has been shown to have some prognostic impact (Syversen et al. 1995, Abrahamsson 1999). These observations were the rationale for studying neuroendocrine activity as a prognostic factor for RCC.

Serotonin immunoreexpression is absent in metastatic RCC (Edgren et al. 1996), which was confirmed in our study population (data not shown). In light of the results of previous studies, the prognostic impact of serotonin seems to be
contradictory in cancer. In prostate cancer, both increased and decreased serotonin immunoexpression have contributed to advanced or aggressive disease (Bostwick et al. 2002, Dizeyi et al. 2004). On the one hand, by reducing tumour blood flow, presumably through its vasoconstrictive effect, serotonin inhibits tumour growth (Vicaut et al. 2000). On the other hand, serotonin is a growth factor either directly or through synergy with other growth factors and it stimulates DNA synthesis and cell proliferation and differentiation (Vicaut et al. 2000, Siddiqui et al. 2005). The tumour growth promoting impact of serotonin has been established in several malignancies (Vicaut et al. 2000) and 5HT antagonists, in turn, inhibit growth and cell division in a variety of tumours (Tutton & Barkla 1987, Siddiqui et al. 2005). In high-grade prostate carcinoma, serotonin stimulates cancer cells and serotonin receptors are overexpressed. In prostate cancer cell lines serotonin activates PI3K/Akt signalling pathways, for example. (Dizeyi et al. 2004, Dizeyi et al. 2011) However, the tumour-promoting effect seems to be dose dependent. High doses of serotonin may have a direct mitogenic effect on tumour cells whereas low doses may reduce tumour growth (Vicaut et al. 2000). Suppressed plasma serotonin levels have been detected in metastatic RCC (Jungwirth et al. 2008), as well as a correlation between elevated serotonin plasma levels and longer survival (Edgren et al. 1996). In a way, our observation of longer RCC-specific survival being related to serotonin immunoexpression supports these previous reports. It is possible that our population was too small and serotonin expression too rare to show statistically significant differences in the survival analysis. However, based on our results and on previous observations of metastatic RCC, the role of serotonin in RCC seems to be more of a suppressive one than a promotional one.

We confirmed a previous observation of CD56 expression in less than 20% of RCCs (Daniel et al. 2003). There are some observations in the literature linking CD56 expression to unfavourable clinical outcomes of cancer, for example metastasis (Koukoulis et al. 1998, Daniel et al. 2000, Daniel et al. 2001). In RCC, CD56 immunopositivity is associated with advanced stage, aggressive Fuhrman grade and poor survival rates (Daniel et al. 2003). In our study population the relationship between CD56 expression and a higher T class was almost significant (p=0.05) and the survival analysis suggested an unfavourable RCC-specific prognosis in CD56-immunopositive tumours.

Neurone-specific enolase is the most sensitive and unspecific neuroendocrine marker. Based on the results of previous studies, NSE expression is extremely frequent in RCC which may indicate that NSE is produced by the RCC tumour itself (Haimoto et al. 1986, Edgren et al. 1996, Rasmuson et al. 1999). The
discrepancy in NSE expression observed in the present study can be explained, at least partly, by the use of formalin-fixed, paraffin-embedded tumour samples compared to cryostat sections (Haimoto et al. 1986) and by a different stage distribution and larger study population (Edgren et al. 1996). In addition, these previous studies reported staining patterns other than the pure cytoplasmic staining found in our study. Nevertheless, NSE immunopositivity was more common in metastatic than localized RCC in our material, as in previous studies, although the difference was not statistically significant (data not shown). In RCC, an increase in serum NSE is a common phenomenon which is associated with advanced disease and a poor patient outcome. A decline in serum NSE can be observed after the treatment of RCC. (Haimoto et al. 1986, Takashi et al. 1989, Yaman et al. 1996, Rasmuson et al. 1999) As epithelial cells of the proximal tubules where RCC originates do not normally express NSE, NSE expression is believed to occur during renal oncogenesis (Haimoto et al. 1986). Although NSE expression was somewhat related to decreased RCC-specific survival in our study population, the immunoexpression of NSE does not seem to be a statistically significant prognostic factor in RCC based on our material.

We were not able to detect any chromogranin A immunoexpression, which is in line with the results of previous reports where absent or extremely rare chromogranin A expression was reported in RCC (Edgren et al. 1996, Rasmuson et al. 1999). Increased serum chromogranin A levels have been found in RCC patients, which presumably reflects sources of chromogranin A other than RCC tumours (Edgren et al. 1996, Rasmuson et al. 1999).

According to the results of our study specific neuroendocrine differentiation is rare in RCC since there were only two synaptophysin positive tumours (1%) in our material. To the best of our knowledge, this was the first study to detect the immunoexpression of synaptophysin in RCC. In a small previous study on metastatic RCC, no immunoreactivity was found for synaptophysin at all (Edgren et al. 1996). The synaptophysin-expressing tumours in the present study had TNM classifications of T1aN0M0 and T3aN0M0 and excellent RCC-specific survival as the patients were alive after 10 and 17 years of follow-up, respectively. There are no reports in the literature of an association between synaptophysin expression and more favourable cancer survival. Instead, synaptophysin immunoreactivity is associated with a higher tumour grade in breast carcinoma (Makretsov et al. 2003). In undifferentiated endometrial carcinoma, synaptophysin expression did not seem to have any prognostic value (Taraif et al.}
2009). It would be fascinating to discover whether this indolent clinical behaviour is related to the neuroendocrine differentiation observed in these RCC tumours.

### 6.8 Challenges in searching for prognostic biomarkers for renal cell carcinoma

There are several factors that could explain the difficulties encountered in attempting to discover prognostic biomarkers suitable for clinical practice in RCC. The studies in this field are mostly single institutional retrospective studies with a limited number of patients and a relatively short follow-up period (Nogueira & Kim 2008, Volpe & Patard 2010). Compared to previous studies with a similar research framework and objective, the advantages of our study were its long and detailed follow-up period and the rather large study population. Due to the retrospective nature of our study not all anamnestic and clinical data were available afterwards, which may have biased our results, particularly concerning the prognostic value of preoperative symptoms and signs, as well as some of the laboratory findings. The style of documentation was more undefined in the 1990s than it is nowadays and, in some cases, collecting all of the data we required was laborious since several different sources had to be checked. However, the most relevant data were available and each patient was appropriately staged. In addition, the follow-up was completed in every case. Some discrepancies between our reports and previous studies can be explained by the different patient cohorts. In our material, stage and grade distribution deviated slightly from generally reported. Although the number of patients was rather large compared to other studies, the study cohort might still have been too small to distinguish minor statistical differences. However, the patient cohort was large enough to point out the most relevant prognostic factors, such as stage.

A fundamental problem in this kind of study is the lack of recognition of distinct histological subtypes of RCC in the analysis (Eichelberg et al. 2009). Although each histological subtype was analysed separately in our study, we primarily analysed the whole population mainly because of the low number of cases of each of the histological subtypes other than clear cell carcinoma. In our study population the results from the separate survival analysis were non-conclusive. The molecular markers we used are “generic” in carcinogenesis (Eichelberg et al. 2009), which justified studying them in the whole RCC population as well. According to the separate analyses performed, the biomarkers studied seemed to be suitable for prognostic factors regardless of the histological
subtype. In the future, study populations must be larger if separate analyses for papillary, chromophobic and collecting duct carcinomas are to be performed.

The traditional method used in this kind of study for investigating prognostic tissue markers is the immunohistochemistry of archival tumour material, which addresses certain concerns (Di Napoli & Signoretti 2009). The results of immunohistochemical studies can be influenced by numerous parameters from conditions during surgery such as anaesthesia, blood pressure variations, blood loss and renal artery clumping to variables in tissue handling, including preparation in the pathology department, such as room temperature, tissue drying and oxidation, fixation-related variables including the type of fixative, its volume and concentration, as well as specimen size, and variables during storage such as temperature and length (Di Napoli & Signoretti 2009, Eichelberg et al. 2009). In the immunohistochemical staining procedure there are numerous variables that are not standardized and can influence the immunoexpression of proteins of interest, such as the antibody used and its dilution, pretreatment and staining techniques (Chetty & Serra 2008, Volpe & Patard 2010). In archival tumour material the type and duration of fixation can influence antigen preservation and recognition by a specific antibody. Protein immunoexpression has been shown to be higher in fresh tumour material than in archival formalin-fixed, paraffin-embedded tissue samples. (Abrahamsson et al. 1987, Di Napoli & Signoretti 2009). Our stainings were performed in the paraffin-embedded and formalin-fixed archival material. As some previous studies used freshly harvested tumour specimens the results are not fully comparable. Antigen retrieval by heating and enzymatic treatment such as that used in the present study partly reverse the formalin-fixation effect (Di Napoli & Signoretti 2009). Although the maximum storage length was more than 20 years, the immunoexpression of the different markers studied was generally quite well preserved as the results were in line with those of previous studies. One concern regarding our study material was preoperative embolization, which was performed for one fourth of the patients and possibly somehow influenced the immunoexpression of the markers studied. However, the tumours were selected in the multitissue block from a vital, well-preserved tumour area.

Renal cell carcinomas are often large, necrotic and heterogeneous tumours and therefore the selection of the most representative tumour area for immunohistochemical study is not simple (Di Napoli & Signoretti 2009). The preparation of multitissue blocks demands patience, skill and experience. Due to technical aspects, tumour necrosis or cut-off of the tumour area, for example,
some samples are always lost during immunohistochemical procedures (sampling error). When multiple multitissue block series are prepared the tumour area is not identical in each slide. In the present study, some samples were excluded from the analysis of each series because of the above-mentioned reasons.

The evaluation and quantification of staining results can be important pitfalls in the immunohistochemical procedure. When an evaluation is performed by eye, it is not particularly reproducible or objective. Different scoring procedures lack standardization. (Di Napoli & Signoretti 2009, Volpe & Patard 2010) In addition, immunohistochemical assessments of proteins do not necessarily correlate with their biological activity (Frank et al. 2000). These sources of error also concern this study.

The lack of reproducibility was a fundamental problem among all of the studies that tried to discover a prognostic biomarker for RCC (Volpe & Patard 2010). Although one succeeded in finding an independent prognostic factor, the others were not able to replicate the results or observations were contradictory. Many of these above-mentioned problems can be solved by better study designs. There is a need for large, prospective, multicenter studies in which different histological types are separated (Eichelberg et al. 2009). The immunohistochemical procedures used should be standardized so that others can replicate the procedure and externally validate the results (Crispen et al. 2008, Lam et al. 2008). Although there is much criticism against retrospective, single institutional immunohistochemical studies they provide important basic information about potential prognostic tissue markers that could later be externally validated in a larger series.

6.9 Future aspects

Based on our results, the expression of Keap1, myosin VI, HuR and TLR9 should be evaluated in other RCC cohorts to assess their reproducibility and significance as prognostic markers in RCC. The future goal is to develop a prognostic tool that combines traditional prognostic factors such as stage and grade with the molecular and genetic profile of RCC tumours to provide prognostic information that is not available with classical prognostic factors alone or with current nomograms. We suggest that TLR9, HuR and myosin VI may be promising candidates for novel nomograms since, particularly when combined, they improved the prognostic accuracy of the multivariate survival analysis. Ongoing research recognizes the enormous amount of potential biomarkers, which first
have to be evaluated at a basic level in immunohistochemical studies such as ours, and then the most promising markers can be further re-evaluated and validated in a larger, prospective series. The biomarkers evaluated thus far are primarily based on their expression in tumour tissue; in the future, however, we should attempt to find markers that can also be used in blood or urine samples so that such analyses would be suitable for daily clinical practice, and also be cost-effective. The discovery of “PSA for RCC” needs diligent work and, among other things, a certain amount of luck, too.
7 Summary and conclusions

The traditional prognostic factors are not sufficient to accurately predict the clinical outcome of each RCC patient and there are no prognostic biomarkers for RCC suitable for daily clinical use. As we want to treat our patients in the best way possible, a more precise prognostication of individual patients than is currently possible is necessary. In this study, novel tissue markers were examined and evaluated as prognostic markers for RCC.

The main findings of the current study with 152 RCC patients are as follows:
1. The immunoexpression of Keap1 is related to advanced stage and poorer disease-specific survival in RCC.
2. Cytoplasmic myosin VI immunoexpression is an independent marker of reduced RCC-specific survival and is associated with lower nuclear grade. Nuclear myosin VI and beta-catenin are associated.
3. Cyclooxygenase-2 expression is regulated by HuR in RCC. Cytoplasmic HuR expression is associated with reduced RCC-specific survival.
4. The immunoexpression of TLR9 is common in RCC and does not associate with stage or grade. The lack of TLR9 expression predicts short RCC-specific survival and TLR9 expression is an independent prognostic biomarker in RCC.
5. Although neuroendocrine activity is detectable in RCC, the markers studied, i.e. serotonin, CD56, NSE, chromogranin A and synaptophysin, are not potential prognostic markers in RCC.

In conclusion, several potential prognostic tissue markers for RCC were identified in this study. In particular, cytoplasmic TLR9 and myosin VI, which were acknowledged as independent prognostic factors for RCC-specific survival, should be further investigated in large, prospective series and be validated externally. As RCC is an immunogenic malignancy, the more favourable prognosis associated with TLR9 expression is intriguing and warrants further investigation. The underlying, presumably immunologic responses should be determined. In addition, it should be discovered whether or not TLR9 expression predicts the therapeutic response of TLR9 agonist treatment in advanced RCC. Myosin VI predicted the outcome of RCC-patient also in the subgroup of well-differentiated tumours, which are thought to have a favourable prognosis, making it an interesting marker from a clinical point of view. The central role of HuR in carcinogenesis and its prognostic significance we observed in RCC are also fascinating and may contribute to the therapeutic aspects of RCC.
So far, the prognosis of an individual RCC patient is mainly determined by staging in clinical practice. Although we introduced several potential biomarkers that could help, especially when combined, to predict the clinical outcome of RCC, based on the results of the present study cancer stage is still the most important single prognostic factor for RCC. Thus, all proposed novel prognostic factors should be compared to stage and proven to possess more predictive accuracy than staging.
References


Apopa PL, He X & Ma Q (2008) Phosphorylation of Nrf2 in the transcription activation domain by casein kinase 2 (CK2) is critical for the nuclear translocation and transcription activation function of Nrf2 in IMR-32 neuroblastoma cells. J Biochem Mol Toxicol 22(1): 63–76.


Merikallio H, Pääkkö P, Kinnula VL, Harju T & Soini Y (2011) Nuclear factor erythroid-derived 2-like 2 (Nrf2) and DJ1 are prognostic factors in lung cancer. Hum Pathol Epub Sep 22.


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