PROGNOSTIC FACTORS IN RENAL CELL CARCINOMA

Evaluation of Erythropoietin and Its Receptor, Carbonic Anhydrase IX, Parathyroid Hormone-related Protein and Osteopontin

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To all patients with Renal Carcinoma and their families

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PAPER I-IV

ABSTRACT

A prognostic factor is a marker or a feature that can be used to estimate the risk of recurrence of disease, metastatic spread and clinical outcome. Despite intensive search for more sophisticated markers in renal cell carcinoma (RCC), few have added prognostic information to earlier described factors like stage of disease, nuclear grade, tumour type, and in metastatic disease: performance status, anaemia, hypercalcaemia and increased erythrocyte sedimentation. In the dominating tumour type, clear cell renal RCC (cRCC), hypoxia is common, leading to an up-regulation of hypoxia inducible factor (HIF). The majority of cRCC have a mutation in the von Hippel Lindau gene (VHL-gene), which regulates HIF and in turn leads to up-regulation of a number of target genes for potential growth factors. The aim of the study was to evaluate the possible prognostic information of a few factors associated to pVHL/HIF, anemia and/or hypercalcaemia in RCC; Erythropoietin (EPO) and it´s receptor (EPO-R); carbonic anhydrase IX (CA IX); parathyroid hormone-related protein (PTHrP) and osteopontin (OPN).

Patients diagnosed with RCC between 1982-2007 were included in the studies. The tumour tissue expressions of EPO, EPO-R and PTHrP were assessed using immunohistochemistry. Serum/plasma levels of EPO, CA IX, PTHrP and OPN were also analyzed using immunometric methods.

Our study demonstrated that the expression of EPO and EPO-R were related, and the expressions differed significantly between RCC types. The serum EPO levels did not associate to the tumour expression of EPO or EPO-R, indicating that circulating EPO derives from other sources than tumour cells. EPO-R expression was more frequent in advanced stages of disease, but neither EPO, nor EPO-R, were independent prognostic factors for survival.

Serum CA IX levels were higher in cRCC compared to papillary RCC (pRCC). In cRCC, the CA IX serum levels correlated positively to TNM stage, but serum CA IX did not add independent prognostic information.

PTHrP is a cause of hypercalcaemia in malignancy, and we observed that circulating PTHrP related to hypercalcaemia in RCC. The tumour expression of PTHrP associated positively to serum PTHrP, but not to serum calcium. We found an association between PTHrP and OPN in plasma, and both plasma PTHrP and OPN were positively associated to TNM stage. Neither serum/plasma PTHrP nor tumour expression of PTHrP were independent prognostic factors for survival. The serum OPN levels were higher in pRCC but no impact on survival was observed in this RCC type. In contrast, plasma/serum OPN was an independent prognostic factor for disease-specific survival in cRCC.

Our results support a role for these factors in RCC. The expressions vary between tumour types, which can be explained by different gene aberrations. Some of the factors have a close relation to para-malignant symptoms like hypercalcaemia. Most of the factors correlate positively to TNM stage, reflecting a relation to advanced disease. Although expression of EPO, EPO-R, PTHrP and CA IX did not add independent prognostic information, the results might contribute to greater understanding of important mechanisms and associations in RCC.

Osteopontin is a strong independent prognostic factor in cRCC, and should be further evaluated as a tool in the clinic when treating RCC patients.

Keywords: Renal cell carcinoma, EPO, EPO-R, CA IX, PTHrP, OPN, prognosis

SVENSK SAMMANFATTNING

Njurcancer drabbar ca 1000 personer varje år i Sverige. Medelåldern vid insjuknande är vanligtvis 60-65 år men kan också drabba yngre personer och även barn. Det är ungefär en och en halv gång så vanligt hos män som hos kvinnor. När sjukdomen är begränsad till njuren är kirurgi den viktigaste behandlingen och kan leda till bot. Vid spridd njurcancer kan medicinska behandlingar vara effektiva för att bromsa sjukdomen eller lindra symtom. Förloppet vid spridd sjukdom är väldigt olika hos olika personer, vissa kan leva i flera år med eller utan behandling, medan överlevnaden för andra är kort.

En prognostisk faktor är en markör eller egenskap som kan användas till att uppskatta risken för återfall av en tumörsjukdom, liksom risken för att utveckla dottertumörer och ha betydelse för överlevnaden. Trots intensivt letande efter mer specifika markörer vid njurcancer så har få tillfört prognostisk information till tidigare beskrivna faktorer såsom tumörtyp, tumörutbredning vid diagnos, tumörcellsmognad, hemoglobin- och kalkvärde i blodet, sänka och dåligt allmäntillstånd.

Vid njurcancer är det vanligt med syrebrist i delar av tumören. Lågt syretryck leder till uppreglering av ett protein kallat hypoxi-inducerad factor (HIF). HIF regleras av von Hippel Lindau-proteinet (pVHL) genom att pVHL binder till HIF som snabbt sönderfaller. Detta sker inte i syrefattig miljö vilket gör att HIF då ökar. Dessutom är ofta genen som kodar för pVHL muterad eller tystad vid klarcellig njurcancer vilket också leder till att HIF ökar. HIF i sin tur stimulerar andra gener som leder till ökad produktion av ett flertal proteiner som kan ha betydelse för tumörcellsöverlevnad, kärl- och tumörtillväxt och tumörspridning.

Målet med våra studier var att studera betydelsen för prognosen av några av dessa proteiner som helt eller delvis regleras av pVHL och HIF: Erytropoetin (EPO) och dess receptor (EPO-R), Karbanhydras IX (CA IX), Parathyroideahormon-liknande protein (PTHrP) och Osteopontin (OPN).

I studierna som ingår i avhandlingen har tumörvävnad och blodprover använts från patienter som diagnosticerades med njurcancer i Umeå mellan 198 och 2007. Information om ålder, kön, tumörtyp, sjukdomsutbredning, tumörcellsmognad, rutinblodprover, kirurgisk behandling, sjukdomsåterfall och överlevnad inhämtades för statistisk analys.

För att kunna analysera ett stort antal tumörvävnader gjordes så kallade tissue microarrayer (TMA), vilket innebär att man samlar flera små tumörbitar från olika tumörer på samma glas för färgning och mikroskopisk bedömning. Uttrycket av EPO, EPO-R och PTHrP i tumörvävnad analyserades genom immunhistokemisk färgning och blodnivåer av EPO, CA IX, PTHrP och OPN mättes.

Vi fann att de flesta tumörer uttryckte EPO och EPO-R och att det fanns ett samband mellan uttrycken i tumör men inte till EPO-nivåerna i blodet. Detta kan bero på att EPO som mäts i blodet produceras av andra celler än tumörcellerna. Det var också skillnad mellan uttrycken av EPO och EPO-R i olika njurtumörtyper. Starkt uttryck av EPO-R korrelerade till sjukdomsutbredning och patienter vars tumörer hade ett starkt uttryck hade tendens till kortare överlevnad. Varken uttryck av EPO, EPO-R i tumörer eller EPO nivåer i blod var oberoende prognostiska faktorer.

I tidigare analyser av CA IX-uttryck i njurtumörer har man konstaterat att patienter med lågt tumöruttryck har sämre prognos och att starkt uttryck är vanligare vid klarcellig njurcancer. Vi analyserade CA IX i blod och fann att värdet var högre vid klarcellig än vid papillär njurcancer, vilket överensstämmer med tidigare resultat i tumörvävnad. Nivåerna var högre vid utbredd tumörsjukdom men det förelåg ingen skillnad i överlevnad mellan patienter med höga och låga CA IX-nivåer i blod.

PTHrP anses vara den vanligaste orsaken till förhöjda kalciumnivåer i blodet vid cancer.

Högt kalciumvärde är en känd negativ prognos-

tisk faktor vid spridd njurcancer. Därför användes också kalciumnivåer som mätts vid diagnos för jämförelse med uttryck av PTHrP i tumörvävnad och blod. I ca hälften av tumörerna uttrycktes PTHrP men i blodet var PTHrP endast förhöjt hos ca 15% av patienterna och förhöjda kalciumnivåer sågs också hos ca 15%. Det fanns ett positivt samband mellan PTHrP och kalcium i blodet vilket talar för att PTHrP är en av orsakerna till förhöjt kalcium vid njurcancer. Däremot hade uttrycket av PTHrP i tumörerna inget samband till kalciumnivåerna i blodet. Höga kalcium- och PTHrP-nivåer i blodet var vanligare vid avancerad tumörsjukdom medan uttryck av PTHrP i tumör var vanligare vid begränsad sjukdom. Skillnaden mellan tumöruttryck, blodnivåer av PTHrP och kalcium kan tyda på att PTHrP i blodet delvis bildas av andra celler än tumörceller.

I djurmodeller har man tidigare sett ett samband mellan PTHrP och OPN och OPN har visat sig ha prognostisk betydelse vid flera tumörsjukdomar. Detta ledde oss till att även analysera OPN i blodet och jämföra med våra PTHrP-data.

Vi kunde visa att det fanns ett samband mellan nivåerna av OPN och PTHrP i blodet vilket kan tala för att de regleras av samma mekanismer eller att det ena proteinet stimulerar till produktion av det andra. Vidare fann vi att OPN hade ett starkt samband till avancerad sjukdom och att patienter med höga OPN-nivåer i blodet hade kortare förväntad livslängd. Dessutom var OPN en oberoende prognostisk faktor vid klarcellig njurcancer.

Sammanfattningsvis stödjer våra resultat att de studerade faktorerna har en roll vid njurcancer. Det finns en skillnad mellan tumöruttryck och nivåer i blod mellan olika njurtumörtyper vilket talar för att olika mekanismer är av betydelse. Flera av faktorerna har ett samband med tumörutbredning vilket speglar en relation till avancerad sjukdom. Även om EPO, EPO-R, CA IX och PTHrP inte gav någon oberoende prognostisk information kan våra resultat förhoppningsvis tillföra ökad kunskap om mekanismer och samband vid njurcancer. Osteopontin är en stark oberoende prognostisk faktor vid klarcellig njurcancer och bör utvärderas ytterligare.

ABBREVIATIONS

AKT	protein kinase B
bFGF	basic fibroblast growth factor
BRAF	serine/threonine-protein kinase
CA IX	carbonic anhydrase 9
CD	cluster of differentiation
chRCC	chromophobe renal cell carcinoma
cKIT	cytokine receptor: CD117
cRCC	clear cell renal cell carcinoma
CT	computed tomography
DAB	3,3`-diaminobenzidine
EDTA	ethylenediaminetetraacetic acid buffer
ELISA	enzyme-linked immuno-sorbent assay
EpCAM	epithelial cell adhesion molecule
EPO	erythropoietin
EPOR	erythropoietin receptor
Flt	FMS-like thyrosine kinase
GLUT	glucose transporter protein
Hb	haemoglobin
HIF	hypoxia-inducible factor
HLPRCC	hereditary leiomyomatosis papillary renal cell carcinoma
HPRCC	hereditary papillary renal cell carcinoma
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
IRMA	immuno-radiometric assay
kDA	kilodalton
LPS	lipopolysaccharide
MAP	mitogen-activated protein
MET	proto-oncogene
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
MVD	microvessel density
OPN	osteopontin
PCNA	proliferating-cell nuclear antigen
PDGF	platelet-derived endothelial growth factor
PDGFR	platelet-derived endothelial growth factor receptor
PI3K	phosphatidylinositol 3-kinase
pRCC	papillary renal cell carcinoma
PTEN	phosphatase and tensin homolog
PTH	parathyroid hormone
PTHR	parathyroid hormone receptor
PTHrP	parathyroid hormone-related protein
pVHL	von Hippel Lindau protein
RCC	renal cell carcinoma
rhEPO	recombinant human erythropoietin
s-Ca	serum calcium
SIBLING	small integrin-binding ligand N-linked glycoprotein
STAT	signaltransducer and activator of transcript
TIMP 1	metalloproteinase 1
TMA	tissue microarray
TNF	tumour necrosis factor
TNM	tumour-node-metatasis
UICC	union for international cancer control
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VHL	von Hippel Lindau

ORIGINAL PAPERS

This thesis is based on the following papers, and referred to by their roman numerals in the text:

I *Parathyroid hormone-related protein and serum calcium in patients with renal cell carcinoma*
Karin Papworth, Kjell Grankvist, Börje Ljungberg, Torgny Rasmuson
Tumour Biology 2005;26:201-206

II *Expression of erythropoietin and its receptor in human renal cell carcinoma*
Karin Papworth, Anders Bergh, Kjell Grankvist, Börje Ljungberg,
Torgny Rasmuson
Tumour Biology 2009;30:86-92

III *Soluble carbonic anhydrase IX is not an independent prognostic factor in human renal cell carcinoma*
Karin Papworth, Johanna Sandlund, Kjell Grankvist, Börje Ljungberg,Torgny Rasmuson
Anticancer research 2010;30:2953-2958

IV *Osteopontin and parathyroid hormone-related protein in human renal cell carcinoma*
Karin Papworth, Anders Bergh, Kjell Grankvist, Börje Ljungberg, Johanna Sandlund
and Torgny Rasmuson
Manuscript

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INTRODUCTION

It is now almost 200 years since renal carcinoma was first described by Miril and Koenig.

Since then, this malignancy has been better characterised into different histological types, genetic mutations associated with the disease, hereditary predisposition, risk factors for developing renal cancer and prognostic factors. In many other cancers, specific markers in tumour and serum have been discovered and are used in daily practice for diagnosis, prognosis estimation, choice of and evaluation of treatment. Renal cell carcinoma presents with symptoms in an advanced stage and once spread, the disease is incurable. The natural progress is diverse; some patients live for many years without treatment whereas, in others, the survival time is very short. Specific markers giving more information about the outcome have, up to date, been limited. In the last few years, some progress has been made in the therapy of unresectable disease. However, there is still a great lack in knowledge concerning what factors are important for the prognosis, choice of treatment and therapy response. This work is an evaluation of some tumour-associated proteins as potential prognostic biomarkers in renal cell carcinoma.

Epidemiology

Renal cell carcinoma (RCC) accounts for about 2-3% of all cancer. In Sweden, there are about 1000 new cases each year and about 550 deaths from the disease. It is almost twice as common in men as in women and the incidence was in 2008 14/100 000 cases for men and 8/100 000 cases for women[1]. The median age at diagnosis is approximately 60-65 years, but the disease also appears in younger people and even in children. The incidence is increasing worldwide with approximately 2% yearly, though a decrease has been seen in Sweden. The reason for this decline is not fully understood.

Risk factors

Several risk factors for the development of RCC have been identified. The incidence rate of renal cell carcinoma differs internationally which could be due to a strong role for exogenous factors [2]. No single risk factor explaining the development of all renal cell cancers has been found.

Cigarette smoking has been identified as a risk factor in RCC, and the risk increases with dose. In heavy smokers, the risk is up to 2-fold compared to non-smokers[3]. The mechanisms are not yet clarified, but mutagens have been found in the urine of smokers, and the excretion correlate to the tar content [4].

Obesity is another independent risk factor described in several epidemiological studies, and the risk increases with an increased body mass index, both in men and women[5,6]. As with smoking, the mechanisms are not fully understood. It has been suggested that changes in levels of steroid-hormones, insulin-like growth factor-1, cholesterol, vitamin D, adipose tissue derived cytokines and hormones, caused by obesity, are all involved in cancer development[7,8].

Hypertension has also been identified as a risk factor in several studies, but few of these have included women, and because of the extensive use of anti-hypertensive medication, the results have been difficult to interpret. An American case-control study showed that the risk was associated to high blood pressure in both men and women, neither group having been under anti-hypertensive treatment[9]. In a European prospective study, including both men and women, hypertension was found to be an independent risk factor when adjusted for anti-hypertensive medication, and the risk increased with both increased diastolic and systolic blood pressure[10]. Hypothesis concerning the mechanisms include increased sensitivity to carcinogens due to functional changes in the renal tubules and up-regulation of hypoxia-inducible factors (HIF). These established risk factors, cigarette smoking, obesity and hypertension can explain less than half of all RCC.

The roles of other earlier described risk factors like analgesics, exposure to asbestos, gasoline or trichloroethylene, and protective effects of alcohol, fruit and vegetables are less clear[2].

Heredity and genetics

Most renal cell carcinomas are sporadic, but can also occur in hereditary forms.

Approximately 3-4% can be explained by genetic predisposition[11]. Hereditary renal cancer forms often occur earlier in life and are more frequently multiple and bilateral than in sporadic cancers. In the last decade, a number of syndromes caused by mutations in genes important for renal cancer development have been discovered. Tumour-suppressor genes control cell growth, and a mutation in these genes can lead to dysfunction and imbalance in cell-growth control and in turn to cancer development. Proto-oncogenes stimulate cell growth and are normally well regulated. A mutation in these genes can promote cancer development by damaged control mechanism or up-regulation of gene copies.

Von Hippel Lindau (VHL) disease is a syndrome that leads to clear cell RCC (cRCC), as well as neoplasm in the brain, spinal cord, eye, pancreas, epididymis and adrenal glands. It is associated with mutation in the tumour-suppressor gene, VHL, which is linked to the short arm on chromosome 3, and characterized by loss of chromosomal sequences[12,13]. This mutation is also common in sporadic cRCC.

In papillary RCC (pRCC), two hereditary forms have been identified; hereditary papillary renal cell cancer (HPRCC), associated with a mutation of the MET proto-oncogene on chromosome 7[14], and hereditary leiomyomatosis papillary renal cell carcinoma (HLPRCC), associated with the fumarate hydratase gene on chromosome 1[15]. Furthermore, loss of the Y chromosome, and trisomies of chromosomes 3q, 7, 8, 16, 17 and 20 are common in pRCC[16].

Chromophobe RCC (chRCC) and other renal tumour types, are seen in Birth-Hogg-Dube´syndrome, where a mutation in the Folliculin-gene on chromosome 17 has been found[17]. Familial renal oncocytoma has also been described[18].

Molecular biology of RCC

As discussed above, a number of mutations in proto-oncogenes and tumour suppressor genes have been identified in RCC. Up to 70% of sporadic clear cell RCC have a VHL somatic mutation and loss of heterozygosity[19,20]. The VHL-gene product, the VHL-protein (pVHL), is a regulator of the hypoxia inducible transcription factors (HIF)[21]. When normal oxygen tension is experienced in the microenvironment, pVHL binds to the HIF-α subunits, leading to degradation of the subunits. When cells experience low oxygen tension, hypoxia, no binding of pVHL occurs and the HIF levels increase. Different HIF-α subunits have different functions[22]. HIF-1α forms a complex with HIF-1ß, which leads to expression of a number of target-genes including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), endoglin, erythropoietin (EPO), glucose transporter protein-1 (GLUT-1), carbonic anhydrase IX (CA IX)[22, 23,24] (figure 1). These products are involved in angiogenesis, erythropoiesis, cell survival or death, metabolism, regulation of pH, adhesion, extracellular remodelling and migration. HIF-2α up-regulates other target genes, such as parathyroid hormone-related protein (PTHrP)[25].

A mutation in the VHL-gene results in deficient regulation of HIF and in turn up-regulation of HIF target genes.

Hypoxia is a common feature in RCC as well as in other solid tumours, and is associated with tumour progression, metastasis, resistance to chemo- and radiotherapy and poor prognosis[26,27,28,29]. HIF-1α has a key role in the adaption to a hypoxic environment.

Osteopontin (OPN) is another hypoxia inducible gene of importance with multi-functions involved in cell migration, cell survival, regulation of immune cell function and tumour metastasis[30,31].

Although HIF-regulation probably is the most important function of pVHL it is apparently not unique. Von Hippel Lindau-protein has been demonstrated to negatively regulate the transcription of PTHrP at posttranscriptional level[32].

In pRCC, mutation in the MET-gene is of importance, leading to constitutive activation or up-regulation of the MET tyrosine kinase receptor. Its signalling results in activation of the Ras/Raf/Mek and PI3K pathways and activation of gene transcription factors, which promote cell proliferation, invasi-

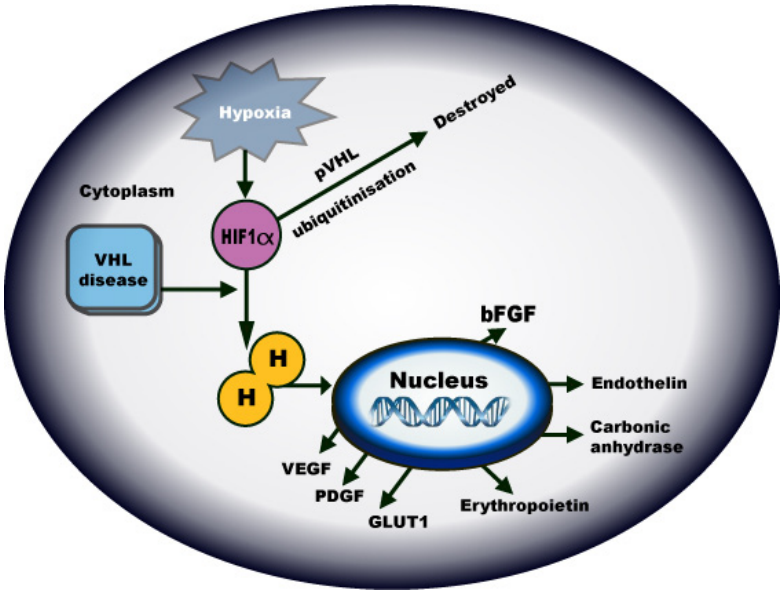


Figure 1. Mechanisms in RCC. Hypoxia or defect pVHL leads to increased levels of HIF and up-regulation of target-genes and synthesis of growth factors.
Illustration by Nigel Papworth

on and cell survival[33]. Furthermore, hypoxia activates the MET promoter via HIF, leading to up-regulation of the receptor. In HLPRCC, loss of fumarate hydratase function, an enzyme involved in the Kreb´s cycle, leads to HIF stabilization[34,35].

Molecular markers in the present study

EPO and EPO-R

Erythropoietin (EPO) is a glycoprotein consisting of 165 amino acids. The protein is synthesised in response to low oxygen tension in the kidney and is regulated by HIF and upregulated in anaemia[36,37]. The protein is a haematopoietic growth factor controlling red blood cell production by promotion of cell survival, proliferation and differentiation of erythroid progenitors[38,39]. Erythropoietin acts by binding the EPO-receptor (EPO-R), a receptor belonging to the type 1 cytokine receptor family. This induces intracellular signalling via the Ras/MAP pathway, involved in cell proliferation[40]. Other pathways activated by the EPO-R have also been described such as signal transducer and activator of transcription (STAT)[36,41]. The receptor is located on haematopoietic cells and also on other cells such as renal cells, megakaryocytes, placenta, vascular endothelial cells, skeletal myoblasts, cardiac myocytes, and neural cells[42,43,44,45,46,47]. Furthermore, EPO and EPO-R are expressed in a broad spectrum of tumour cells, and can stimulate tumour growth and angiogenesis and down-regulate apoptosis[48,49,50,51,52].

In RCC, increased serum levels of EPO have been demonstrated, and 1-5% of the patients present with erythrocytosis, which may be due to tumour EPO production[53]. The production of EPO in cultured RCC cells has been demonstrated in several studies[54,55,56], and EPO-R-expression has also been demonstrated in RCC, as well as co-expression of EPO and EPO-R[57,58,59]. Previous studies have indicated that both increased serum EPO levels and immunohistochemical expression of EPO in RCC are associated with worse prognosis[60,61].

The use of recombinant human EPO (rhEPO) in the treatment of anaemia in cancer patients has been debated and has raised concern. A randomized, doubleblind, trial in anaemic head-and-neck

cancer patients receiving radiation therapy showed that local control and survival decreased in the group treated with rhEPO[62]. In a trial investigating the benefit of rhEPO treatment in non-anaemic metastatic breast cancer patients, a higher mortality was seen in the treatment group[63].

CA IX

Carbonic anhydrase IX (CA IX) is a membrane bound zinc metallo-enzyme consisting of 459 amino acids with a large extracellular N-terminal part and an intracellular C-terminal end[64]. The enzyme regulates pH by converting carbon dioxide to carbonic acid at the extracellular side. This results in maintenance of a neutral intracellular pH, which protects the cell from death, and persistent acidosis in the extracellular microenvironment, which promotes production of growth factors, increases genomic instability, disturbs cell-cell adhesion and facilitates tumour spread[65]. Carbonic anhydrase IX is often expressed in hypoxic areas and the most important regulator is HIF-1 transcription factor[66]. As mentioned above, HIF-1α is controlled by pVHL, and loss of functional protein influences CA IX expression.

Carbonic anhydrase IX has other functions besides enzyme activity, it is also a cell adhesion molecule that mediates attachment to non-adhesive solid support and is involved in intercellular adhesion[67,68]. Moreover, CA IX appears to act in a growth factor manner by phosphorylation and activation of the AKT-mTOR pathway, which induces HIF-1α and in turn CA IX expression[69]. In normal tissue, CA IX is only sparsely expressed and limited to a few organ sites; the mucosa of the stomach and gallbladder, in lower levels in the intestinal epithelium, epithelia of the pancreatic ducts, and the male reproduction organs [70,71,72].

Carbonic anhydrase IX is more frequently expressed in tumours including carcinomas of breast, cervix uteri, oesophagus, head-and-neck, lung and kidney[73,74,75,76,77]. In the corresponding normal tissue the CA IX expression is low.

In a number of malignancies increased CA IX expression has been correlated to poor prognosis[78,79]. The tumour expression of CA IX in RCC has been reported in several studies and the expressions differ between renal tumour types[69,80,81]. High CA IX expression has, in contrast to other cancers, been associated with a more favourable outcome in clear cell RCC, although the prognostic value has been evaluated with diverting results[81,82,83,84].

Even though CA IX is a cell membrane bound enzyme, a soluble form has been identified in culture medium and can be measured in blood and urine in humans. Elevated levels have been reported in cRCC[85]. In addition, Li *et al.* showed that high levels of serum CA IX in cRCC were correlated to short recurrence-free survival[86].

PTHrP

Human parathyroid hormone-related protein (PTHrP) is a poly-protein presented in three isoforms consisting of 139-173 amino acids, and various peptides are generated through post-translational proteolysis[87]. The homology to parathyroid hormone (PTH) is located in the amino-terminal part where 8 of 13 amino acids are identical[88].

The protein derives from normal and malignant cells and is essential for foetal development[89,90]. The various peptides regulate a number of cellular functions including muscular relaxation of vascular and non-vascular smooth muscle, trans-epithelial calcium transport in placenta, renal tubules and breast, regulation of cell proliferation, cell differentiation and cell death[87,91]. The mechanisms of action are cell- and tissue specific, for example PTHrP can promote or suppress apoptosis depending on setting[92,93].

Parathyroid hormone-related protein is expressed /over-expressed in many different neoplasm in humans and has been demonstrated to stimulate adhesion, migration and proliferation and/or apoptosis in breast-, lung- and prostate cancer cells in animal models through interaction with the PTH1-receptor or by nuclear translocation of PTHrP[94,95,96].

In an animal model, Massfelder *et al.* showed that blocking of PTHrP or antagonizing the PTH1-receptor inhibited the expansion of human cRCC and total regression was seen in a large number of implanted tumours in nude mice[32]. This was demonstrated

to have been caused by apoptosis. Moreover, it was shown that pVHL negatively regulated PTHrP expression at post-transcriptional level, by decreasing mRNA stability.

Hypercalcaemia is a common paraneoplastic symptom in patients with RCC and is seen in approximately 3-15% of the patients. It is often associated with poor prognosis[97]. Parathyroid hormone-related protein is thought to be the main reason for hypercalcaemia in advanced malignant disease in absence of skeletal metastasis[98]; by binding the PTH1-receptor it stimulates bone resorption and the reabsorption of calcium in the kidney.

In clinical studies, conflicting results concerning the role of PTHrP for prognosis have been found. In breast cancer, expression of the protein is correlated to reduced disease-free survival, whereas in RCC tumour expression of PTHrP has been associated to better prognosis[99,100].

Osteopontin

Osteopontin (OPN) is a secreted phosphoprotein consisting of 314 amino acids. It was first characterized in 1979 in malignant epithelial cells[101]. It belongs to the small, integrin-binding, ligand N-linked glycoprotein family (SIBLING) including a number of proteins[102]. Osteopontin is expressed in several normal tissues such as bone, blood vessels, epithelial cells of the bronchi, gallbladder, gastrointestinal tract, inner ear, kidney, mammary gland, reproductive and urinary tracts, salivary glands and sweat ducts[103]. It is a ligand for the α_Vß integrin and CD44 receptor families, and activation leads to regulation of the formation and remodelling of mineralized tissue, regulation of immune cell function, neovascularisation and involvement in cell migration and metastasis [30,31,104,105,106].

The expression of OPN is up-regulated by a variety of stimuli such as 1,25-dihydroxyvitamin D, bFGF, TNFα, IL-1, IFN-ϒ and LPS, though the signalling pathways causing the up-regulation are still not fully understood[107,108]. In a mouse model, hypoxia was demonstrated to highly induce OPN expression via a Ras-activated enhancer[109]. The function of OPN in a hypoxic environment seems to be cytoprotective[110]. Furthermore, an inverse relation to the VHL-gene has been demonstrated [111].

A number of studies have shown that OPN expression in tissue/plasma is associated to tumour invasion, progression or metastasis in several cancers such as, breast, colon, head-and-neck, liver, lung, prostate and stomach. OPN has also been identified as a strong prognostic marker in colon cancer[111,112,113,114,115,116,117]. In RCC OPN over-expression has been observed in both cRCC and pRCC, and tumour over-expression and elevated levels in plasma has been associated with poor prognosis[118,119,120].

Tumour classification

Histological subtype

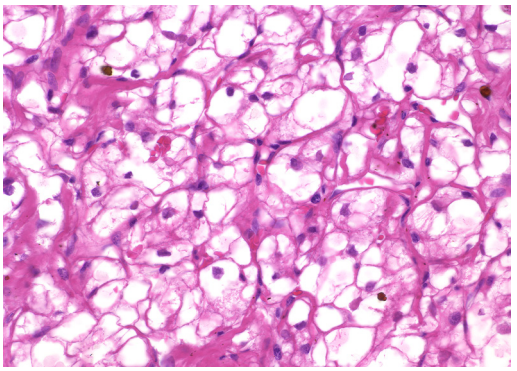
Malignant parenchymal neoplasm of the kidney is classified into five subtypes according to the Heidelberg criteria[121].

Clear cell renal cell carcinoma (cRCC) is the most common type and constitutes about 70-75% in surgical specimen. In routine sections, the cytoplasm is often clear due to high lipid and glycogen content but the cytoplasm can also be more granular eosinophilic. There is a variability in growth pattern which may be solid, cystic, papillary, trabecular, or tubular.

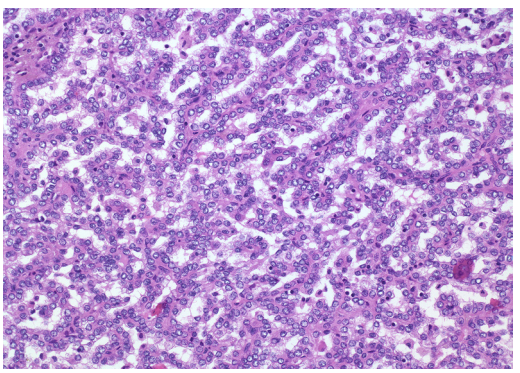
Papillary renal cell carcinoma (pRCC) accounts for about 10-15% of all RCC. Two subtypes have been described that differ morphologically, clinically and genetically[122,123]. Type 1 consists of small cells, characterized by small oval nuclei with a diffuse nucleoli, pale cytoplasm and papillary or tubular growth pattern. Type 2, is represented by large cells, with eosinophilic cytoplasm, and large spherical nuclei with prominent nucleoli. The growth pattern is papillary.

Chromophobe renal cell carcinoma (chRCC) makes up approximately 5%. The cytoplasm is pale, eosinofilic granular or mixed and the cells may be large and polygonal shaped. The tumours often grow in large solid sheets.

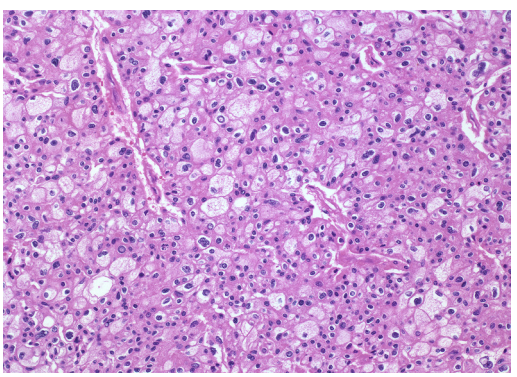
Collecting duct carcinoma accounts for 1% of renal tumours and are morphologically characterized by irregular channels lined by atypical epithelium, sometimes with a hobnail appearance[121]. The



cRCC



pRCC



chRCC

Figure 2. Histopathological appearances of the three most common renal cell carcinoma types, clear cell renal cell carcinoma (cRCC), papillary renal cell carcinoma (pRCC) and chromophobe renal cell carcinoma (chRCC) (Printed with permission from Ramnani, D.M, Virginia Urology Pathology Laboratory, USA)

remaining 3-5% are categorized as *renal cell carcinoma, unclassified* and do not fit into any of the other subtypes. Tumours with sarcomatoid morphology, mucin production, mixture of epithelial and stromal elements and unrecognizable cell types are stratified into this group. (Figure 2)

TNM stage

Tumour stage describes the anatomical extension of the disease and is assessed according to the TNM-system, UICC, 2002 (Table 1)[124]. It is based on clinical examination and radiological findings. The pathological tumour stage (pT stage) is assessed after histopathological examination of local distribution, vessel breakthrough and involvement of adjacent organs. Lymph node involvement (N stage) is assessed by histopathological examination of surgical specimen, and distant metastases (M stage) are assessed by radiological methods.

Table 1. TNM classification and stage grouping **(UICC 2002, 6th edition)**

T - primary tumour

TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
T1	Tumour ≤ 7 cm in greatest dimension, limited to the kidney
T1a	Tumour < 4 cm
T1b	Tumour > 4 cm
T2	Tumour > 7 cm in greatest dimension, limited to the kidney
T3	Tumour extends into major veins or directly invades adrenal gland or perinephric tissue, but not beyond Gerota´s fascia
T3a	Invasion directly invades adrenal gland or perinephric tissue but not beyond Gerota´s fascia
T3b	Tumour extends into major veins or the vena cava below the diaphragm
T3c	Tumour grossly extended into vena cava or it's wall above the diaphragm.
T4	Tumour directly invades beyond Gerota´s fascia

N-regional lymph nodes

NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in one single regional lymph node
N2	Metastasis in more than one regional lymph node

M –distant metastasis

MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Stage grouping

I	T1	N0	M0
II	T2	N0	M0
III	T1-2	N1	M0
	T3	N0-1	M0
IV	T4	N0-1	M0
	Any T	N2	M0
	Any T	Any N	M1

Nuclear grade

The tumours are classified as to nuclear grade, at present according to Fuhrman, and previously according to Skinner (Table 2)[125,126]. Four nuclear grades (1-4) are defined in order of increasing nuclear size, irregularity and nuclear prominence.

Table 2. Nuclear grade according to Skinner and Fuhrman

Grade	Skinner	Fuhrman
1	Nuclei are small non-distinguishable from normal tubular cells.	Nuclei are round and uniform, diffuse or absent nucleoli
2	Nuclei are irregular and pycnotic, slightly enlarged, without abnormal nucleoli	Nuclei are irregular and slightly enlarged with small nucleoli
3	Nuclei are moderately enlarged, irregular, and pleomorphic with large nucleoli, no bizarre forms	Nuclei are very irregular and large, with prominent nucleoli
4	Nuclei are large, numerous and the forms are bizarre	Nuclei are extremely irregular, often multi lobular, with aggregated chromatin

Clinical presentation

Renal cell carcinoma presents symptoms at a late stage and the tumour can grow to a big mass in the retroperitoneum unnoticed. The most common symptoms at diagnosis are haematuria, seen in approximately 60%, palpable resistance in the abdomen 45%, abdominal pain 45%, weight loss 30%, anaemia 20%, hypercalcaemia 3-15% and erythrocytosis 3%[126].

Less than half of the patients have localized disease at diagnosis, and about 30% have distant metastasis. The most common sites for metastases are lung, bone, brain and liver[127]. The use of more sophisticated radiological examinations in the clinic has led to an increased incidental discovery of renal tumours.

Treatment

Surgery

Small renal tumours, ≤ 4 cm, are treated with nephron-sparing surgery, since the result is similar as with radical nephrectomy in these small lesions[128,129]. For tumours over 4 cm in diameter, radical nephrectomy is recommended in most cases, but in selected cases nephron-sparing surgery can still be considered in tumours as large as 7 cm[130]. Tumours greater than 7 cm should always be considered for radical nephrectomy, and may also require resection of adjacent structures, isolation and occlusion of regional vasculature, and venous thrombectomy[131,132]. Patient with metastatic disease may also benefit from radical nephrectomy, if the performance status is good, and metastasectomy is sometimes performed in limited metastatic disease, e.g. solitary lung metastasis[133,134].

Systemic therapy

No systemic treatment has as yet shown any improvement of outcome in the adjuvant setting, and for patients without metastatic disease clinical follow up is the standard of care.

In contrast to many other solid tumours, chemotherapy is ineffective in RCC, and only a limited number of patients respond to immunotherapy, such as interferon-α and IL-2[135].

Recently, angiogenesis inhibitor drugs have shown efficacy with improved progression-free survival.

Sunitinib, Sorafenib and Pazopanib are multi-targeted tyrosine kinase inhibitors of VEGFR, PDGFR, c-Kit and Flt 3, and Sorafenib also of B-RAF, inhibiting angiogenesis and tumour growth[136,137,138]. These drugs have shown effect on progression free survival in phase III studies and are used as first and second line treatment of metastatic renal cell carcinoma[139,140,141].

Bevacizumab, a monoclonal antibody that binds circulating VEGF-protein, has also been identified as an active drug in RCC, especially in combination with interferon, and is also used as first-line treatment[142].

The mammalian target of rapamycin (mTOR) is an intra-cellular kinase enzyme in the down-stream signalling pathway of several growth factors. It is involved in the regulation of proliferation and survival of tumour cell and angiogenesis[143]. Temsirolimus, an inhibitor of mTOR, can prolong time to progression in patients with RCC, especially in patients with at least three risk factors (performance status < 80%, lactate dehydrogenase >1.5 x N, anaemia, hypercalcaemia, time from diagnosis to treatment less than 1 year and at least three metastatic sites)[144]. Mammalian TOR inhibitors are used as first- and second-line treatment.

Prognostic factors

A prognostic factor is a feature or marker that can be used to predict the risk of recurrence, metastatic spread, survival and sometimes the probability of adjuvant or palliative treatment benefit. In the last decades a vast number of possible factors have been tested in the hope to find more sophisticated markers for better prediction of prognosis, but in spite of growing knowledge about the molecular mechanisms in RCC, little information has been added to earlier established prognostic factors.

Tumour associated factors

Histological subtype

Histological subtype has been identified as a prognostic factor. Generally patients with chromophobe RCC have the best outcome followed by papillary RCC, clear cell RCC and unclassified RCC[145,146] (Table 3). Two subtypes of papillary RCC have been describe and they differ in outcome. Type 2 is more frequent in younger patients and presents with higher TNM stage and nuclear grade, with worse prognosis compared to type 1[122]. In chromophobe RCC, a small subset of patients progress, and other factors such as high pT stage, tumour necrosis and sarcomatoid change are of importance for the prognosis[147].

Table 3. Disease-specific survival in different RCC types

Tumour type	5-year disease-specific survival
Chromophobe RCC	85 - 100%
Papillary RCC	65 - 85%
Clear cell RCC	55 - 75%
Unclassified RCC	~ 25%

TNM stage

Several studies have confirmed that TNM stage is one of the strongest prognostic factors for predicting survival with statistical significance, taking in account tumour size, vascular spread, lymph node involvement and metastasis as described in detail above. The 5-year disease-free survival is for TNM stage I-IV approximately 90-100%, 75-80%, 55-70% and 10-30%, respectively[146,148,149].

Nuclear grade

Another strong prognostic factor in RCC is nuclear grade, which also has been verified in a number of studies[125,146,150,151]. The 5-year disease-free survival for grade 1-4 is approximately 75-95%, 60-75%, 40-55% and 15 - 30%, respectively.

Factors related with the patient

In advanced disease, some other factors have been identified being of importance for survival. Poor performance status, metastasis in several organs, hypercalcaemia, anaemia, increased erythrocyte sedimentation rate, inflammation, increased lactate dehydrogenase and vein invasion are all associated with poor survival[152,153,154]. Nephrectomy could improve the survival despite metastatic disease in patients without these risk factors[133].

Molecular factors

Many different molecular markers, especially associated with tumour proliferation, chemo-resistance, apoptosis, angiogenesis, cell adhesion and cytogenetic abnormalities, have been investigated as potential prognostic markers in RCC such as bFGF, HIF-1α, VEGF, CA IX, CA XII, p53, Vimentin, PTEN, Ki67, EpCAM, Gesolin, CD44, CD95, PCNA, TIMP 1, loss of heterozygosity. A few show some potential, either individually or in combination, but so far none has meet the criteria for a clear prognostic marker in RCC[81,83,155,156,157,158,159,160,161].

AIMS OF THE STUDY

The overall aim was to investigate the expression and possible prognostic impact of some tumour associated proteins with growth factor potential, mainly or partially regulated by the von Hippel Lindau protein, in human renal cell carcinoma

Specific aims:

I To evaluate the expression of Parathyroid Hormone-related Protein (PTHrP) in serum, the relation to serum calcium, and the possible prognostic information in human RCC.

II To compare the expression of Erythropoietin (EPO) and the Erythropoietin receptor (EPO-R) in tumour tissue, to evaluate their relation to serum EPO and blood haemoglobin levels, and to assess their prognostic impact in human RCC.

III To evaluate the prognostic information of soluble carbonic anhydrase IX (CA IX) in serum in human RCC.

IV To evaluate the association between osteopontin (OPN) in plasma/serum and PTHrP in plasma/serum and tumour tissue, and to assess the prognostic value of OPN and PTHrP in human RCC.

Patient material

Tumour tissue and serum/plasma from patients diagnosed with RCC during the period 1982-2007 were used in the studies. Blood samples were collected at diagnosis before surgical treatment, and serum or plasma was stored at -80 °C until analyses were performed. Tumour specimens were collected at nephrectomy or at diagnostic biopsy and before treatment, and were formalin-fixed and paraffin-embedded.

The number of patients varied between the studies. This was due to the time of analysis, loss of tissue in the preparations, and plasma was only recently collected. The patient groups were stratified for gender and age (Table 4). Tumour type, TNM stage and nuclear grade were assessed as described above[121,124,125,126].

Immunohistochemistry (IHC)

In brief, the tissue microarray blocks were sliced into 4-µm sections, de-paraffinized and rehydrated according to standard procedures. Citrate or EDTA buffers were used as antigen retrieval solutions and heat was applied by microwave oven for 20 minutes. Immunostaining with antibodies against EPO, EPO-R and PTHrP was performed using an automatic IHC machine (Ventana 320 ES, Ventana Medical Systems, Tucson, USA) with iView DAB detection kit. The antibodies used are described in Table 5.

Table 4. Summary of the patient material in study I-IV

Study	Number of patients	Years of diagnosis	Men	Women	Median age
I	243	1982 - 2001	143	100	66
II	195	1982 - 1997	114	81	65
III	339	1982 - 2007	199	140	65
IV	189 /80	1982 - 1997/ 2001 - 2006	109 /44	80 /36	64.5

Tumour tissue analyses

Tissue microarray (TMA)

The tissue microarray technique has enabled analysis of markers in a large number of specimen and is well established[162]. Tissue microarrays were used for immunohistochemical (IHC) staining of tumour tissue in paper II and IV. These arrays included patients diagnosed during the period 1982-1997.

Representative tumour tissue was selected from formalin-fixed and paraffin-embedded tissue blocks and two tissue cores from each tumour, with a diameter of approximately 0.6 mm, were placed in a new paraffin block with a total of 98 tissue cores representing up to 49 tumours in each block. Initially 258 tumours were prepared in the tissue microarrays (Figure 3).

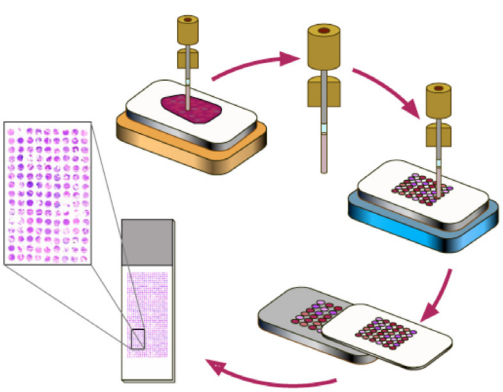


Figure 3. The principles of tissue microarray preparation
Illustration by Nigel Papworth

Table 5. Summary of antibodies used for IHC in different studies

Study / Specificity	Antibody	Dilution	Retrieval solution
II / EPO	MAB287 R&D systems, USA	1:25	Citrate buffer
II / EPO-R	MAB307 R&D systems, USA	1:25	EDTA buffer
IV / PTHrP	T-5045 Bachem, Switzerland	1:1000	Citrate buffer

As positive controls human placenta and normal renal tissue were used for EPO, EPO-R and PTHrP[42,163,164,165,166,167].

The immunostaining was evaluated in a light microscope by one of the authors, and in addition a second author evaluated part of the material for comparison. The samples were coded and all information was unknown to the examiner at the time of evaluation.

The entire tissue core was evaluated at 10x and 25x magnification. For all three markers, the immunostaining was cytoplasmatic and uniform within the tumours. The expression was categorized into three groups:
No (0), week (+) or strong (++) IHC expression.

Plasma and serum analyses

IRMA

Serum PTHrP (paper I) was analysed using the PTHrP 65T kit, two-site immunoradiometric assay (IRMA), from Nichols Institute Diagnostics.Two different polyclonal antibodies against the PTHrP molecule, one labelled with biotin binding to the mid section (aa 60-72) and the second labelled with ^{125}I binding the N-terminal (aa 1-40), were added to sera and the samples were incubated. A solid phase avidin-coated plastic bead, with high affinity to biotin, was added and the beads were washed to remove unbound components. The radioactivity bound to the solid phase was measured on a Vitros 950 chemist system (Ortho-Clinical Diagnosis). The counts were proportional to the PTHrP concentration.

Plasma PTHrP (paper IV) was analysed using the PTHrP DSL8100 kit from Immunotech, also a two-site immunoradiometric assay following the principles above, but instead of plastic beads, the solid phase consisted of pre-coated tubes.

ELISA

Enzyme linked immuno-sorbent assays (ELISA) were used to detect serum EPO (paper II), serum CA IX (paper III) and plasma OPN (paper IV).

For CA IX and OPN the Quantikine assays were used, supplied by R&D systems.

A monoclonal antibody specific for either CA IX or OPN was pre-coated onto a microplate. Serum or plasma was added and incubated for 2 hours. The wells were washed to remove unbound substance. An enzyme linked polyclonal antibody specific for the protein was then added, followed by a wash and then addition of a substrate solution, and colour developed. The optical density was measured with spectrophotometry and was proportional to the protein concentration. For the serum EPO analysis (paper II), the automatic Immulite 2000 system (Siemens) was used, following the principles for ELISA assays described above.

Statistical analyses

Analysis of the data was performed with the computer software SPSS version 13.0 (study I-III) and version 18.0 (Study IV). Non-parametric Kruskal-Wallis test and Mann-Whitney U-test were used for analysing different mean values between different groups, and $\chi 2$-test was used for assessing associations between categorical variables. Correlation between different variables was analysed with the Spearman correlation test. Survival time from diagnosis between different groups was compared using the log rank test, and disease-specific survival was assessed and illustrated with the Kaplan-Meier method. Multivariate regression analyses of prognostic factors for survival were performed by Cox proportional hazards model.

As described above, the basis of this thesis was to evaluate the expression of some tumour associated proteins, EPO, CA IX, PTHrP and OPN, and their possible prognostic information in human RCC. These proteins have potential growth factor-like properties and are possibly regulated through the same mechanism, the VHL/HIF system. The proteins have previously been investigated in human RCC, but this has been either in a limited number of patients, or with different patient characteristics or end points than in our studies. Our patient material has been collected from 1982 onwards, and is therefore large in comparison to most other studies. Many variables have been collected in a database enabling us also to assess some interesting associations. The follow-up times stretch over long periods, which is essential when evaluating prognosis for disease-specific survival.

This material has been used previously for different studies, and we were therefore also able to use some earlier published data of interest for comparison. Because of the large number of patients, TMAs were prepared for the IHC tumour tissue staining. The TMAs consisted of approximately 200 patients diagnosed 1982-1997, and were used in two of the studies (II and IV). Serum samples were available in most of these patients, and in some of the studies in this thesis, serum was also analysed in other patients to get an even larger cohort. In addition, plasma was available from 2001 onwards. At the time of analysis, the number of samples available varied and therefore, there is a discrepancy in the number of patients between and within the different studies.

Erythropoietin and its receptor

In study II, tumour tissue immunohistochemistry (IHC) expression of erythropoietin (EPO) and the EPO-receptor (EPO-R) were evaluated in TMA including patients diagnosed 1982-1997. The expressions were compared to EPO levels in serum analysed in 1992 on samples collected 1982-1991, and in 2004 on samples from 1992-1997, both using ELISA, as earlier described. Results from previous serum analyses were published in 1992[60]. Some of these samples were re-analysed in 2004 and the levels were equal in the two runs, indicating that the analyses were compatible and the protein stable during the long storage period. Haemoglobin levels in blood, analysed at diagnosis, were used for comparison.

Originally 195 patients were included in the study and serum EPO was analysed in all samples. The IHC EPO expression was evaluable in 187 tumours and the IHC EPO-R expression in 172 tumours. For the statistical analysis, tumours with no and weak expression were pooled and compared to tumours with strong expression (Figure 4).

Both EPO and EPO-R expressions were cytoplasmatic, and strong EPO-expression was found in 83% of the tumours whereas only 56% expressed EPO-R strongly, though a positive association was found between the two (p=0.028). The expression varied

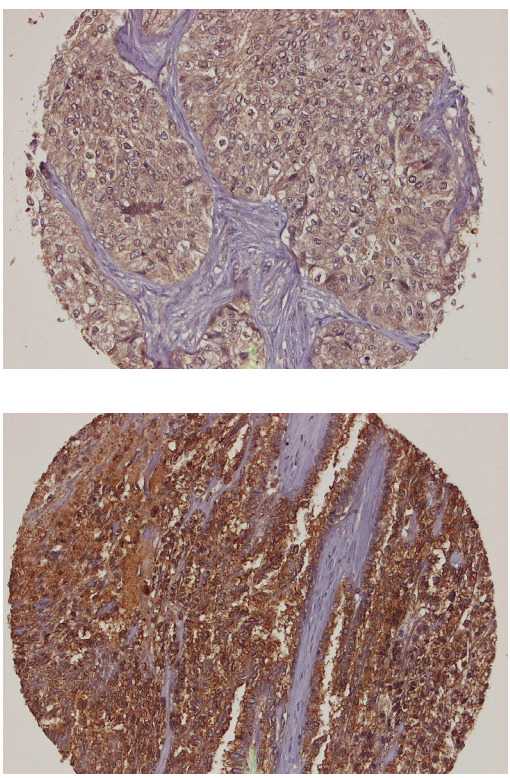


Figure 4. No (above) and strong (below) immunohistochemical staining of EPO in RCC

between tumour types. Strong EPO expression was significantly more frequent in cRCC (86%) compared to pRCC (64%). When evaluating the EPO-R expression, the reverse relationship was found, strong expression was more common in pRCC (81%) than in cRCC (54%). The number of other tumour types was too low to evaluate.

There was a positive association between TNM stage and EPO-R expression ($p<0.005$) whereas no association between EPO expression and TNM stage could be demonstrated ($p=0.57$). Neither EPO nor EPO-R tumour expression correlated to age, gender, nuclear grade or serum EPO levels.

The median follow-up time, for surviving patients, was 190 (106-289) months.

Patients with tumours expressing EPO-R strongly, had a tendency for shorter survival time but the results did not meet significance ($p=0.068$). There was no difference in survival between patients with tumours expressing EPO or not.

Elevated serum EPO levels were observed in 37% of the patients, and 41% had anaemia at diagnosis. Six percent of the patients had erythrocytosis.

As expected, and shown in earlier studies by Ljungberg *et al.*[60], the serum EPO levels were higher in patients with advanced disease and tumours of high nuclear grade, and serum EPO correlated inversely to Hb levels in blood ($r=-0.491$, $p<0.001$). Also, as earlier shown, patients with levels of EPO above median had a significantly shorter survival time; 31 (21-40) months, compared to those with low levels, where the median survival time was 97 (0-197) months ($p<0.001$).

To evaluate the prognostic impact, tumour EPO and EPO-R expression, serum EPO, haemoglobin, age, gender, TNM stage and nuclear grade were included in multivariate analyses. After final step analysis only TNM stage and nuclear grade remained independent prognostic factors for survival.

Discussion

Erythropoietin binds to the EPO-R, which leads to proliferation and differentiation of erythroid cells, but it has also been demonstrated that EPO stimulates to tumour growth and angiogenesis and inhibits apoptosis[48,49,50,51]. Erythropoietin expression has earlier been described in RCC with diverting results. Similar expression to our results was shown by Clark *et al.*[55] who found that the majority of clear cell RCC expressed EPO, but in opposite to our results, they demonstrated higher expression in papillary RCC, though the number of patients in their study was limited. There is a VHL mutation or silencing in the majority of cRCC, in contrast to pRCC where other mutations are more common. Defect VHL-protein function or absence of the protein leads to a stabilization and increase of HIF-1α and in turn to stimulation of EPO production. This could be an explanation of higher expression in cRCC than in pRCC as demonstrated in our study. In contrast to our results, a study by Michael *et al.* demonstrated that only a minority of the tumours expressed EPO[61]. They also showed that patients with tumours expressing EPO had shorter survival. This role of EPO for prognosis could not be confirmed by our study, where no impact on prognosis was observed. The diverting results could be explained by the use of different antibodies with different specificity for the IHC staining, a common dilemma when comparing results from different studies.

The expression of EPO-R in tumours has raised concern as recombinant human EPO (rhEPO), acting via the receptor, has been frequently used in the treatment of anaemia in cancer patients. Two clinical trials, in breast and head-and-neck cancer respectively, have shown unfavourable effect of rhEPO treatment [62,63].

We demonstrated that a majority of tumours strongly expressed EPO-R and the frequency was significantly higher in patients with advanced disease. Furthermore, in patients with clear cell RCC, there was a non-significant tendency for shorter survival when the tumour expressed EPO-R. These results may indicate that the use of rhEPO in RCC could be of disadvantage for the patients. The specificity of anti-EPO receptor antibodies has been questioned and the actual size of the functional EPO-R has been debated [168,169,170]. The antibody used in our study was not evaluated in those analyses. We found an association between EPO and EPO-R expression, which indicates that it is the EPO-receptor that is expressed.

There was an inverse correlation of serum EPO and blood Hb and approximately 50% of the elevated EPO levels could be explained by anaemia. Anaemia is common in RCC and there are several causes; bleeding from the tumour, inflammatory reaction, deficient iron and folinic acid metabolism, and renal failure[171].

A number of mechanisms suppress EPO production such as increased catabolism, renal failure and cytokines produced by inflammatory cells or neoplasm, a plausible explanation to the moderate correlation between serum EPO and Hb[172,173]. Expression of EPO was observed in the majority of tumours but no correlation was found to the serum levels. This opposes the assumption that circulating EPO originates from the tumour and suggests that serum EPO originates from other sources, which could be tumour-induced hypoxic renal tissue, and is in line with earlier theories[174]. Increased production of EPO could lead to erythrocytosis. This was only observed in approximately 5%, whereas serum EPO was increased in about one third of the patients. This could indicate that some of the circulating EPO measured in serum is not biologically active, or that the bone marrow cannot respond to EPO due to catabolic metabolism.

In summary, we could demonstrate that neither EPO, EPO-R expression in tumour tissue, nor serum EPO are independent prognostic factors in RCC. The serum EPO levels are dependent on a number of factors making it difficult to interpret and therefore less useful as a prognostic tumour marker. The EPO-receptor expression in tumour may be of most interest and could be of importance for the survival and the use of rhEPO treatment.

Carbonic anhydrase IX

Previous studies on tumour expression of CA IX have indicated an impact on prognosis in cRCC, and a soluble form of the protein has been detected in serum in RCC patients. As the tumour expression was analysed previously in TMA in this patient material[81], we wanted to assess CA IX in serum, to compare the levels to the tumour tissue expression and to assess the possible prognostic information.

Soluble CA IX in serum was evaluated in study III. Patients diagnosed with RCC between 1982 and 2007 were included, in total 339 patients. There were 287 clear cell, 40 papillary and 12 chromophobe RCC. Twenty-two patients with oncocytomas, a benign tumour, were used as a control group. Serum CA IX was measured with ELISA and the median level, 141 (2-4181) pg/mL, was set as cut-off point for statistical analyses. In 125 patients with cRCC, the serum levels were compared to the IHC expression previously assessed in TMA[81]. The serum CA IX levels were significantly higher in cRCC compared to pRCC ($p<0.001$) and oncocytomas ($p=0.002$). When relating the serum CA IX levels to TNM stage, there was a positive correlation in cRCC ($p=0.002$) but not in pRCC, and no correlation to nuclear grade, age or gender was found in any RCC type.

The median follow-up time for surviving patients was 60 (12 -317) months, measured from time of surgery or diagnosis. Patients with levels above median had a non-significant tendency for shorter disease-specific survival time ($p=0.12$)

In the previous study by Sandlund *et al.*, evaluating tumour IHC expression of CA IX, the tumours were divided into three groups according to percentage of positive cells; 0–10%, 11–90% and 91–100%. The serum levels in the different groups were evaluated and there was a trend towards lower levels in patients with tumours with low expression, but the results did not meet significance ($p=0.15$).

The prognostic impact of serum CA IX was evaluated in a multivariate analysis, including serum CA IX, age, gender, TNM stage and nuclear grade, and after final step analysis only TNM stage and nuclear grade were independent prognostic factors.

Discussion

Carbonic anhydrase IX is a membrane bound enzyme, regulating pH in tumour cells in hypoxic conditions protecting the cells from cell death. It is also thought to have the ability to act in a growth factor receptor manor, activating pathways leading to tumour cell growth. A soluble form has been detected and can be measured in blood and urine. It is thought to constitute a cleaved off extracellular part of the protein[85].

CA IX expression is common in a broad spectrum of malignant tumours and has been described as one of the most interesting potential tumour markers in RCC, and a potential target for therapy[175]. The enzyme is only expressed in a few normal tissues, mainly in the gastrointestinal tract. High tumour expression has been associated with worse prognosis in many tumour types, and expression has mainly been found in hypoxic tumour areas[79]. In clear cell RCC, the expression is more uniform within the tumour, and the tumour expression has been evaluated in several studies[81,84,176].

Those studies have demonstrated that most clear cell RCC highly express CA IX. This has been explained to be due to mutation in the VHL-gene leading to up-regulation of CA IX production. In cRCC, low tumour expression is associated to worse prognosis, which could reflect a more malignant phenotype when CA IX is not expressed. In papillary and chromophobe RCC, the tumour expression has been demonstrated to generally be low[80,81].

The soluble form of CA IX in RCC has not been as extensively studied as the tumour expression. Li *et al.* demonstrated that the serum levels correlated to TNM stage in cRCC, though the number of patients with stage IV disease was limited[86]. We could confirm, in a large number of patients, that the serum levels of CA IX was significantly higher in advanced disease in cRCC, whereas no difference in serum CA IX levels was seen in pRCC at the different stages. In their study they also found that high levels were associated to postoperative recurrence, and in a univariate analysis, they demonstrated that serum CA IX was an independent prognostic factor for recurrence-free survival in cRCC. We evaluated the impact of serum CA IX on disease-specific survival in cRCC. Patients with high levels had a tendency for shorter survival, although not statistically significant, which could support Li´s findings. In our study only TNM stage and nuclear grade were independent prognostic factors for disease-specific survival according to a multivariate analysis.

In a previous study, our group found that high tumour expression was associated to more favourable prognosis in cRCC, whereas the expression in other RCC types had no impact on prognosis[81]. We compared serum CA IX levels to the tumour expression and could not demonstrate a significant correlation between the tumour expression and the serum levels of CA IX.

When comparing different tumour types, the serum levels were significantly higher in cRCC compared to pRCC and oncocytoma, and these findings are in line with the findings in tissue where the expression is significantly higher in cRCC compared to other RCC types. This indicates that there is a relationship between tumour expression and the corresponding levels in serum.

All serum samples had measurable levels of CA IX, and high levels were found in all tumour types. In pRCC and chRCC this could be explained by increased production of CA IX due to tumour necrosis, which has been associated to worse outcome in chRCC[177]. It is therefore not excluded that serum CA IX also could be of interest in chRCC. The number of patients with chRCC was to limited in our study to provide an answer to this question.

Altogether we could demonstrate that the levels of serum CA IX differ between different RCC types, which could be used as a diagnostic tool. Even though serum CA IX does not seem to be an independent prognostic factor in RCC, the serum levels are higher in advanced disease and this indicates that CA IX could be a useful marker when treating patients, and should be further investigated.

Parathyroid hormone-related protein and Osteopontin

In study I, the aim was to evaluate parathyroid hormone-related protein (PTHrP) expression in serum, to assess the relation to serum calcium (s-Ca) and to evaluate the prognostic information. Serum PTHrP was evaluated in 243 patients diagnosed with RCC in 1982-2001. The serum levels were compared to s-Ca, analysed in 220 patients in the cohort at diagnosis, and according to routine procedures. Serum PTHrP was elevated in 37/243 patients (15%), and 15 percent (33/220) had hypercalcaemia (≥ 2.60 mmol/L). No association of serum PTHrP to RCC type, TNM

stage or nuclear grade was found. Serum calcium correlated positively to TNM stage (p=0.001), but not to nuclear grade (p=0.067).

There was no difference in survival time in relation to serum PTHrP levels (p=0.16). In contrast, the survival time was significantly shorter in patients with hypercalcaemia (p<0.001).

The number of samples with elevated PTHrP levels decreased with time. When excluding samples stored for longer than 15 years, a positive association to serum calcium levels was found (r=0.349, p=0.01). A multivariate analysis showed that hypercalcaemia and TNM stage, but not serum PTHrP, were independent prognostic factors for disease-specific survival.

In study IV, one-hundred and twenty-four of the samples from study I were used for comparison.

The aim was to evaluate the expression of PTHrP in tumour tissue and to relate the results to the serum levels analysed in study I. Furthermore, we wanted to investigate the association to another protein possibly related to PTHrP, osteopontin (OPN). The aim was also to assess the prognostic information of the two proteins.

Parathyroid hormone-related protein IHC tumour tissue expression was evaluated in 189 patients diagnosed 1982-1997. There were 153 cRCC, 23 pRCC and 13 chRCC in this cohort. Weak and strong IHC expression of PTHrP were pooled and compared to no expression for statistical analysis. Serum OPN was evaluated in 148 of the patients and compared to the PTHrP IHC tumour tissue expression and to serum PTHrP levels from study I.

Tumour expression of PTHrP was more frequent in low TNM stages (p=0.013), whereas no association to RCC type, nuclear grade or survival time was found. A positive association to serum PTHrP was observed (p=0.048).

Massfelder et al. had previously reported a possible anti-angiogenic effect of PTHrP. A marker of microvessel density, CD31, previously evaluated in the same cohort by Sandlund *et al.*, was used for comparison in 166 patients[32,178]. An inverse relationship between tumour expression of PTHrP and CD31 was found (p=0.029), indicating an anti-angiogenic effect of PTHrP.

Serum OPN was measurable in most samples, the median value was 1.57 (0-8.83) ng/mL. No association of serum OPN and serum PTHrP could be demonstrated. Neither serum OPN nor s-Ca were associated to PTHrP IHC tumour tissue expression. Serum OPN levels correlated positively to TNM stage, nuclear grade and negatively to survival time in cRCC, in accordance with the results in plasma described below. The serum OPN levels were significantly higher in papillary RCC compared to other RCC types (p=0.009). In contrast to the findings in cRCC, no correlation of serum OPN to TNM stage, nuclear grade or survival time was found in pRCC.

According to the manufacturers, PTHrP and OPN should be analysed in plasma. Therefore, as plasma was available from 2001, plasma PTHrP and plasma OPN were analysed in 80/76 patients with clear cell RCC diagnosed in 2001-2006. Plasma PTHrP was elevated in 16 of 80 samples (20%). OPN was measurable in all 76 samples, and the median level was about three times higher in plasma than in serum. There was a positive association between plasma PTHrP and plasma OPN (p=0.009), and plasma PTHrP associated positively to TNM stage (p=0.035) and to serum calcium (p=0.002), but no association to survival time could be demonstrated. Plasma OPN was positively associated to TNM stage (p=0.001), nuclear-grade (p=0.005), and negatively to survival time (p<0.001) (Figure 5). No correlation of plasma OPN and serum calcium was found. A multivariate analysis showed that TNM stage and plasma OPN were independent prognostic factors for disease-specific survival in cRCC (Table 6).

Three postoperative plasma samples were available in patients with limited disease, who all had elevated plasma OPN levels at diagnosis. The plasma OPN levels were dramatically lower when measured postoperatively 1.44 (1.27-1.77) ng/mL compared to in the preoperative samples, 9.3 (8.4-10.8) ng/mL.

Table 6. Multivariate analysis of prognostic factors for survival, according to Cox, in 76 patients with cRCC diagnosed in 2001-2006

Factor		Exp(B)	95% CI	*p* value
Age (y)	≤ 64,5	1		
	> 64,5	0.638	0.29 -1.40	0.262
Gender	male	1		
	female	1.280	0.60-2.72	0.522
Nuclear grade	1-2	1		
	3-4	1.336	0.48-3.70	0.578
TNM-stage	I-II	1		
	III-IV	4.254	1.48-12.2	0.007
Plasma OPN (ng/mL)	≤ 4.8	1		
	> 4.8	6.410	2.02-20.3	0.002

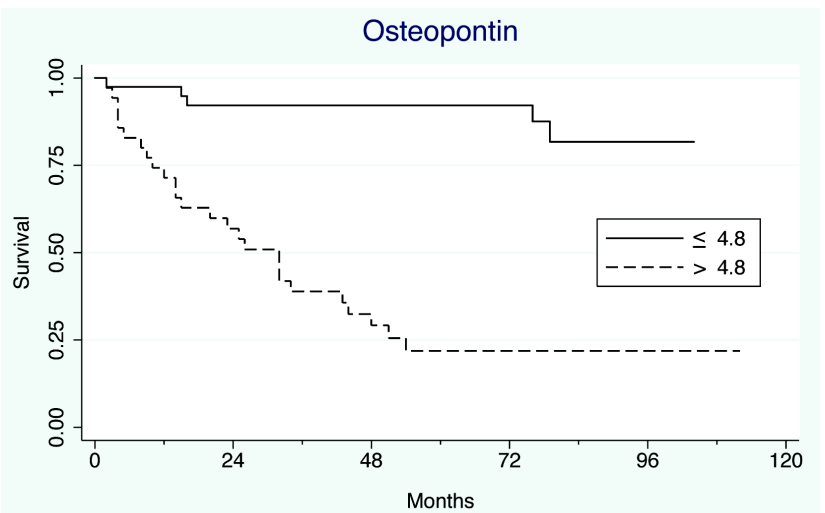


Figure 5. Disease-specific survival in patients with cRCC with low (≤ 4.8 ng/mL) and high (> 4.8 ng/mL) plasma OPN levels.

Discussion

Hypercalcaemia is relatively common in RCC and is associated with advanced disease and poor prognosis[97,179]. Parathyroid hormone-related protein has been described as one of the most important causes of hypercalcaemia in malignancy acting via the PTH1-receptor, increasing the resorption of calcium from bone and the reabsorption in the kidneys[180]. Our results in study I and IV show that there is a positive association between both serum and plasma PTHrP levels and serum calcium levels, and this indicates that PTHrP is one of the causes of hypercalcaemia in RCC. However, less than half of the cases with hypercalcaemia could be explained by elevated PTHrP levels. Therefore other causes of hypercalcaemia are plausible, such as vitamin D analogues, IL 1 or 6, transforming growth factors (TGF) or excessive parathyroid hormone production. Elevated levels of serum PTHrP were observed in 15%, whereas 52% of the tumours expressed PTHrP. A positive association between the two could be demonstrated, but no association between PTHrP tumour expression and serum calcium levels was observed, which supports the results of Gotoh *et al.*[167]. They demonstrated that tumour PTHrP expression was common in RCC, but no correlation to serum calcium was found in their study. This could indicate that circulating PTHrP derives from other sources than tumour cells. In study I, we found that serum PTHrP is unstable when stored for a long time. Furthermore, the method is recommended for analysis on plasma samples. In the cohort of study I, plasma samples were not collected, and therefore serum was used. This could be one explanation to the instability and low number of elevated samples and could also make the results difficult to interpret. As plasma was available from 2001 onwards, plasma PTHrP was analysed in 80 of the more recently diagnosed patients. However, the proportion of patients with elevated plasma PTHrP in this cohort were only slightly larger compared to that in the serum cohort (20% compared to 15%).

The stability of PTHrP in whole blood and plasma has also previously been evaluated [181,182]. They concluded that samples collected in EDTA tubes should be separated within 15 mins and then frozen. This is a restriction when evaluating the protein in plasma as a prognostic marker, and could be an explanation to the moderate correlation to serum calcium in our studies.

Elevated plasma PTHrP levels were more frequent in patients with high TNM stages, which indicates an association to advanced disease. In the serum analysis in study I, there was a non-significant tendency for shorter survival time in patients with elevated PTHrP levels. Parathyroid hormone-related protein has been identified as a prognostic factor for poor survival in lung cancer[183], but has been associated to both impaired and improved survival in breast cancer[99,184]. The protein has been demonstrated to exist in different isoforms and to have different functions in different cell types, having both pro and antitumoural effects[185]. In RCC, Iwamura *et al.* demonstrated that the expression of PTHrP in tumour associated to low TNM stage and longer recurrence-free survival. In our study, expression of PTHrP was more frequent in low TNM stages and this is in line with Iwamura´s findings.

The relation to CD31, a marker of microvessel density (MVD), was also assessed, as Massfelder *et al.* proposed that PTHrP has an anti-angiogenic effect in RCC[32]. We found that PTHrP and CD31 expressions were inversely correlated, which could indicate that PTHrP has an inhibiting effect on angiogenesis. Whether this is of advantage or disadvantage in RCC is not clarified, although low MVD has been associated to worse outcome in RCC[186].

Neither tumour expression of PTHrP, nor serum or plasma PTHrP were independent prognostic factors in RCC in our studies and the role for PTHrP as a prognostic factor for survival remains unclear.

Osteopontin is a secreted protein expressed in a vast number of different cell types such as bone cells, breast, epidermal cells, renal cells, macrophages and T-lymphocytes[103.] The protein is involved in boneresorption, inflammation, ischemic re-prefusion, as well as tumour invasion, progression and metastasis in several cancer forms[30,31,104,105,106].

Osteopontin has earlier been demonstrated to

have an association with the PTHrP/PTH1-receptor-signalling system. In a rat model, where hypercalcaemia was induced using PTHrP, the OPN expression increased in the kidney[187], and Ono *et al.* demonstrated that OPN regulates the PTH1-receptor signalling in osteoblasts[188]. These previous findings lead us to also analyse OPN in serum/plasma, and we could demonstrate that there was a positive association between plasma PTHrP and plasma OPN. In Yasui´s study on rats, the expression of OPN in tissue was enhanced. Our findings could indicate that OPN expression is enhanced by PTHrP, or that the proteins are regulated by common mechanisms, e.g. the VHL/HIF-system. Le *et al.* found an inverse correlation between OPN and VHL gene expression in head-and-neck cancer and Massfelder *et al.* demonstrated that pVHL negatively regulates PTHrP expression in cRCC. Furhermore, both OPN and PTHrP are induced by hypoxia which is common in RCC[25,32,111].

Plasma OPN correlated positively to TNM stage, nuclear grade, and negatively to survival time and we could demonstrate that OPN is a strong independent prognostic factor for disease-specific survival in cRCC. These findings confirm the results by Ramankulov et al. who demonstrated that high plasma OPN levels were associated to distant metastasis and poor prognosis[120]. Osteopontin is a ligand for the CD44 receptor family and α_Vß integrins, mediating migration and invasion of tumour cells, promoting angiogenesis and facilating metastatic spread, and could therefore potentially have a negative effect on the outcome for cancer patients[30,104,105,106,189].

We could also demonstrate a dramatic decrease in plasma OPN levels in a few post-operative samples, which could indicate that plasma OPN is a usable tumour marker when monitoring patients during treatment and as a follow-up tool. This also indicates that OPN derives from the tumour and is supported by the results of Matusan *et al.*, who demonstrated that patients with OPN-positive tumours had worse prognosis[119]. Osteopontin is present in a broad range of normal cells and is enhanced by a number of conditions apart from cancer, such as inflammation, infection and ischemia and this must be taken into consideration when validating OPN for prognostic use. As IHC tumour tissue expression of PTHrP was assessed in an older cohort, where only serum samples were available, we also analysed OPN in serum for comparison. The levels of OPN were lower in serum compared to plasma, but associations to TNM stage, nuclear grade and survival time were comparable to the results in the plasma sample cohort. Serum OPN did not associate to IHC PTHrP tumour tissue expression. In contrast to the results of Ramankulov *et al.,* serum OPN was significantly higher in pRCC than in other RCC types. However, in pRCC serum OPN did not correlate to TNM stage, nuclear grade or survival time. These results are in accordance with previous studies on tumour tissue OPN expression in pRCC[118], and suggests that other mechanisms are of importance for OPN regulation in pRCC.

Altogether, OPN seems to be one of the most interesting plasma markers for prognosis in cRCC.

CONCLUSIONS

I Serum PTHrP is one of the causes of hypercalcaemia in RCC. Serum calcium, but not serum PTHrP, correlates to high TNM stage and poor prognosis.

II Tumour expression of EPO and EPO-R are related, and the expressions differ significantly between RCC types. The expressions of EPO and its receptor in tumour tissue are not associated to the serum EPO levels.

The EPO-R expression in tumour is positively associated to TNM stage, but neither EPO-R nor EPO tumour expression adds prognostic information in RCC.

III Serum CA IX levels are higher in cRCC compared to other RCC types. In cRCC, the serum levels are positively correlated to TNM stage, but serum CA IX is not an independent prognostic factor for survival in cRCC.

IV Plasma OPN is associated to plasma PTHrP. Circulating OPN does not associate to PTHrP tumour tissue expression, but is positively correlated to TNM stage and nuclear grade, and negatively correlated to survival time.

Serum OPN is significantly higher in pRCC compared to other RCC types. In cRCC, circulating OPN is an independent prognostic factor for survival.

ADDITIONAL REMARKS

Renal cell carcinomas affect about 1000 people in Sweden every year, and over 30% of the patients have distant metastasis at diagnosis. No curative treatment is available once the disease is spread, although some advancement in the treatment of metastatic disease has been made in the last few years, with improvement of progression-free survival. The natural progress of RCC is diverse, and the factors that are of importance for recurrence and progression of the disease are not clear. It is therefore of interest to find more specific prognostic factors to select patients for adjuvant and palliative treatment, to enhance decisions about follow-up and possibly also to find new targets for therapy.

In cRCC, the mechanisms regulated by the tumour suppressor gene product, pVHL, are important for tumour development. Mutation in the gene is common, leading to increased levels of HIF, which stimulates synthesis of a number of growth factors. The therapeutic targets available today are, therefore, aimed to inhibit some of these growth factors, their receptors or the signalling pathways activated from them. Probably other mechanisms, less well studied, are also acting in cRCC. In other RCC types, different mechanisms are of importance, but also hypoxia and tumour necrosis leading to increased HIF levels may play an important role.

In our studies, we wanted to further investigate some proteins earlier described as potential prognostic markers in RCC. These have all been shown to have growth factor-like properties in cancer and also, at least partially, to be regulated by the VHL/HIF-system.

We discovered that PTHrP was not stable in serum and we therefore concluded that this protein is less useful as a marker when analysing stored samples. We found that plasma PTHrP associated to advanced disease and hypercalcaemia, and analysis on fresh samples, could therefore be of interest in future studies to further explore the prognostic information of circulating PTHrP. We did not assess the main target of PTHrP, the PTH1-receptor. Possibly, the receptor expression is more important than the actual protein and could be of interest for further investigation.

Serum EPO is induced by hypoxia, and dependent on many different mechanisms and therefore difficult to interpret as a prognostic marker, even though elevated levels were associated to advanced stages of disease. The EPO-receptor expression was significantly more frequent in advanced disease and could be of importance when considering rhEPO treatment in patients with RCC. To confirm this, further research is needed.

Parathyroid hormone-related protein, EPO and EPO-R expression in tumour were all evaluated in TMAs with IHC staining. Tissue microarrays enable analysis in a large number of samples but have limitations. The sample volume is small and does not give information of the adjacent tissue or the expression in different areas in the tumour. Furthermore, the IHC technique has both advantages and limitations; it is fairly quick to perform compared to other methods, e.g. western blot or RT-PCR, and can be performed on tissue stored for a long time, but the method relies on the specificity of the antibodies used and the sensitivity, and could give both false positive and false negative information. Therefore, using other techniques to evaluate the expressions could be of value.

Elevated levels of CA IX in serum were associated to more advanced disease and the levels were significantly higher in cRCC compared to other RCC types and could therefore be useful as a diagnostic tool. Previous studies have demonstrated that the CA IX levels decrease after nephrectomy[85], and although serum CA IX does not seem to be an independent prognostic factor, it would be of interest to find out whether serum CA IX could be used as an early indicator of recurrence of RCC.

Plasma OPN has previously been identified as a prognostic factor in cRCC and we could confirm that OPN is an independent prognostic factor for survival in cRCC. Furthermore, we found that the levels decreased dramatically after nephrectomy. This was only analysed in a few samples and we therefore intend to do some further analysis on this matter. The

tumour expression and the receptors on which OPN acts, α_Vß integrins and the CD44 receptors, would also be interesting to investigate further, to relate their expression to the circulating protein levels, and to assess their prognostic information. Serum OPN was significantly higher in pRCC, the importance in this RCC type should be further evaluated in a larger number of patients.

Osteopontin seems to be one of the most interesting plasma markers in cRCC. Along with our results, two independent studies have confirmed that OPN is a strong independent prognostic factor for survival. The protein, its receptors, and the down-stream signalling pathways could be possible targets for therapy in RCC, and have previously been discussed[189]. Osteopontin should be further evaluated as a prognostic marker and follow-up tool in prospective studies in RCC.

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