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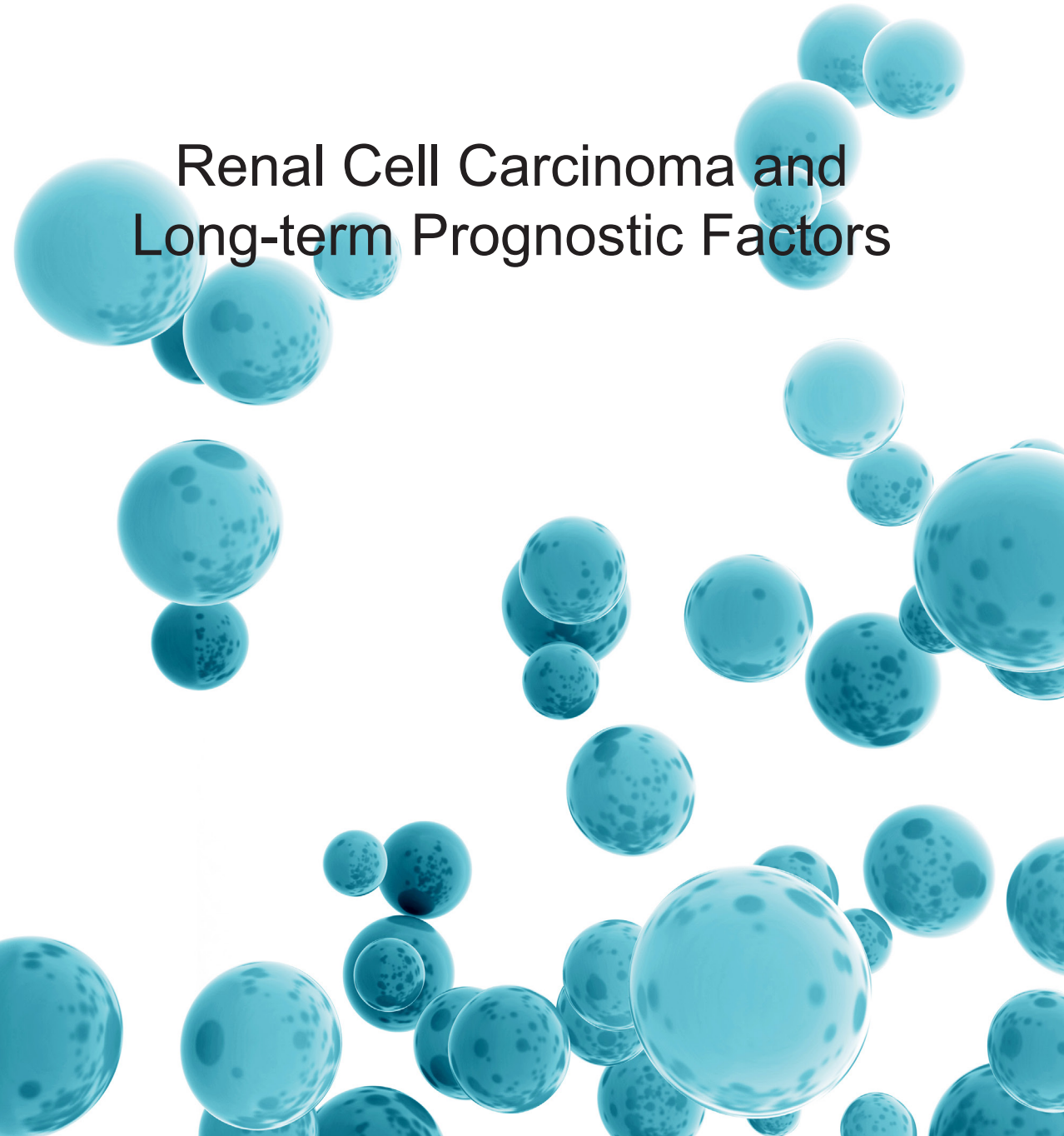
Renal Cell Carcinoma and Long-term Prognostic Factors



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Renal Cell Carcinoma and Long-term Prognostic Factors

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ACADEMIC DISSERTATION
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the Board of the School of Medicine of the University of Tampere,
for public discussion in the small auditorium of building M,
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UNIVERSITY OF TAMPERE

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CONTENTS

TIIVISTELMÄ

Paikallisen munuaissyövän ennuste on hyvä, mutta levinneen munuaissyövän ennuste on edelleen huono. Vuoden 2005 jälkeen on tullut uusia verisuonikasvun estoon vaikuttavia lääkkeitä ja levinneen syövän ennuste on parantunut, silti ennuste on pysynyt edelleen huonona. Munuaissyövän ilmeneminen on lisääntynyt, koska kuvantamistutkimukset ovat myös lisääntyneet ja sen seurauksena sattumalöydöksenä munuaissyöpiä löytyy yhä enemmän. Tyypillisesti nämä sattumalta löytyneet syövät ovat pienempiä ja siten näiden syöpien ennuste on myös yleensä parempi. Syövän histologinen erilaistumisaste, koko ja levinneisyys sekä potilaan kliininen tila ovat vahvoja ennusteeseen vaikuttavia tekijöitä. Erilaisia muita ennusteellisia merkkitekijöitä on tutkittu munuaissyövässä, mutta mikään näistä ei ole saavuttanut itsenäistä asemaa ennustetekijänä. Munuaissyöpäpotilaiden seurannasta ei ole käytössä yhteneväisiä suosituksia ja uudet hoidot asettavat haasteita, kuinka potilaita pitäisi seurata ja mikä hoito olisi kenellekin potilaalla hyödyllisin. Tarvitsemme uusia merkkiaineita, joilla voidaan ennustaa tarkemmin potilaiden selviytymistä, sekä arvioida vastetta käytössä oleviin lääkkeisiin.

Tämän tutkimuksen tarkoituksena oli tutkia mahdollisia ennusteellisia kasvainnäytteiden merkkitekijöitä erikseen sekä näiden yhdistelminä ja niiden yhteyttä jo tiedossa oleviin ennustetekijöihin sekä yhteyttä potilaiden elossa oloon. Tutkimusasetelma oli retrospektiivinen ja tutkimukseen sisällytettiin potilaat, joiden munuaissyöpä oli diagnosoitu vuosien 1985 - 1995 välillä ja munuainen oli poistettu Tampereen yliopistollisessa sairaalassa tai Hatanpään sairaalassa. Tutkimus sisälsi 224 potilasta, joiden munuaissyöpien tyypitys ja erilaistumisaste määritettiin uudelleen kokeneen uropatologin toimesta. Edustavista näytteistä tehtiin rinnakkaiset monikudosblokit, joista tehtiin analyyseihin käytettävät leikkeet. Kaikki nämä vaiheet tehtiin sokkoutetusti, tietämättä potilaan potilashistoriaa tai selviytymistä. Seuranta-aika ulottui pisimmillään 21,7 vuoteen ja kaikki potilaat hoidettiin samalla tavalla tuolloin käytössä olevan hoito- sekä seurantakäytäntöjen mukaisesti.

Tämä tutkimus osoitti, että syöpäsolujen erilaistumisaste ja syövän levinneisyys olivat merkittävimmät ennusteeseen vaikuttavat tekijät. Soluväliproteiineina toimivilla klaudiineilla ja verisuonikasvuun vaikuttavalla VEGFR3:lla ei ollut yhteyttä kuolleisuuteen. Verisuonikasvuun vaikuttavan tekijän Angiopoetiini-2:n (Ang-2) lisääntynyt ilmentyminen ja solunjakautumistekijän MIB-1:n vähäinen ilmentyminen olivat yhteydessä potilaiden parempaan ennusteeseen. Verisuonitiheyttä kuvaavan CD31:n ja solukuolemaa estävän Bcl-2:n lisääntyneet ilmentymiset olivat myös yhteydessä potilaiden parempaan ennusteeseen. Monimuuttuja-analyyseissä saatiin itsenäisiksi ennustetekijöiksi aiempien tunnettujen tekijöiden lisäksi MIB-1 ja Ang-2. Tutkittujen merkkiaineiden yhdistetyissä analyyseissä saatiin tulokseksi, että MIB-1:n ja Bcl-2:n ilmentymiset olivat kuolleisuuteen vaikuttavia tekijöitä.

Loppupäätelmänä tutkimuksessa saatiin, että syövän levinneisyys ja histologinen erilaistumisaste ovat edelleen vahvimpia ennusteeseen vaikuttavia tekijöitä ja näiden lisäksi MIB-1 ja Ang-2 olivat itsenäisiä

ennustetekijöitä. Klaudiineilla ja VEGFR3:lla ei ollut yhteyttä potilaiden selviytymiseen. Lisäksi CD31 ja Bcl-2 olivat yhteydessä potilaiden kuolleisuuteen, mutta eivät olleet itsenäisiä ennustetekijöitä.

ABSTRACT

The prognosis of patients with localized renal cell cancer (RCC) is good, but remains poor in patients with metastatic RCC (mRCC). The survival of mRCC patients has improved since 2005, when the antiangiogenesis target therapies became available in RCC treatment, but it is still poor compared to lower stage disease. The incidence of RCC has risen partly due to increased imaging, but cancers incidentally found are usually smaller and thus patients have a better prognosis. Tumor histologic grade, tumor stage and clinical presentation are known to be the most significant prognostic factors in RCC. Numerous promising molecular markers have been examined for the prognosis of RCC, but none of them has achieved status as an independent prognostic factor. In clinical practice follow-up protocols for RCC are not clear and we have lack awareness which patients benefit most from available targeted drugs. We urgently need new markers for better patient selection of the available treatments and for tailoring to follow-up individually.

The aim of this study was to investigate possible prognostic factors in primary tumors as single factors alone and in combination, and their association with classical indicators and long-term survival in RCC patients. This retrospective study involved 224 consecutive RCC patients whose nephrectomy was performed between 1985-1995 at Tampere University Hospital or Tampere Hospital, in Finland. All tumor samples were re-classified and re-graded, before immunohistological stainings and analyses. Two parallel tissue microarrays (TMA) were performed for further immunohistochemical analyses. All these measures were carried out without knowledge of patients history, in other words blinded. All patients had the same follow-up and treatment protocols according to clinical practice during the study period. In addition, all patients were treated at the time without new anti-angiogenesis target therapies. Long-term survival analysis used Cox-regression hazard models and Kaplan-Meier survival estimation methods, the longest follow-up being 21.7 years.

This study showed, that tumor stage and grade were both significant prognostic factors in RCC among studied markers. However, examination of single marker expressions showed high expression of an angiogenetic marker, angiopoetin-2 (Ang-2) and low expression of a proliferation marker, MIB-1 expression to which were associated with better survival and retain their significance also in a multivariate analysis performed with stage and grade. High expressions of microvessel density marker CD31 and antiapoptosis marker Bcl-2 were associated with better survival in univariate analysis, but lost their significance in multivariate analysis. The evaluation of combined marker expression showed that Bcl-2 and proliferation marker MIB-1 expressions had the strongest influence on patients survival.

In conclusions, tumor stage, tumor grade, Ang-2 and MIB-1 expressions showed significant association with long-term survival in RCC patients. In addition, Bcl-2 and CD31 expressions showed both an association with survival, but were not independent prognostic factors.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals.

I Virman J, Soini Y, Kujala P, Luukkaala T, Salminen T, Sunela K, Kellokumpu-Lehtinen P-L (2014): Claudins as prognostic factors in renal cell cancer. Anticancer Res 34(8): 4181-4188.

II Virman J, Bono P, Luukkaala T, Sunela K, Kujala P, Kellokumpu-Lehtinen P-L (2015): VEGFR3 and CD31 as prognostic factors in renal cell cancer. Anticancer Res 35(2): 921-927.

III Virman J, Bono P, Luukkaala T, Sunela K, Kujala P, Kellokumpu-Lehtinen P-L (2016): Combined angiogenesis and proliferation markers' expression as long-term prognostic factors in renal cell cancer. Clinic Genitourin Cancer 14(4):e 283-289.

IV Lampinen A, Virman J, Bono P, Luukkaala T, Sunela K, Kujala P, Saharinen P, Kellokumpu-Lehtinen P-L (2016): Novel angiogenesis markers as long-term prognostic factors in renal cell cancer. Accepted for publication in Clinic Genitourin Cancer.

ABBREVIATIONS

AJCC	American Joint Committee on Cancer
Ang	Angiopoetin
ARCD	Acquired renal cystic disease
Bcl-2	B-cell lymphoma 2 protein, regulates apoptosis
BDH	Birt-Hogg-Dubé syndrome
BMI	Body mass index
CAIX	Carbonic anhydrase IX
ccRCC	Clear cell renal cell carcinoma
CD31	Platelet endothelial cell adhesion molecule-1, panendothelial marker
chRCC	Chromophobe renal cell carcinoma
CI	Confidence interval
CN	Cytoreductive nephrectomy
c-*MET*	proto-oncogene encoding hepatocyte growth factor receptor
COX-2	Cyclooxygenase-2
CSS	Cancer-specific survival
CT	Computed tomography
DM	Diabetes mellitus
ECOG	Eastern Cooperative Oncology Group
ESRD	End-stage renal disease
5-FU	5-Fluorouracil
HIF	Hypoxia-induced factor
HLRCC	Hereditary leiomyamatosis renal cell cancer
HPRCC	Hereditary papillary renal cell carcinoma
IHC	Immunohistochemistry
IMDC	International Metastatic RCC Database Consortium
ISUP	International Society of Urological Pathology
KM	Kaplan-Meier
KPS	Karnofsky performance status
LD	Lactate dehydrogenase
MIB-1	Murine monoclonal antibody-1 against Ki-67 antigen
MSKCC	Memorial Sloan-Kettering Cancer Center – prognostic model
mRCC	metastatic renal cell carcinoma
MRI	Magnetic resonance imaging

mTOR	Mammalian target of rapamycin
MVD	Microvascular density
OS	Overall survival
PD-1	Programmed death-1, a cell-surface co-inhibitory
PD-L1	Programmed death-ligand 1
PET-CT	Positron emission tomography – Computed tomography
pRCC	Papillary renal cell carcinoma
PS	Performance status
RCC	Renal cell carcinoma
SDH	Succinate dehydrogenase
SSIGN	Stage size grade and necrosis – prognostic model
Tie	Tyrosine kinase receptor
TKI	Tyrosine kinase inhibitor
TNM	Tumor node(s) metastase(s)
TSC	Tuberous sclerosis complex
TTR	Time to recurrence
UICC	International Union Against Cancer
UISS	University of California Los Angeles Integrated Staging System
US	Ultrasound
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	Von Hippel-Lindau syndrome

1. INTRODUCTION

Renal cell carcinoma (RCC) accounts for approximately 2-3% of all adult malignancies worldwide and the incidence rate has increased (Cho, Adami, Lindblad 2011; Rini, Campbell, Escudier 2009). Worldwide estimated 338 000 new cases of RCC are diagnosed each year and approximately 30% of patients present with metastatic disease at the time of diagnosis (Ferlay et al. 2015; Fisher, Gore, Larkin 2013). The incidence of RCC has risen partially by reason of the increasing utilization of cross-sectional imaging, leading to incidental diagnosis without any symptoms of RCC (Cho, Adami, Lindblad 2011). The precise etiology of RCC is not clear, but smoking, obesity and hypertension have identified as risk factors of RCC (Decastro and McKiernan 2008).

Ten hereditary renal cell carcinomas have been described, the most common is being the clear cell renal cell type, which is related to von Hippel—Lindau (VHL) disease (Verine et al. 2010). Inactivation of the VHL gene leads to constitutive activation of hypoxia-inducible factor 1-alpha (HIF-1α) and in response to hypoxia the expression of vascular endothelial growth factor (VEGFR) increases (Linehan, Srinivasan, Schmidt 2010; Sato et al. 2013). Knowledge of this pathway has improved finding of new drugs such as currently used angiogenesis inhibitors in treatment of RCC (Finley, Pantuck, Belldegrun 2011). In everyday clinical practice there are five drugs targeted on antiangiogenesis, two focused on inhibition of the mammalian target of rapamycin (mTOR-inhibitor) and a novel immune checkpoint inhibitor used in the treatment of metastatic RCC (mRCC) (Motzer et al. 2015; Mourad, Dutcher, Ennis 2014). The prognosis of metastatic RCC has improved with the use of these targeted therapies (Abe and Kamai 2013; Vaishampayan et al. 2014).

Tumor stage, grade and clinical presentation are acknowledged to be the most powerful prognostic factors in patients with RCC (Escudier et al. 2014; Ljungberg et al. 2015). However, several molecular markers have been studied for the prognosis of RCC, but none has proven to be as an independent indicator (Funakoshi, Lee, Hsieh 2014; Lam et al. 2008; Ngo, Wood, Karam 2014). We need new markers to predict RCC patients' survival more accurately and to tailor treatments and follow-up individually.

The purpose here was to evaluate expression of several cells' adhesion, proliferation and angiogenesis markers in primary tumors of RCC and to define their significance for long-term survival and association with tumor stage and grade.

2. REVIEW OF THE LITERATURE

2.1 Epidemiology of renal cell carcinoma

2.1.1 Incidence and prevalence

Renal cell cancer (RCC) is the 9th most common cancer worldwide and approximately 337 860 new cases were diagnosed in 2012 (Jonasch, Gao, Rathmell 2014). The incidence varies geographically, being highest in developed countries (Jonasch, Gao, Rathmell 2014). During the last ten years the incidence of RCC has risen slightly, 1.7% in men and 0.6% in woman in Finland (Nordcan a). There were 534 new cases among men (incidence 10.3/100 000) and 401 among women (incidence 6.3/100 000) in Finland in 2013 (Nordcan b; Nordcan c). RCC was 9th in men (3.3% of all tumors) and 12th in women (2.6% of all tumors) cancer disease in Finland in 2013 (Nordcan d; Nordcan e). At the beginning of the year 2014 the prevalences were 3807 in men and 3359 in women in Finland (Nordcan f; Nordcan g). The five-year proportional survival were 63% in men and 63% in woman among patients with RCC diagnosed between 2005-2012 (Cancer.fi).

2.1.2 Etiology

Cigarette smoking is an established risk factor for RCC (Ljungberg et al. 2011). The relative risk is low but ever-smokers have a higher risk of RCC compared to never-smokers (HR 1.38, CI 95%= 1.27-1.50) and heavier smoking increases the risk even more (Hunt et al. 2005). Ten years after quitting smoking the risk of RCC seems to be reduced to the similar level than in non-smokers (Hunt et al. 2005; Parker et al. 2003).

High BMI (body mass index) has been established in several studies as a risk factor for RCC (Renehan et al. 2008). Every 5 kg/m^2 increase in BMI elevates the risk 1.24 in men and 1.34 in women (Renehan et al. 2008). On the other hand, RCC patients with normal or excess body mass at the time of diagnosis had better survival compared to patients with low BMI (Sunela, Kataja, Kellokumpu-Lehtinen 2013).

Two large prospective studies have shown elevated blood pressure or use of antihypertensive drugs to be associated with the risk of RCC (Chow et al. 2000; Weikert et al. 2008). Use of acetaminophen and non aspirin non-steroidal anti-inflammatory have also associated with risk of RCC (Choueiri, Je, Cho 2014).

Patients with end-stage renal disease (ESRD) or on long-term hemodialysis have a risk of acquired renal cystic disease (ARCD). ARCD patients have been shown to have a three to six times risk of RCC (Ljungberg et al. 2011). Diabetes mellitus (DM) type 2 increases the risk of many cancers but has not been demonstrated an association with RCC (Zucchetto et al. 2007).

The incidence of RCC varies substantially in worldwide and this is suggested to be caused by lifestyle, especially diet (Ljungberg et al. 2011). Both men's and women's daily intakes of fat and protein are

associated with RCC but the type of fat and its linkage to the condition has yielded conflicting result in the literature (Ljungberg et al. 2011). Vegetable and fruit consumption has been shown to reduce the risk of RCC (Lee et al. 2009).

Occupational exposure to different chemical substances may involve a potential risk of carcinogenesis. Exposure to trichloroethylene (TCE), lead, glass fibers, wool fibers, brick dust, blast-furnace or working coke-oven, iron or steel industry have shown associations with an increased insidence of RCC (Boffetta et al. 2011; Moore et al. 2010; Sim et al. 2009).

2.1.3 Genetics

Approximately 2-3% of RCCs are familial and first-degree relatives of patients with RCC have an approximately double risk of RCC (Ljungberg et al. 2011). Four main familial types of epithelial renal cell cancer are described in the literature (Crino, Nathanson, Henske 2006; Linehan et al. 2009).

The most common genetic alteration in RCC is the von Hippel-Lindau (VHL) gene germline mutation, which also elevates the risk of cysts and tumors e.g. hemangioblastomas of the central nervous system, retinal angiomas and pheochromocytomas (Ljungberg et al. 2011). A mutation in the VHL gene has been found in very high portion in sporadic clear cell renal cell carcinoma (ccRCC), while other RCC types have not been observed (Gnarra et al. 1994; Shuin et al. 1994). Yao et associates showed that sporadic ccRCC patients with a mutation in the VHL gene had better survival rates compared with RCC patients without mutated VHL gene (Yao et al. 2002). The prevalence of VHL is about 1 in 36,000 and RCC is found in 40-50% of VHL gene mutation carriers (Ljungberg et al. 2011; Maher et al. 1991). The VHL gene is located in chromosome 3 and it has an important role in angiogenesis. When both alleles of this tumor suppressor gene are mutated, abnormal VHL protein function induces an effect similar to that of hypoxia (Lonser et al. 2003; Pfaffenroth and Linehan 2008). As a result of this, HIF (hypoxia-induced factor) accumulates, leading to increased expression of growth, mitogenic and angiogenic factors such as vascular endothelial factor (VEGF) (Lonser et al. 2003). Increased VEGF plays an important role in angiogenesis, which is important for tumor growth and spreading (Roy, Bhardwaj, Yla-Herttuala 2006; Tugues et al. 2011)

The *MET* proto-oncogene mutation in chromosome 7 has been established to be linked to the hereditary papillary renal cell carcinoma (HPRCC) (Schmidt et al. 1999). The *MET* proto-oncogene encodes receptor tyrosine kinase, *c-MET,* which is important in many physiological functions, but could also promote malignant transformation when overexpressed (Appleman 2011). This syndrome causes only type 1 papillary RCC (pRCC) (Coleman 2008). Patients with HPRCC carry a risk of bilateral and multifocal malignancy, estimated up to 3,000 lesions per kidney (Pfaffenroth and Linehan 2008).

Patients affected with hereditary leiomyamatosis renal cell cancer (HLRCC) are a risk of RCC but also cutaneous and uterine leiomyomas (Pfaffenroth and Linehan 2008). Type 2 papillary RCC appears in HLRCC and its tumor biology is aggressive (Pfaffenroth and Linehan 2008).

Birt-Hogg-Dubé (BHD) syndrome develops to renal cancer in 20% to 40% of cases. The most typical variant is chromophobe type of RCC (chRCC), but ccRCC has also been observed (Cohen and Zhou 2005). Renal tumors may appear bilaterally or multifocally, and the histology can vary even within the same kidney (Pfaffenroth and Linehan 2008).

Hereditary paraganglioma/pheochromocytoma is characterized by germline mutation of three of the four subunits of succinate dehydrogenase (SDH) implicated in the Krebs cycle (Linehan et al. 2009). Germline mutation of SDHB (one of SDH subunits) had reported patients with either early onset or bilateral, multifocal ccRCC or chRCC (Linehan et al. 2009).

Patients with tuberous sclerosis complex (TSC) have similar risk of RCC compared to general population, but the RCC is diagnosed at younger age in patients with TSC patients (Crino, Nathanson, Henske 2006). A special feature of TSC associated with RCC is that ccRCC, pRCC and chRCC have all been reported in patients with RCC (Crino, Nathanson, Henske 2006).

2.2 Diagnostic of renal cell carcinoma

The first possible renal cell tumor was reported by Daniel Sennert in 1613, but the first unequivocal case report of RCC published by Miril in 1810 (Bhatt and Finelli 2014). Nowadays radiological examinations such as abdominal ultrasound (US), computed tomography (CT) and magnetic resonance imaging (MRI) are performed increasingly. Over 50% of RCC cases are currently detected incidentally and the proportion of small tumors has increased significantly (Escudier et al. 2014). The classical clinical triad of RCC, flank pain, palpable abdominal mass and gross hematuria, has decreased due to increased radiological imaging (Sunela et al. 2014), but is still present in some patients. Metastatic RCC may cause paraneoplastic syndromes such as unexplained fever, erythrocytosis or wasting syndromes, and in addition bone pain or lung nodules may occur (Escudier et al. 2014). Laboratory tests such as serum creatinine, haemoglobin, leucocyte and platelet counts, lactate dehydrogenase (LD) and serum-corrected calcium should be made, if RCC is suspected (Escudier et al. 2014). Some of these laboratory tests correlate with survival and are used for risk assessment (Escudier et al. 2014).

According recommendations, initial radiological examination for diagnosing RCC is US and CT is performed for assessment of local invasiveness, lymph node involvement or other metastases. MRI may be used when intravenous contrast medium is contraindicated. It is recognized to be superior to CT in detecting local advancements and venous involvements of tumor thrombus (Escudier et al. 2014). Abdominal and chest CT or MRI should be done for accurate staging of RCC. Bone scan or brain CT are not recommended unless the patient has clinical symptoms. PET-CT could be useful in detecting extra-renal metastasis rather than renal lesion (Wang et al. 2012).

A tissue specimen from nephrectomy should be used when available to determine the final histological diagnosis, classification, grading and evaluation of prognosis factor. A diagnostic biopsy could be performed when ablative therapies have been planned or before commencing systemic therapies for patients with metastatic RCC (Escudier et al. 2014).

2.3 Classification of renal cell carcinomas

2.3.1 Histopathology and grading

The first classification of renal tumors was published as early as 1826 by Koenig (Bhatt and Finelli 2014). In the past decade it has been realized that all RCCs are not related and it is no longer reasonable to place these tumor types in the same category (Jonasch, Gao, Rathmell 2014). RCC is not a single disease, but rather many cancers occurring in the kidney. In 1997 the Heidelberg classification of RCC was issued by the International Union Against Cancer (UICC) and the American Joint Committee on Cancer (AJCC). This classifies RCC into five groups according to histological features and genetic alterations, as described below (Kovacs et al. 1997). In the International Society of Urological Pathology (ISUP) 2012 Concensus Conference additional recommendations were made (Delahunt et al. 2013). According to it, morphotypes of RCC have prognostic significance, e.g. papillary types 1 or 2 and tumor necrosis are of prognostic significance, and in addition a chromophobe RCC should not be graded. In our study, the histology of RCCs was classified according to the Heidelberg classification and the different types of RCC are described below (Kovacs et al. 1997). Classification is based on morphological characteristics. Genetic alterations are linked to morphological features, but not normally available in clinical practice.

Clear cell renal carcinoma (ccRCC) is most frequent subtype of RCC, accounting 75% of cases. Tumor cells have a predominantly clear cytoplasm. Cells grow in solid, trabecular, tubular and cystic patterns, and additionally focal papillary growth may be seen. These tumors evince a highly specific deletion of chromosome 3p and mutation of the VHL gene is typical. In addition, deletion of chromosome arms 6q, 8p, 9p and 14q and duplication of chromosome band 5q22 may be typically observed.

Papillary renal cell carcinoma (pRCC) is the second most common type of RCC, compromising 10% of cases in surgical series. pRCC cells may be small with scanty cytoplasm, but may also have abundant cytoplasm with basophilic, eosinophilic or pale staining incidence. A papillary growth pattern predominates, though solid and tubulopapillary architecture have also been seen. Characteristic alternations are a trisomy of 3q, 7, 8, 12, 16, 17 and 20 and a loss of the Y chromosome.

Chromophobe renal cell carcinoma (chRCC) comprises 5% of all RCCs. Cells have pale and eosinophilic granular cytoplasm and cytoplasmic microvesicles can be seen in the electronic microscope. Genetically, loss of heterozygosity at chromosome 1, 2, 6, 10, 13, 17 and 21 and hypoploid DNA content are characteristic of this variant.

Collecting duct carcinoma accounts for approximately one per cent of RCCs. It has characteristically an atypical epithelium, sometimes with hobnail appearance and irregular channel lines. The channels are located in an inflamed stroma and focal mucin can be seen. Genetic abnormalities have not observed in the collecting duct type of RCC.

Unclassified renal cell carcinoma constitutes a diagnostic category which other RCC type criteria do not readily identify. To this category belong 4-5% of RCCs.

RCC grading: The histological differentiation of RCC is classified according to the Fuhrman system to four categories (grades 1-4) (Fuhrman, Lasky, Limas 1982). The classification is based on tumor cell nuclear size, irregularity and prominence (Delahunt et al. 2013), as shown in Table 1.

Table 1. Renal cell grading system according to the International Society of Urological Pathology.

Grade 1	Tumor cell nucleoli invisible or small and basophilic at 400 x magnification
Grade 2	Tumor cell nucleoli conspicuous at 400 x magnification but inconspicuous at 100 x magnification
Grade 3	Tumor cell nucleoli eosinophilic and clearly visible at 100 x magnification
Grade 4	Tumors showing extreme nuclear pleomorphism and/or containing tumor giant cells and/or the presence of any proportion of tumor showing sarcomatoid and/or rhabdoid dedifferentiation

2.3.2 TNM classification and staging

The TNM classification (TNM) by the American Joint Committee on Cancer (AJCC) and the International Union Against Cancer (UICC) is the most commonly used staging system for RCC. It contains three components: T (tumor) describes the size of the primary tumor and the extent of invasion; N (Node) the status of metastasis in regional lymph nodes and M (metastasis) indicates the status of metastasis or absence of it. The first TNM system for RCC dates from 1974 (Eggener 2010) and the 10th edition was last revised in 2010 (Sobin LH, Gorpodarowich MK, Wittekind CH 2009). The tumors in our study are classified according to TNM 2002 Classification of Malignant tumor (Sobin LH 2002), shown in Table 2. The anatomic stage (I-IV) of RCC is obtained from TNM information and it is used for RCC patient prognosis as described in Table 3. RCC patients with stage I disease have a five-year disease-specific survival of about 80-95% and patients with stage II have some 80 % (Elmore et al. 2003). If stage I-II RCC patients have invasions of the urinary collecting system, the five-year survival is decreases to 60% compared with over 90% without invasion (Verhoest et al. 2009). Patients with stage III disease have a five-year cancer-specific survival (CSS) of around 60%. Before the use of new targeted agents, RCC patients with stage IV disease had a five-year CSS of only 10% with a median overall survival (OS) of 10-15 months (Jonasch, Gao, Rathmell 2014). However, median OS has extended beyond two years since targeted agents have been available for the treatment of RCC (Jonasch, Gao, Rathmell 2014).

Table 2. TNM classification of renal cell cancer according to TNM classification of malignant tumors 2002.

T	**Primary tumor**
T1a	Organ confined, ≤ 4 cm
T1b	Organ confined, 4-7 cm
T2	Organ confined, ≥ 7 cm
T3a	Perinephric tissue, renal sinus, or contiguous into adrenal gland
T3b	Renal vein or vena cava below diaphragm
T3c	Vena cava above diaphragm
T4	Beyond Gerota's fascia
N	**Regional lymph nodes**
Nx	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in regional lymph node(s)
M	**Distant metastasis**
Mx	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Table 3. Anatomic stage of renal cell cancer.

Stage I	**T1**	**N0**	**M0**
Stage II	T2	N0	M0
Stage III	T1/T2	N1	M0
	T3	N0/N1	M0
Stage IV	T4	Any N	M0
	Any T	Any N	M1

2.4 Treatment of renal cell carcinoma

2.4.1 Surgical treatment

The first recorded nephrectomy was performed in 1862, in fact accidentally as in the surgery Wolcott assumed the mass be hepatic (Bhatt and Finelli 2014). Gustav Simon performed the first planned successful nephrectomy in Heidelberg in 1869 (Bhatt and Finelli 2014). At present surgery continues to play an important role in RCC treatment, and it is the only effective treatment for localized RCC. Nephrectomy can be performed with either radical or partial techniques (Escudier et al. 2014).

Localized RCC: For tumors < 7 cm (T1) partial nephrectomy is recommended (Escudier et al. 2014). The operation can be performed via open, laparoscopic or coeliscopic robot-assisted approaches. Laparoscopic radical nephrectomy is recommended if partial surgery is not technically possible (MacLennan et al. 2012). One study comparing hand-assisted laparoscopic partial nephrectomy and open partial nephrectomy found no difference in overall survival or recurrence-free survival, and the first-mentioned technique involved fewer intra- and postoperative complications (Nisen et al. 2015). Laparoscopic radical nephrectomy for tumors > 7 cm (T2) is the preferred option (Escudier et al. 2014). Open radical nephrectomy is the preferred option in locally advanced RCC (T3 and T4) tumors, although laparoscopic surgery can be considered (Escudier et al. 2014). If there is no evidence of adrenal or lymph node invasion in CT, extensive lymph node dissection or adrenalectomy is not recommended (Escudier et al. 2014).

Metastatic RCC: In the period of cytokinetherapy for RCC, cytoreductive nephrectomy was recommended in patients with good performance status (PS) (Flanigan et al. 2004). Now in the era of targeted therapy, cytoreductive (CN) nephrectomy is recommended for good PS RCC patients with a large primary tumor and with limited metastasis and for patients with a symptomatic primary tumor (Escudier et al. 2014). In one study, the median survival for CN patients was 20.6 compared to 9.6 months for patients without CN (Heng et al. 2014). A new study showed that patients underwent CN and targeted therapy had favorable OS compared to patients with targeted therapy alone, 17.1 months versus 7.7 months (Hanna et al. 2016). However, CN is not a preferred option for RCC patients with poor PS. Metastasectomy of all metastatic lesions has been shown to contribute to improvement of clinical prognosis (van der Poel et al. 1999). Embolisation is recommended before resection of hypervascular bone or spine metastasis (Ljungberg et al. 2010). Embolisation could be considered for patients with painful bone or paravertebral metastases in relieving symptoms (Forauer et al. 2007).

2.4.2 Cryotherapy

For patients with a small (< 3cm) cortical tumor, especially those with high surgical risk, solitary kidney, compromised renal function, hereditary RCC or multiple bilateral tumor radiofrequency or cryoablative treatments could be considered (Escudier et al. 2014). Cryoablation treatment causes direct cell damage by an argon gas based system and was first described in humans for renal cell cancer in 1995 (Bhatt and Finelli 2014; Uchida et al. 1995). These treatments have low recurrence rates and excellent cancer-specific survival (CSS) (Psutka et al. 2013).

2.4.3 Oncological treatments

2.4.3.1 Chemotherapy and radiotherapy

RCC has been shown to be a somewhat chemorefractory tumour type. Overexpression of the multidrug-resistance gene (*MDR-1*) has been connected to this chemoresistance (Mignogna et al. 2006). Vinca-alcaloids have been used alone or combined with interferons with a low response rate, from 2.5% to 16.5% (Pyrhonen et al. 1999). In addition, 5-fluorouracil (5-FU) has been studied but results have been poor. Kish et al. showed that low-dose 5-FU infusion had an overall response rate of 5.2% (Kish et al. 1994). Today immunotherapy has replaced chemotherapy in the treatment of mRCC.

Radiotherapy has only a limited role in the treatment of RCC. The approach can be used for unresectable local or recurrent disease with the aim of improving local control and in addition, palliative radiotherapy may be used for bone and brain metastases (Escudier et al. 2014). There is no randomized trial where radiotherapy has been applied as adjuvant or neoadjuvant treatment in RCC (Escudier et al. 2014).

2.4.3.2 Cytokine therapy

Before 2005, when angiogenesis and the mammalian target of the rapamycin (mTOR) pathway inhibitor were not available in the treatment of RCC, systemic generally used agents were cytokines (Quinn and Lara 2015). These agents (interferon alfa and interleukin-2) have modest efficacy and significant toxicity, and have therefore been replaced by the novel targeted agents (Quinn and Lara 2015). However, some patients evincing durable complete response to high-dose interleukin-2 , are in most cases cured (Klapper et al. 2008; McDermott et al. 2005). Interleukin-2 can be used in first-line treatment for patients with metastatic ccRCC as the ESMO 2014 guideline recommends (Escudier et al. 2014). Interleukin-2 has a major effect on the expansion and activation of the tumor-directed killer cell population, along with a cascade of proinflammatory cytokines (Mourad, Dutcher, Ennis 2014). Interferon alfa (IFN-α) modulates several proteins and is known to activate dendrite cells (antigen-presenting cells) (Mourad, Dutcher, Ennis 2014). Some RCC patients treated with IFN-α have shown durable response and

excellent survival (Parton, Gore, Eisen 2006). IFN-α is used in first-line treatment with bevacizumab for the good or intermediate risk group of ccRCC patients (Escudier et al. 2014).

2.4.3.3 Targeted therapies

Seven registered targeted systemic drugs are currently widely used in advanced RCC (Motzer et al. 2015). Five of them (bevacizumab, sunitinib, sorafenib, pazopanib, axitinib) are targeting vascular endothelial growth factor and angiogenesis. They prevent new vessel growth in tumors (Mourad, Dutcher, Ennis 2014). Temsirolimus and everolimus are directed toward inhibition of the mammalian target of rapamycin (mTOR-inhibitors) and they effects through antiangiogenesis, antimetabolism and impairment of protein synthesis (Mourad, Dutcher, Ennis 2014). The selection of the drug is based on the patient's individual prognosis. These drugs and their features are described in Table 4.

2.4.3.4 Checkpoint inhibitor therapy

Nivolumab is a new drug currently entering for the treatment of mRCC in second line (Table 4). It is a human lgG4 monoclonal antibody against programmed death 1 (PD-1) protein and serves as an immune checkpoint inhibitor (Motzer et al. 2015). PD-1 is expressed on activated T cells and PD-1 ligand (PD-L1) and 2 (PD-L2), which are expressed on immune and tumor cells (Motzer et al. 2015).

2.4.3.5 Other treatments

Corticosteroids can be used for patients with symptoms of brain metastases (Escudier et al. 2014). Biphosphonate therapy with zoledronic acid is used for patient with bone metastases of RCC, but osteonecrosis is an undesirable adverse event (Escudier et al. 2014). Denosumab is a monoclonal ligand antibody which has proved to be effective in preventing skeletal events in different type of bone metastasis cancers. However, osteonecrosis is an unwanted side-effect (Lipton et al. 2016).

Table 4. Drugs used in the management of advanced renal cell cancer.

Drug	Target	Dose	Effect	Side effects
Bevacizumab (Escudier et al. 2007)	Human monoclonal antibody against VEGF growth factor	10 mg/kg iv every 2 weeks with interferon 9 000 000 k.y 3 times in week s.c	First-line PFS 10.2 months, responses 31%, OS 18.3 mo	proteinuria, hypertension, thromboembolisms
Sunitinib (Motzer et al. 2007)	VEGFR- and PDGFR- TKI	50 mg p.o every day for 4 weeks and two weeks break	First-line PFS 9.5-11 months, responses 31%, OS 29.3 mo	diarrhea, nausea, hypertension
Sorafenib (Escudier et al. 2009)	Raf-kinase, VEGFR1-,-2,-3, PDGFR-beta, Ftl3, c-kit, RET-TKI	400 mg x 2 po every day continually	Second-line PFS 5.5 months, responses 10%, OS 16.6 mo	diarrhea, eczema, fatigue
Pazopanib (Motzer, McCann, Deen 2013)	VEGFR1,-2,-3, PDGFR-alfa, -beta, c-kit, FGFR1,-3, IL-2 induced T-cell kinase, Transmembrane glycoprotein receptor TKI	800 mg x1 po every day continually	First-line PFS 8.4 months, responses 31%, OS 28.4 mo	diarrhea, eczema, fatigue, hypertension, elevated liver enzymes, hair color changes
Axitinib (Motzer et al. 2013; Rini et al. 2011)	VEGFR1, -2, -3-TKI	5 mg po twice in day, option raise to 10 mg twice in day	Second-line PFS 6.7 months, responses 19%, OS 20.1 mo	diarrhea, hypertension, nausea, dysphonia, fatigue
Temsirolimus (Hudes et al. 2007)	m-TOR inhibitor	25 mg iv once in week	First-line PFS 3.8 months, responses 8.6%, OS 10.9 mo	fatigue, anemia, nausea, dyspnea, diarrhea, eczema, edema, stomatitis, elevated glucose and cholesterol, pneumonitis
Everolimus (Motzer et al. 2010)	m-TOR inhibitor	10 mg x 1 po every day continually	Second-line PFS 4.9 mo, partial responses 1.8 %	infections, dyspnea, fatigue, pneumonitis
Nivolumab (Motzer et al. 2015)	PD-1 inhibitor	3 mg/kg iv every 2 weeks	Second-line PFS 4.6 mo, responses 25%, OS 25 mo	fatigue, nausea, pruritus

2.5 Prognostic factors

At present, evaluation of RCC patients' prognosis is based on clinical factors such as tumor size and biological factors such as laboratory parameters and their combinations (Leibovich et al. 2003; Ljungberg et al. 2010; Patard et al. 2004). Several molecular markers have also been studied, but none has achieved status as an independent prognostic factor and they are therefore not recommended in routine practice (Ljungberg et al. 2010).

2.5.1 Stage, grade and histology

The most important and powerful prognostic factor continues to be clinical classification (Stage I-IV) according to the TNM system in RCC (Delahunt et al. 2002; Fergany, Hafez, Novick 2000; Mejean, Oudard, Thiounn 2003; Zisman et al. 2002). Tumor stage represents the size and spreading of a tumor.

Fuhrman and associates present a grading system for cell histological differentiation according to nuclear features and presence of nuclear pleomorphism, used since 1982 (Fuhrman, Lasky, Limas 1982). The system is widely accepted as an independent prognostic factor (Ljungberg et al. 2010; Mejean, Oudard, Thiounn 2003), although several problems have been identified. For example, nuclear prominence is more subjective and nuclear pleomorphism is poorly defined (Cheville et al. 2003). The prognostic value of this grading system for pRCC and chRCC has been debated, but it has been shown to constitute an independent prognostic factor for ccRCC in many studies (Cheville et al. 2003; Kallio et al. 2004; Klatte et al. 2010).

Clear cell RCC is reported to present higher tumor stage and nuclear grade compared to chRCC and pRCC. Furthermore, chromophobe and papillary types of RCC have a lower CSS rate when compared to ccRCC despite similar tumor stage (Delahunt et al. 2013). Cheville and colleagues found no difference in CSS between pRCC and chRCC ($p=0.91$) (Cheville et al. 2003). Papillary-type RCC is divided into type 1 and type 2 according to morphologic criteria (Leroy et al. 2002). Patients with type 2 pRCC have a poorer prognosis compared to type 1 RCC. In addition, type 2 pRCCs usually have higher tumor stage and grade (Delahunt et al. 2013). Collecting duct RCC is a less common morphotype of RCC and usually represents high tumor grade. When collecting duct RCC is diagnosed, one third to half of patients already have metastases (Delahunt et al. 2013). Sarcomatoid morphological changes can be found in all histologic subtypes of RCC. Sarcomatoid RCC has a very poor prognosis, a median survival between 4 to 9 months after diagnosis (Shuch et al. 2012).

Histological necrosis shows an association with poorer prognosis in ccRCC and chromophobe RCC, but not with pRCC (Cheville et al. 2003).

2.5.2 Clinical presentation

The first multivariate model for the prediction of renal cell cancer specific mortality was published in 1988 (Elson, Witte, Trump 1988). Currently for localized tumors there are two widely accepted systems to assess risk of disease progression: the SSIGN (stage size grade and necrosis – prognostic scoring system) model based on tumor stage, size and necrosis (Leibovich et al. 2003) and the University of California Los Angeles Integrated Staging System (UISS) based on tumor stage, grade and patient's Eastern Cooperative Oncology Group (ECOG) performance status (Patard et al. 2004). The systems are set out in Tables 5 and 6. The UISS system can also be used to predict survival for patients with advanced RCC. SSIGN and UISS systems categorize patients into low, intermediate and high risk groups for estimating CSS.

Table 5. SSIGN scores for localized renal cell cancer.

FEATURE		SCORE
Stage (TNM 2002)	pT1a	0
	pT1b	2
	pT2	3
	pT3a-4	4
Regional lymph node (TNM 2002)	pNx or N0	0
	pN1 or pN2	2
Tumor size (cm)	< 10	0
	≥ 10	1
Nuclear grade	1 or 2	0
	3	1
	4	3
Histological necrosis	No	0
	Yes	1
SCORES	Group	5-year metastasis-free survival (%)
0-2	Low risk	97.1
3-5	Intermediate risk	73.8
≥ 6	High risk	31.2

Table 6. UISS system for localized and advanced renal cell cancer.

Patient group		Prognostic group			
		Stage	Fuhrman's grade	EOCG status	5-year DSS (%)
Localized RCC	Low risk	1	1-2	0	91.1
	Intermediate risk	1	1-2	1 or more	80.1
		1	3-4	Any	
		2	Any	Any	
		3	1	Any	
		3	2-4	Any	
	High risk	3	2-4	1 or more	54.7
		4	Any	Any	
Metastatic RCC	Low risk	N1M0	Any	Any	32
		N2M0/M1	1-2	0	
	Intermediate risk	N2M0/M1	1-2	1 or more	19.5
			3	0,1 or more	
			4	0	
	High risk	N2M0/M1	4	1 or more	0

Metastatic RCC prognostic models were first devised, when cytokinetherapy was the standard therapy and the Memorial Sloane Kettering Cancer Centre (MSKCC) model was the first and standard system (Escudier et al. 2014). Currently, when targeted therapies are used as treatment in RCC, the MSKCC has been validated and updated, now known as the Heng or International Metastatic RCC Database Consortium (IMDC) criteria (Escudier et al. 2014; Heng et al. 2009). These systems classify patients into three groups (favourable, intermediate and poor) according to five criteria: Karnofsky performance status (KPS), time from diagnosis to treatment (< 1 year or > 1 year), hemoglobin, calcium, neutrophil count and platelet count as described in Table 7.

Table 7. Heng's or IMCD criteria and survival for advanced renal cell cancer

Number of risk factors	Risk Group	Median OS (months)	2-year survival (%)
0	Favourable	43	75
1-2	Intermediate	27	53
3-6	Poor	8.8	7

2.5.3 Proliferation and apoptosis markers

MIB-1 (murine monoclonal antibody-1 against Ki-67 antigen). Ki-67 expression is detectable during the G1 phase, it increases during the S phase and rapidly decreases after mitosis and is absent in the G0 phase (Gerdes et al. 1984; Mejean, Oudard, Thiounn 2003). The corresponding gene is located in chromosome 10 and MIB-1 assays reflect an equivalent amount of Ki-67 antigen (Cattoretti et al. 1992). Consequently, high MIB-1 expression indicates increased mitotic activity and MIB-1 is agreed to be an excellent marker of proliferation in immunohistochemical analyses (Mejean, Oudard, Thiounn 2003). The Ki-67 index has proved to be associate with tumor stage (Kankuri et al. 2006; Sakai et al. 2009; Tannapfel et al. 1996), but Kallio and coworkers found MIB-1 expression not to be associated with tumor stage (Kallio et al. 2004). In addition, there are contrary results in the literature as to an association between tumor grade and Ki-67/MIB-1 expression (Hofmockel et al. 1995; Kallio et al. 2004; Kankuri et al. 2006; Sakai et al. 2009). Many studies have shown that high MIB-1 expression has an association with poorer survival in RCC patients compared to those with low MIB-1 expression, but MIB-1 expression has not yet gained acceptance as an independent prognostic factor (Abel et al. 2014; Cheville et al. 2002; Gayed et al. 2014; Hofmockel et al. 1995; Kallio et al. 2004; Kankuri et al. 2006; Kramer et al. 2005; Sakai et al. 2009; Tannapfel et al. 1996; Visapaa et al. 2003). In addition, low pre-therapeutic Ki-67 expression had been associated with better PFS and OS in mRCC patients treated with sunitinib

compared patients with high Ki-67 expression (10.6 months vs. 6.5 months and 28.5 months vs. 15.7 months) (Rautiola et al. 2016).

Bcl-2, Bax and survivin. Bcl-2 family has an important role in regulating cell apoptosis, first identified in the 1980s (Strasser, Cory, Adams 2011; Westphal, Kluck, Dewson 2014). The Bcl-2 protein is known as an anti-apoptotic marker protecting mitochondria, while Bax is an apoptotic marker causing damage to mitochondria (Strasser, Cory, Adams 2011; Westphal, Kluck, Dewson 2014). Survivin is an apoptosis inhibitor expressed in fetal and tumor tissues but not detactable in most adult tissue (Wenzel et al. 2000). Apoptosis proteins are frequently downregulated in cancer while an anti-apoptosis protein such as Bcl-2 is overexpressed (Leibowitz and Yu 2010). Some studies have shown an association between tumor grade and Bcl-2 expression, but the results of some studies are conflicting showing an association of Bcl-2 expression with tumor grade as well as tumor stage (Kallio et al. 2004; Lipponen, Eskelinen, Syrjanen 1995; Sakai et al. 2009; Sejima and Miyagawa 1999). Kallio et al. showed that RCC patients with high Bcl-2 expression had better survival compared to those with low expression, but many studies have found no association between Bcl-2 and survival in RCC patients (Kallio et al. 2004; Lee et al. 2003; Lipponen, Eskelinen, Syrjanen 1995; Sakai et al. 2009; Sejima and Miyagawa 1999; Skolarikos et al. 2005). In contrast, high Bax expression has been shown to be associated with poorer survival in RCC patients (Kallio et al. 2004). Vasavada and associates found no association of Bcl-2 or Bax expressions with survival in RCC patients (Vasavada, Novick, Williams 1998). Negative survivin expression is found to have an association with better survival and lower tumor stage and grade in ccRCC patients (Shi et al. 2015), while Baytekin et al. observed that survivin expression was higher in lower tumor grades and had no association with tumor stage or survival in RCC patients (Baytekin et al. 2011).

2.5.4 Cell adhesion proteins

Claudins 1-5, 7 and fascin. Claudins are tight junction proteins, maintaining cell polarity and regulating the passage of ions, water and macromolecules through paracellular spaces (Lee, Huang, Ward 2006; Sawada et al. 2003). These proteins are abnormally expressed in several cancer types, for example breast, colon, gastric, hepatocellular, head and neck squamous, ovarian, pancreatic, prostate and thyroid papillary cancers (Morin 2005; Soini 2005). Fascin is an actin cross-linking protein and important for cell adhesion and motility. It is expressed at low levels or not expressed in normal tissues, while highly expressed in many carcinomas (Hashimoto, Skacel, Adams 2005). Only few studies of claudin expression and its associations with survival in RCC patients have been published (Fritzsche et al. 2008; Lechpammer et al. 2008). Patients with cRCC and positive claudin-1 expression have proved to have poorer survival compared to negative claudin-1 expression in a univariate analysis (3-year survival rate 72.6% vs 84.7%) (Fritzsche et al. 2008). In the same study Kaplan-Meier (KM) survival curves yielded the same result ($p=0.009$), but, interestingly, patients suffering from pRCC with positive claudin 1

expression have a trend towards better survival (p=0.064). Lechpammer and colleagues found that RCC patients with low expression of claudins 3 and 4 had better survival while expression of claudins 1, 7 and 8 had no association with survival (Lechpammer et al. 2008).

Higher fascin expression has been shown to be associated with higher tumor grade, stage and poorer survival in RCC patients (Tsai et al. 2007). Zigeuner et al. likewise found an association between higher tumor stage and grade and high fascin expression, but high fascin was an independent predictor for mRCC (Zigeuner et al. 2006).

2.5.5 Angiogenesis markers

Angiogenesis is necessary for tumor growth and it is especially important in RCC, which is a highly vasculated tumor type (Kim and Kaelin 2004). There are five mammalian VEGF ligands (VEGF) and three VEGF receptors (VEGFR) (Tugues et al. 2011). The angiopoetin (Ang)/Tie ligand receptor system is important regulator in vessel remodeling and maturation and thus an attractive therapeutic target (Gerald et al. 2013). This Ang family has four members: Ang-1, Ang-2, Ang-3, Ang-4 and two related receptors Tie-1 and Tie-2 (Hu and Cheng 2009). Angiogenesis markers have been studied in both tissue-based and blood-based assays (Maroto and Rini 2014).

VEGFs and VEGFRs. High VEGF expression has been shown to correlate with tumor stage, grade of necrosis and CSS in ccRCC (Jacobsen et al. 2004; Paradis et al. 2000; Rioux-Leclercq et al. 2007). VEGFR3 and VEGF-C are vital to lymphangiogenesis, activating in cancer while being largely inactivated in normal physiology (Alitalo and Detmar 2012). Expression of VEGFR3 has shown no association with tumor stage, grade or survival in patients with ccRCC or pRCC (Bierer et al. 2008). Higher VEGF expression is reported to be associated with shorter survival time and higher tumor stage and grade (Yilmazer, Han, Onal 2007).

A group DePrimo studied soluble angiogenesis marker levels in patients with mRCC treated with sunitinib. Significantly greater changes were observed in soluble plasma levels in VEGF (increased), VEGFR2 (decreased) and VEGFR3 (decreased) in patients exhibiting an objective tumor response compared with those exhibiting disease progression or stable disease (Deprimo et al. 2007). In another study where mRCC patients were treated with sunitinib the VEGFR2 level was observed to decrease, but had no association with PFS (Gruenwald et al. 2010). Clinical benefit is seen in sunitinib-treated mRCC patients whose plasma VEGF increase were lower compared to patients with progressed disease after two cycles of treatment (Kontovinis et al. 2009). Low baseline VEGF-A and VEGFR3 have been associated with better survival in mRCC patients in the sunitinib group, while in IFN-α the group no association is noted with survival (Harmon et al. 2014). In the same study baseline VEGF-C was not associated with survival either in the sunitinib arm or in the IFN-α arm (Harmon et al. 2014).

A marked decrease in the soluble VEGFR2 concentration in axitinib-treated patients with mRCC is

associated with a higher objective response rate (ORR) and longer PFS (Tomita et al. 2011).
A high baseline VEGF level has been associated with higher MSKCC score (poorer prognosis) and poorer PFS and OS when mRCC patients were treated with sorafenib (Escudier et al. 2009).
Rini and colleagues showed that patients with low baseline VEGFR3 and VEGF-C levels had better PFS and objective response rate (ORR) when they received sunitinib after their mRCC disease progressed after bevacizumab-based therapy (Rini et al. 2008). In contrast, the baseline level of VEGF-A has no association with survival. In contrast, low VEGFR3 expression has shown an association with poorer PFS after treatment with sunitinib (Garcia-Donas et al. 2013).

Ang-2 is a molecule which destabilizes vascular networks, thus supporting neoangiogenesis (Wang et al. 2014). Ang-1 and Ang-2 with their endothelial specific tyrosine kinases (Tie1 and Tie2), and VEGFs and VEGFRs regulate normal vascular remodeling and development, thereby tendering this system particularly interesting as a potential targeted therapy (Augustin et al. 2009; Bach, Uddin, Burke 2007; Eklund and Saharinen 2013; Gerald et al. 2013). Circulating Ang-2 levels are low in normal homeostasis, but high Ang-2 levels have been found in many diseases, for example sepsis and cancers (Milam and Parikh 2015; Sato et al. 2013). Higher Ang-2 plasma levels have been recorded in patients with RCC compared to benign disease. In addition, it was observed that higher Ang-2 plasma levels were associated with higher tumor stage and grade (Gayed et al. 2015). Wang et associates showed that Ang-2 expression was 11.3-fold higher in ccRCC than in normal kidney tissue ($p=0.0074$) (Wang et al. 2014). A lower baseline level of Ang-2 is associated with better tumor response in mRCC patients treated with sunitinib (Motzer et al. 2014). Higher Ang-2 level before nephrectomy associated with poorer prognosis (higher MSKCC risk group), but not associated to PFS in sunitinib treated mRCC patients (van der Veldt et al. 2012).

Microvessel density (MDV), CD31 and CD34. Microvessel density is an indicator of angiogenesis and microvessels are usually identified by factors CD31 and CD34 (Cheng et al. 2014). CD31 is a pan-endothelial cell marker and not only associated with newly formed microvessels, and CD34 is often expressed in the pericyte of blood vessels but rarely in normal vessels (Cheng et al. 2014). Higher CD31 expression has shown an association with better survival in patients with ccRCC. In addition, higher CD31 levels in mRCC patients have been proved to be associated with delayed progression (Sharpe et al. 2013). Another study showed no association of CD31 expression with survival, but low CD31 expression was associated with higher tumor stage and grade in RCC patients (Sandlund et al. 2007). Yilzamer and colleagues, for their part, found no association with tumor stage or grade and CD31 (Yilmazer, Han, Onal 2007). In contrast to CD31 expression, ccRCC patients with higher CD34 expression have had poorer disease–free survival (DFS) (Iakovlev et al. 2012), whereas Yildiz and colleagues showed poorer survival in patients with ccRCC and low CD34 expression (Yildiz et al. 2008).

VHL mutation leads to **HIF** accumulation which is critical for tumorigenesis in RCC (Linehan, Srinivasan, Schmidt 2010). VHL mutations and HIF-1α expressions have been studied in patients with RCC, but their reports on association with survival are divergent (Klatte et al. 2007; Lidgren et al. 2006; Schraml et al. 2002; Yao et al. 2002).

2.5.6 Immune system marker PD-L1

Immune cells are activated by RCC tumor cells, but the tumor is able to avoid this immune response (Frankenberger, Noessner, Schendel 2007). This immune system in cancer is complex and not well understood, but some knowledge has accrued as to the T-cell inhibitory pathway regulated by the protein programmed death 1 (PD-1). Nivolumab is a novel immunotherapeutic agent which inhibits the T-cell checkpoint regulator PD-1, making this immune system an interesting research topic in RCC patients (Quinn and Lara 2015). High PD-LI expression has shown an association with higher tumor grade and stage, tumor necrosis and poorer survival compared to low PD-L1 expression (Thompson et al. 2004; Thompson et al. 2006), and the results of a meta-analysis were similar (Xu et al. 2015).

2.5.7 Carbonic anhydrase IX (CAIX)

Sixteen different alpha-CA isoforms are currently known, regulating various physiological processes, including acid-base balance and ion transport (Li et al. 2015). Carbonic anhydrase IX (CAIX) is thought to play a role in regulating pH during hypoxia in tumor cells (Lam et al. 2005). Expression of CAIX is over-regulated by the VHL protein and high CAIX expression has been shown in ccRCC and low CAIX expression in pRCC and chRCC (Stillebroer et al. 2010). Over-expression of CAIX has been found to be associated with better survival in patients with locally advanced disease and patients with metastatic RCC (Bui et al. 2003).

2.5.8 Other markers and factors

Time to recurrence (TTR) has proved a significant predictor of cancer-specific survival in patients with RCC (Brookman-May et al. 2013). However, higher tumor stage and grade as well as poorer survival were associated in the same study. Incidentally discovered RCC has proved to have better prognosis compared to RCC diagnosed in a patient evincing symptoms (Patard et al. 2002). High expression of the cytoplasmic membranous protein cyclooxygenase-2 (COX-2) has shown an association with better prognosis and slow development of metastases (Kankuri et al. 2006).

3. AIMS OF THE STUDY

This retrospective study was conducted to evaluate possible prognostic factors and their associations with long-term survival and the known renal cell cancer prognostic markers, tumor stage and grade, in patients with renal cell cancer.

The specific aims in each study were:

1. To evaluate the associations between claudins 1-5, 7 and the survival of RCC patients.

2. To explore VEGFR3 and CD31 expressions and their associations with prognosis in RCC.

3. To examine the influence of MIB-1, Bcl-2 expressions alone and combined with VEGFR3 and CD31 expression in long-term survival in RCC.

4. To evaluate Ang-2 expression alone and combined with expressions of proliferation (MIB-1 and Bcl-2) and angiogenesis (VEGFR3 and CD31) markers in RCC tumors and their associations with long-term survival.

4. PATIENTS AND METHODS

4.1 Patients

Our retrospective study population comprised 224 of patients undergoing nephrectomy between 1985-1995 at either Tampere University Hospital or Tampere Hospital, in Tampere, Finland. The list of 299 RCC patients from this period was obtained from the Finnish Cancer Registry. Five of these were autopsy samples and were excluded from analyses. The data were collected directly from the patient records of these two hospitals. All tumor samples were re-evaluated and reclassified according to the Heidelberg classification and the Fuhrman grading system by an experienced uropathologist (Paula Kujala, MD, PhD). The patients' clinical characteristics set out in Table 8. The median follow-up was four years IQR 1.27-7.24 in paper I and 5.4 years IQR 1.41-11.9, the longest follow-up reached 21.9 year, in papers II-IV. The reason for the different follow-up times in the individual papers was that patients' data were re-evaluated during this project. After nephrectomy all patients were treated and follow-up was conducted according to standard clinical practice at that time.

4.2 Methods

Multi-tissue blocks were obtained to evaluate and select the highest tumor grade area (1 mm core) in each tumor. These multi-tissue blocks were used for further immunohistochemical analysis. All immunohistochemistry analyses were performed without knowledge of the patients' history. Antibodies used for immunostaining in this study are described in Table 9 and in detail in section 4.3.

Table 8. Baseline Patient Characteristics

Patients (N = 224)	132 men (58.9 %) 92 women (41.1 %)
Mean age at time of diagnosis	63.4 (IQR 55.9-71.9)
TNM classification	
T1	104 (46.4%)
T2	29 (12.9%)
T3	38 (17.0%)
T4	4 (1.8%)
N+	13 (5.8%)
M1	36 (16.1%)
Stage	
1	79 (35.3%)
2	44 (19.6%)
3	61 (27.2%)
4	39 (17.4%)
Histology	
Clear cell renal cell carcinoma	202 (90.2%)
Papillary renal cell carcinoma	12 (5.4%)
Chromophobe renal cell carcinoma	5 (2.2%)
Unclassified renal cell cancer	4 (1.8%)
Collecting duct renal cell carcinoma	1 (0.4%)
Grade	
1-2	22 (9.8%)
3	114 (50.9%)
4	88 (39.3%)

Table 9. Antibodies used in immunostainings.

ANTIGEN	DESCRIPTION	DILUTION	SOURCE
Claudin 1	polyclonal rabbit anti-claudin 1	1:50	Zymed Laboratories Inc.
Claudin 2	monoclonal mouse anti-claudin 2 antibody	1:50	Zymed Laboratories Inc.
Claudin 3	polyclonal rabbit anti-claudin 3	1:50	Zymed Laboratories Inc.
Claudin 4	monoclonal mouse anti-claudin 4	1:50	Zymed Laboratories Inc.
Claudin 5	monoclonal mouse anti-claudin 5	1:50	Zymed Laboratories Inc.
Claudin 7	polyclonal rabbit anti-claudin 7	1:50	Zymed Laboratories Inc.
VEGFR3	monoclonal VEGFR3 mouse antibody	1:100 000	Kari Alitalo, Helsinki, Finland
CD31	mouse antihuman CD31	1:200	Novocastra Laboratories Ltd.
MIB1	monoclonal MIB1 antibody	1:110	Immunotech S.A
Bcl-2	monoclonal mouse antihuman Bcl-2	1:60	DAKO
Ang-2	polyclonal goat antibody	1:50	R&D Systems

4.3 Immunostainings

4.3.1 Claudins 1-5 and 7 (I)

The primary antibodies used for immunostaining were obtained from Zymed Laboratories Inc (South San Fransisco, CA, USA) and were designed for use in formalin-fixed, paraffin-embedded tissues. They were polyclonal rabbit anti-claudin 1 (clone JAY.8), monoclonal mouse anti-claudin 2 antibody (clone 12H12), polyclonal rabbit anti-claudin 3 (clone Z23.JM), monoclonal mouse anti-claudin 4 (clone 3E2C1), monoclonal mouse anti-claudin 5 (clone 4C3C2) and polyclonal rabbit anti-claudin 7 (clone ZMD.241). Before application of the primary antibodies, sections were heated in a microwave oven in 10 mM citrate buffer, pH 6.0, for 10 minutes. After 60 minutes of incubation with the primary antibody (dilution 1:50 for anti-claudin 1, 2, 3, 4, 5 and 7), a biotinylated secondary anti-rabbit antibody and the Histostain-SP kit (Zymed Laboratoris Inc, South San Fransisco, CA, USA) were used on the sample. In all cases, the colour for immunostaining was developed by diaminobenzidine, after which the sections were lightly counterstained with hematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany). Negative control stainings were made by substituting non-immune rabbit or mouse serum and phosphate-buffered saline for the primary antibodies. Immunostaining results were categorized as follows: 0, no immunostaining; 1, weak immunostaining (< 50% membrane-bound positivity); 2, moderate immunostaining (50% to 90% membrane-bound positivity); or 3, strong immunostaining (>90% membrane-bound positivity). Expression of claudins was evaluated only in tumor cells.

4.3.2 VEGFR3 and CD31 assays (II, III and IV)

Immunohistochemistry (IHC) for CD31 (1:200, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) was performed on formalin-fixed, paraffin-embedded tissue sections as a part of a TMA. Briefly, the sections were deparaffinized with xylen and rehydrated in graded alcohol, treated in an autoclave in 10 mmol/l sodium citrate (pH 5.0) for 2 min, and washed with phosphate-buffered saline. Primary antibody was incubated at 4°C overnight, and antibody binding was detected by Vectastain ABC kit reagents (Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine (DAB) was used as chromogen. The slides were counterstained with haematoxylin and eosin, and mounted.

VEGFR-3 was stained with the 9D9 antibody (a mouse mAb against the extracellular domain of human VEGFR-3**;** a kind gift from Professor Kari Alitalo, Helsinki, Finland) at a concentration of 10 μg/ml as described in detail elsewhere (Bono et al. 2004).

Micorovascular density (MVD) was quantified as the number of CD31-positive or VEGFR-3-positive microvessels per high-power field at x 250 (field of view 0.407mm^2, including the whole TMA core) using a Leitz Laborlux 12 bright-field microscope (Leitz Wetzlar GmbH). Two fields with the highest

density of vessels were counted and the average of two scores was reported. Scoring was performed in blinded manner. Two investigators analyzed these specimens independently.

4.3.3 Analysis of MIB-1 and Bcl-2 (III and IV)

Sections were deparaffinized, and antigen retrieval was achieved by heating the sections in a microwave oven for 2 x 7 minutes in 10 mM tris/10 mM ethylenediaminetetraacetic acid (pH 9.0). For acid Ki-67 antigen immunostaining, the monoclonal antibody MIB-1 (IgG1, Immuno-tech S. A., Mareille, France) was used at a 1:110 dilution. Counterstaining was accomplished using 0.4% ethyl green in acetate buffer. The staining of MIB-1 was evaluated by visual estimation and using a computer-assisted image analysis system (CAS-200 Software; Becton Dickinson, Parsippany, New Jersey, USA). The MIB-1 index was defined as the percentage of cells with immunopositivity in the nuclei. We first evaluated patients between 1990 and 1995 by visual estimation; only definitely brown nuclei being recorded as positive, and the same samples were evaluated using a CAS-200. Spearman's correlation between the visual estimation and CAS-200 software was excellent 0.826 ($p<0.001$). Samples from patients seen between 1985 and 1990 were analysed by CAS-200, and the results of computer-assisted image analysis were used for statistical analysis.

Monoclonal mouse antihuman Bcl-2 oncoprotein clone 124 (Dako, Glostrup, Denmark) was used at a 1:60 dilution. Sections were slightly counterstained by haematoxylin, and staining for Bcl-2 was analysed semiquantitatively. Stainings were quantitated by intensity (0 to 3) and the percentage area of expression (0% to 100%), and by multiplying these figures to obtain staining scores (0 to 300).

4.3.4 Assay of Ang-2 (IV)

IHC was performed on deparaffinized (Tissue-Tek, Tissue Clear, Sakura Finetek Europe, AJ Alphen aan den Rijn, NL) and rehydrated sections using the Tyramide Signal Amplification kit (PerkinElmer, Waltham, MA, USA) following the manufacturer's instructions. Antigen retrieval was conducted in 10 mM citrate buffer using 2100-Antigen Retriever (Aptum Biologics Ltd., Southampton, UK) 120°C for 20 min. and cooling for 2 hours. Endogenous peroxidase activity and nonspecific binding sites were blocked using 10% hydrogen peroxidase and a TNB (0.5 %) blocking buffer (PerkinElmer), respectively. The sections were incubated with goat polyclonal antibodies to amino acid residues Asp68-Phe496 of human Angiopoietin-2 (1:50, 0.2 mg/ml, R&D Systems, Minneapolis, MN, USA) diluted in TNB overnight at +4°C, washed with TNT buffer (100 mM Tris pH 7.4, 150 mM NaCl, 0.05% Tween-20), and subsequently incubated with biotinylated anti-goat secondary antibody (1:300, 1.5 mg/ml, Vector Laboratories Inc, Burlingame, CA) in TNB for 30 min at room temperature, washed and detected using the chromogenic visualizing method AEC (3-amino-9-ethylcarbazole) for 8 min. The sections were counterstained with Mayer's Hemalum Solution (Merck KGaA, Darmstadt, Germany) and mounted using Aquatex (Merck KGaA). To confirm the specificity of the Ang-2 staining, Ang-2 antibodies were

incubated with 5x molar excess of recombinant human Ang2- (3µg) (R&D Systems) in TNB for 20 min, and subsequently used for staining of the tissue sections as above.

Ang-2 IHC slides were scanned (The Genome Biology Unit GBU, University of Helsinki, Finland) by Pannoramic FLASH II (3DHISTECH Ltd., Budapest, Hungary) using a 40 x objective, low and high Ang-2 expression are illustrated in Figures 1 and 2. Digital image quantification of positive Ang-2 vessel endothelial area was analyzed using HistoQuant module image software (3DHISTECH).

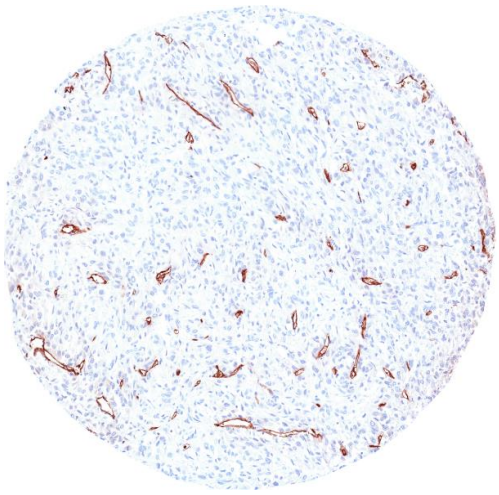

Figure 1. Low Ang-2 expression in RCC tissue.

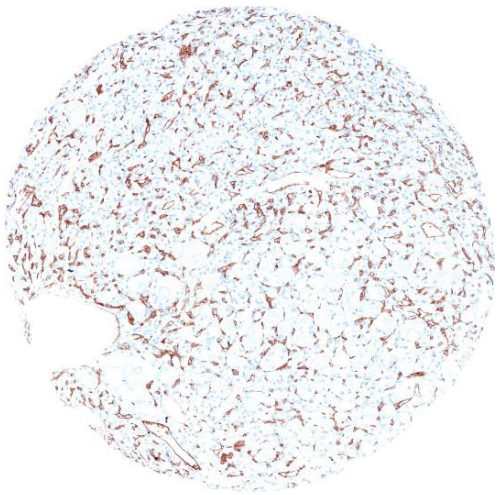

Figure 2. High Ang-2 expression in RCC tissue.

4.5 Ethical statement

The research protocol and use of tumor samples were approved by the Ethics committee at Tampere University Hospital (R94534) and the National Authority for Mediolegal Affairs.

4.6 Statistical methods

The associations of categorial covariates were tested by cross-tabulation and chi-square test or Fisher's exact test (papers I-IV). Continuous variables were tested by Kruskal-Wallis test due to skewed distribution (paper II). Age- and gender adjusted univariate and multivariate survival analyses were made using Cox proportional hazards models (papers I-IV). Kaplan-Meier survival estimation methods were used to illustrate survival in RCC patients (papers I-IV). Statistical analyses were performed using IMB SPSS Statistics for Windows (version 14.0.2 in study I, version 21.0 in study II-IV). All P-values under 0.05 were considered statistically significant.

5. RESULTS

All (Studies I-IV) involved 224 RCC patients where treated and follow-up were conducted in the same clinical practice. The median follow-up was 4 years in the Study I and 5.4 years in the Studies II-IV.

5.1 Claudins 1-5 and 7 (I)

Positive immunoreactivity for claudins 1, 2, 3, 4, 5 and 7 was present in 62.2%, 67.4%, 44.6%, 54.7%, 7.4% and 35.4% of tumor samples, respectively. Negative staining for claudin 1-5 and 7 varied between 32.6% and 92.6% being lowest in claudin 2 and highest in claudin 5. Strong immunoreactivity was observed most in claudin 2 (20.2%), lowest in claudin 5 (2.1%). Expressions of claudins 1-5 and 7 are shown in Table II, page 4183. All papillary and all chromophobe types of RCC were positive for claudin 2, but their percentages among all tumor samples were low, 5.4% and 2.2%. Immunostaining failed in claudins 1, 2, 3, 4, 5 and 7 in 4.5%, 2.2%, 2.7%, 2.7%, 3.6% and 3.1%, respectively, and these cases were excluded from analyses.

Claudins 1 and 2 were associated significantly with tumor grade (Pearson chi-square test; $p<0.001$ and $p<0.009$) while neither claudin had associations with tumor stage. Cross-tabulations between claudins with tumor stage and grade are depicted in Tables III and IV, page 4185.

The strong prognostic factors tumor grade and stage were associated with survival but neither claudin showed association with survival in RCC patients. Age and gender univariate analysis of claudins 1-2 and tumor grade and stage are shown in Table V, page 4186.

5.2 VEGFR3 and CD31 (II)

VEGFR3 expression was observed to be negative in 45.8 % of tumor samples while, only 1.8% of CD31 stainings were negative. Most pRCC samples showed both low expression of VEGFR3 (91%) and CD31 (75%) and furthermore all four chRCC had low CD31 expression. VEGFR3 expression was categorized into low and high groups according to vessels immunoreactivity and CD31 expression classified into low and high according to the median expression value of 18. Only a minority of samples failed in immunostaining and was excluded from statistical analysis; 5.4% in both VEGFR3 and CD31 respectively.

Low VEGFR3 expression was distributed almost significantly according to higher tumor stage, while CD31 expression was not, independent in Kruskal-Wallis test; $p=0.058$ and $p=0.50$), shown in Figures 1 and 2, page 923. In contrast, low CD31 expression had an almost significant association with higher tumor stage, while VEGFR3 had none with tumor stage; Pearson chi-square test showed $p=0.069$ and $p=0.899$ respectively, cross-tabulation described in Table II, page 922.

Age- and gender-adjusted Cox regression univariate analysis showed low CD31 expression and both higher tumor stage and grade to have associations with poorer survival in RCC patients; Cox proportional hazard models are shown in Table III, page 922. In addition, Kaplan-Meier survival analysis also confirmed the same results, KM-curves described in Figures 3-5, pages 924-925. VEGF3 expression was not associated with survival in these analyses.

5.3 MIB-1 and Bcl-2 alone and combined with VEGFR3 and CD31 (III)

All 224 tumor samples MIB-1 immunostainings were successful and only three samples of Bcl-2 immunostaining failed. Only one sample of MIB-1 staining was negative and 33.9% of the Bcl-2 samples were negative. MIB-1 and Bcl-2 expressions were classified into low and high class according to the median values of their stainings (1.36 and 30).

High Bcl-2 expression was associated with pRCC (p=0.037), but other RCC types had no associations with Bcl-2 or MIB-1 expressions. Cross-tabulation showed an association between low Bcl-2 and high tumor stage (p=0.011) and grade (0.002), whereas MIB-1 had no associations with the classical prognostic factors, p=0.19 and p=0.81, respectively.

In univariate analysis both proliferation marker MIB-1 and anti-apoptosis marker Bcl-2 showed associations with survival in RCC. High MIB-1 compared to low MIB-1: HR 1.76, 95% CI 1.16-2.68 and p = 0.008; for low Bcl-2 compared to high BCL-2: HR 2.16, 95% CI 1.42-3.31 and p < 0.001, respectively. Furthermore, Kaplan-Meier survival curves showed their association with survival, illustrated in Figures 1 and 2, page e287. In addition, high MIB-1 expression was associated with poorer survival in multivariate analysis, with both tumor stage and grade and Bcl-2 expression, shown in Table 4, page e286.

Cox regression univariate analysis also performed the combinations MIB-1/Bcl-2, MIB-1/VEGFR3, MIB-1/CD31, Bcl-2/VEGFR3, Bcl-2/CD31 and VEGFR3/CD31 according to classification low/high as described in Study III and Study II. We found that low Bcl-2 was associated with better survival despite Bcl-2 were combined with any studied marker; univariate analysis shown in Table 2, page e286. In multivariate analysis performed both tumor stage and grade survival and with combinations of proliferation markers MIB-1/Bcl-2 and angiogenesis markers VEGFR3/CD31. High MIB-1/low Bcl-2 and low VEGFR3/high CD31 were associated poorer survival compared to low MIB-1/high Bcl-2 (HR 3.20, CI 95% [1.66-6.17] p<0.001 and low VEGFR3/high CD31 (HR 2.48, CI 95% [1.29-4.78] p=0.007) as shown in Table 4, page e287.

5.4 Ang-2 alone and combined with MIB-1, Bcl-2, VEGFR3 and CD31 (IV)

Two hundred (83.9%) of the total tumor sample stainings were successful and median Ang-2 expression was 5.59 range 0.07-25.65. Categorization of Ang-2 into low and high according to median expression showed no association with tumor histological type, while all samples evincing expression higher than 6.40 were ccRCC type. Low Ang-2 expression was associated with both higher tumor stage and grade, Fisher's exact test showed p=0.042 and p=0.019. In addition, high Ang-2 expression had significantly associations with low MIB-1, high Bcl-2 and positive VEGFR3 expressions, as presented in Table 2.

Ang-2 expression was not associated with survival in univariate analysis, Table 3, but high Ang-2 expression was associated with better survival in multivariate analysis; HR 1.89, CI 95% [1.16-3.08] p=0.010, Table 4, page 14. Low Bcl-2 and high MIB-1 combined with low or high Ang-2 expression had a lesser association with survival, as shown in Table 3, page 13. Age and gender multivariate analyses were performed with angiogenesis and proliferation markers together with tumor grade and stage. This showed better survival and lower tumor stage and grade but also in low MIB-1 and high Ang-2 expressions in Table 4, page 14. When Ang-2 expression was combined with other angiogenesis marker (VEGFR3 and CD31) expressions, they did not indicate an association with survival, as seen in Table 3 page 13 and Figures 5-6 pages 21-22. Multivariate analysis and KM curves demonstrated that MIB-1 and Bcl-2 expressions were more powerful prognostic factors as against Ang-2 expression, when they were combined with Ang-2, as shown in Tables 5-6 pages 15-16 and Figures 3-4 pages 19-20. Interestingly, when Ang-2 expression was divided into low/high according to median it had no association with survival (p=0.47), but categorization according to upper quartile showed better survival in the high expression group (p=0.046), as seen Figure 2, page 18.

5.5 Summary of the main results

Our study showed that expressions of Bcl-2 and Ang-2 have an association with tumor stage. Claudin 1 expression was associated most markedly with tumor grade, but also expressions of claudin2, Bcl-2 and Ang-2 had a significant association with tumor grade. High CD31, high Bcl-2 and low MIB-1 were associated with better survival in univariate analysis, but low MIB-1 and very high expression had a significant association with better survival in multivariate analysis including tumor stage and grade. A summary of single markers is set out in Table 10.

Table 10. Single markers and their associations with tumor stage, grade and survival.

markers	tumor stage	tumor grade	survival univariate analyses	survival multivariate analyses
claudin 1	-	+++	-	-
claudin 2	-/+	++	-	-
claudin 4	-	-/+	-	-
claudins 3,5,7	-	-	-	-
VEGFR3	-	-	-/+	-
CD31	-/+	-	+	-
MIB-1	-	-	++	++
Bcl-2	+	++	+++	-
Ang-2	+	+	-	+

- = no association, -/+ borderline association (p 0.05-0.10), + = $p< 0.05$,

++ = $p<0.01$, +++ = $p < 0.001$

6. DISCUSSION

6.1 Methods

This was a retrospective study and all information on patients was collected from patient records. The survival data were obtained from the Finnish Cancer Registry. This study involved all patients, whose RCC was diagnosed in Pirkanmaa, Finland between the years 1985-1995 and thus represents fully this area's RCC cases, but does not necessarily reflect the status in the entire Finnish RCC population during the period. The study cohort is part of our previous larger study of prognosis in RCC patients (Sunela et al. 2009). The classifications of tumor types and grading were also particularly reliable, as all cancer samples were examined and re-classified (blinded for patient history or outcome) by using the Heidelberg classification and Fuhrman grading systems (Fuhrman, Lasky, Limas 1982; Kovacs et al. 1997) by an experienced uropathologist (Paula Kujala). The immunostainings of all samples were carried out likewise without knowledge of patient history or survival. Immunostaining of all samples was not completely satisfactory studied markers for statistical analysis, but the failure rate varied from zero only up to 9.4%. The median follow-up of our patients was 5.4 years and continued up to 21.7 years, which is a strength of this study. All patients had the same follow-up and treatment protocols according to clinical practice during the study period. Only twenty-three patients were treated with interferon, which has some anti-angiogenetic activity, but specific anti-angiogenic drugs or checkpoint inhibitors had not yet been approved for the treatment of RCC at that time.

6.2 Single prognostic markers

Tumor stage and grade have been shown to constitute independent prognostic factors in many studies, and they are used in guidelines to prognosticate survival of RCC patients (Cheville et al. 2003; Delahunt et al. 2002; Escudier et al. 2014; Fergany, Hafez, Novick 2000; Kallio et al. 2004; Ljungberg 2004; Ljungberg et al. 2015; Mejean, Oudard, Thiounn 2003). In our study both tumor stage and grade were the most powerful prognostic factor for survival, thus this patient material is typical for RCC.

High expressions of Bcl-2 and Ang-2 were associated with lower tumor stage and grade, while other single markers, claudin 1-5 and 7, VEGFR3, CD31 and MIB-1, were not associated with tumor stage. In addition, low claudin 1 expression had an association with higher tumor grade, while in the case of claudin 2 expression, the association with tumor grade was vice versa. Fritzsche et al. found that low claudin 1 expression was associated with lower tumor grade and stage, which is contrary to our results, but their study included only 44 patients and the study cohort was selected in that all tumors were pRCC

type (Fritzsche et al. 2008). Claudin 2 expression and its association with RCC stage or grade has not been addressed in the literature.

In our study, lymphangiogenesis marker VEGFR3 expression had no association with the classical prognostic factors, tumor stage and grade, neither with survival as Bierer and associates had previously shown (Bierer et al. 2008). Both higher grade and stage have been shown to associate with lower Bcl-2 expression in two studies (patients in study n=101 and n=138) (Itoi et al. 2004; Kallio et al. 2004) as the result of our study showed. There are also studies demonstrating association only of tumor stage or grade with Bcl-2 expression or neither of them, and in addition the number of patients varied 28-153 (Lee et al. 2003; Lipponen, Eskelinen, Syrjanen 1995; Sakai et al. 2009; Sejima and Miyagawa 1999; Skolarikos et al. 2005; Vasavada, Novick, Williams 1998). Associations of Ang-2 expression with tumor grade, stage and RCC patients' survival had been studied only from plasma samples of RCC patients, but not from tumor tissue. Ang-2 levels are higher in ccRCC than in normal kidney tissues (Wang et al. 2014) and a high Ang-2 plasma level has an association with higher tumor grade and stage (Motzer et al. 2014), while our results showed that high Ang-2 is associated with lower tumor stage and grade. We do not know how plasma levels of Ang-2 correlate with Ang-2 expression in RCC tissue and this provides an interesting study topic in the future.

This study showed that low MIB-1, high Bcl-2, high CD31, and very high Ang-2 expressions are associated with better survival in RCC patients, while other tested single markers have no association with prognosis. The claudins have been suggested as diagnostic markers in a number of cancers and as therapeutic targets in several cancers (Morin 2005; Neesse et al. 2012). Our results showed no association between claudins 1-5 and 7 and survival, implying that target therapy for claudin may not be of benefit to RCC patients, unless future studies show otherwise.

We showed that patients with high CD31 expression had better survival, as Biswas and co-workers also showed in a study of 168 patients with ccRCC (Biswas et al. 2012). In addition, one study showed that mRCC patients evincing higher CD31 expression had better response to anti-angiogenic therapy (Dornbusch et al. 2013). According to previous studies and our results, assays of CD31 expression could be useful in RCC patient. When Ang-2 expression was categorized into low and high according to median, it had no association with survival, as was the case with very high Ang-2 expression. The Ang-Tie signaling system is complex containing four circulating angiopoietin proteins (Ang-1, Ang-2, Ang-3 and Ang-4), which interact with Tie-1 and Tie-2, and this system maintains blood vessel homeostasis (Richey and Hutson 2013). Ang-2 is an antagonist for Ang-1 and activates Tie-2 signaling (Hu and Cheng 2009). Thus, the ratio of Ang-1 to Ang-2 is critical in balancing Tie-2, which signals and regulates vascular homeostasis (Fagiani and Christofori 2013). Examination of Ang-1 expression in RCC and the ratio of Ang-1 and Ang-2 expressions may improve our knowledge of tumor behavior. Very high Ang-2 expression also had a significant association with better survival also in multivariate analysis containing the classic prognostic factors tumor stage and grade.

High expression of anti-apoptosis marker Bcl-2 and low expression of proliferation marker MIB-1 have previously shown an association with better outcome in RCC patients (Abel et al. 2014; Cheville et al. 2002; Hofmockel et al. 1995; Itoi et al. 2004; Kallio et al. 2004; Kankuri et al. 2006; Kramer et al. 2005; Sakai et al. 2009; Tannapfel et al. 1996). Anti-apoptotic proteins such as Bcl-2 are frequently overexpressed in cancer while apoptotic proteins decreased (Leibowitz and Yu 2010). Histological tumor cells necrosis has been proved to associate with worse survival in RCC (Sengupta et al. 2005) while high Bcl-2 expression has shown to prevent necrosis (Nikoletopoulou et al. 2013). This might be one reason for better survival of those RCC patients evincing high Bcl-2 expression. Our study yielded similar results and furthermore the number of patients was higher than in those studies. Bcl-2 showed prognostic significance in univariate analysis, MIB-1 however also in multivariate analysis; MIB-1 would thus appear to be an independent prognostic factor in RCC patients.

6.3 Combined prognostic markers

In addition to the association of single markers with survival, all possible combinations (low/low, low/high, high/low and high/high) of three angiogenesis (CD31, VEGFR3, Ang-2) or cell proliferation markers (MIB-1, Bcl-2) were examined. Similar studies are not to be found in the literature. In univariate analysis Bcl-2 expression seemed to be the strongest significant factor affecting survival. The trend was towards high Bcl-2 expression with low or high expression of other studied marker being associated with better outcome. Similarly low MIB-1 expression tended to be associated better survival when it was combined with other markers. In contrast, angiogenesis markers combined among themselves or with cell proliferation markers had not consistent association with survival.

6.4 Future aspects

Targeted therapies were initially approved in 2005 for RCC treatment and since then overall survival (OS) has improved compared to earlier therapies (Vaishampayan et al. 2014). New drugs for the treatment of RCC are under development and two of them have recently obtained promising results (Quinn and Lara 2015). One is nivolumab, which inhibits the T-cell checkpoint regulator PD-1 and the other is cabozantinib, a multikinase inhibitor targeting VEGFR, MER, RET and AXL (Quinn and Lara 2015). Angiopoetins as therapeutic targets in RCC have recently been debated in the literature and may offer one treatment mode for RCC in future, as our understanding of this area expands (Wang et al. 2014). Our study found that Ang-2 expression is associated with survival, but we need prospective studies to confirm our results.

There is no consensus as to how we should conduct the follow-up of localized RCC after surgery (Escudier et al. 2014). Nearly one third of recurrences are missed, when follow-up proceeds according to

American urological Association or National Comprehensive Cancer Network guidelines (Smith and Milowsky 2014). We are moving toward a better understanding of RCC biology in terms of molecular pathway abnormalities, histological subtypes and new morphological variants, but we need to know how different markers affect survival and recurrence (Gore et al. 2014; Smith and Milowsky 2014). Intensive surveillance increases costs, but in several cancers it brings no benefit in improvements in outcomes and we should also assess how the follow-up affects patients' quality of life (Smith and Milowsky 2014).

New drugs for the treatment of cancer are typically expensive than the older. One study has shown, that the average cost of mRCC from initiation of treatment until death is 32,951€ in Finland and the costs of RCC treatment are still increasing due to adoption of new and more expensive medication, an aging population and improved survival times (Purmonen et al. 2010).

Treatment of RCC presents a number of challenges as mentioned above. We should select from among all RCC patients precisely those, who will benefit from intensive surveillance. In addition, we should choose the appropriate treatment for each patient individually. This retrospective study showed that expressions of Ang-2, MIB-1, Bcl-2 and CD31 are associated with survival in RCC. At least their expressions should be explored in prospective studies in the future.

7. SUMMARY AND CONCLUSIONS

The present research series explored both previously assessed and newer possible prognostic markers in RCC patients treated without antiangiogenetic drugs.

The main results of this study were:

1. Tumor stage and tumor grade were the most powerful prognostic factors compared to prognostic candidates studied.

2. Claudins and VEGFR3 showed no associations with survival.

3. Very high expression of angiogenetic marker Ang-2 was significantly associated with better survival in RCC patients compred to lower expression.

4. Patients evincing high expression of proliferation marker MIB-1 reflected a significant assosiation with poorer prognosis.

5. Improved survival was observed in patients with low antiapoptosis marker Bcl-2 expression, but only in univariate analysis.

6. Microvascular density marker CD31 expression was associated with poorer survival in RCC patients evincing low expression.

7. Expressions of MIB-1 and Bcl-2 proved to have more prognostic value compared to angiogenesis markers in compared expession analysis.

8. RCC patients surviving over ten years after nephrectomy, seemed to have excellent survival despite tumor stage.

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Tampere, August 2016

Juha Virman

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Claudins as Prognostic Factors for Renal Cell Cancer

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Abstract. *Background: Claudins are tight junction proteins and their expression is often different in normal and corresponding tumor cells. In the present study, we determined how the expression of claudins 1-5 and 7 correlated to survival, grade and stage of patients with renal cell cancer (RCC). Patients and Methods: Primary tumor samples were collected retrospectively from 229 RCC patients. Claudins were detected by immunohistochemistry using commercial monoclonal antibodies against claudins 1-5 and 7. Median survival time was 6.5 years confidence interval (CI) (4.5-8.5, n=224). Kaplan-Meier survival estimated method was used in survival analyses. Results: Positive expression was detected in 62%, 67%, 45%, 55%, 7% and 35% of cases for claudins 1, 2, 3, 4, 5 and 7, respectively. High expression of claudin 2 was observed in 20% of cases while high expression of other claudins was less frequent. Claudins were compared to classical prognostic factors. On cross-tabulation, claudin 1 ($p<0.001$) and claudin 2 ($p=0.009$) were significantly associated with lower-grade and higher-grade tumors, respectively. None of the claudins was significantly associated with tumor stage or patient survival. Conclusion: Claudins 1 and 2 were associated with tumor grade. However, none of the claudins was a more powerful prognostic factor than tumor stage.*

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Key Words: Claudins, expression, prognostic factors, renal cell cancer (RCC).

Clinical stage and histological grade are the most powerful prognostic factors in renal cell carcinoma (RCC), although new prognostic markers, including proliferation index (MIB-1), anti-apoptosis regulator (*BCL-2*), apoptosis regulator (*BAX*), Vascular endothelial growth factor (VEGF), and claudins have been promoted (1-4). Although MIB-1 is a proliferation marker generally associated with tumor size, nuclear grade and necrosis, it has not been found to be an independent prognostic factor of RCC (2, 3). The *BCL-2* gene has an inhibitory effect on apoptosis while *BAX* promotes it. Some studies have reported that they have no independent association with the prognosis of patients with RCC (2, 3). In one study, VEGF was an independent prognostic predictor of outcome (4), but the result was not corroborated by a subsequent study, which, however, suggested that VEGF was significantly correlated with tumor stage and grade (5).

Tight intercellular junctions lie adjacent to the apical end of the lateral cell membrane surface. They have two functions: barrier function and fence function. The barrier function regulates the passage of ions, water and macromolecules through paracellular spaces; this function also operates in cancer cells (6). The fence function maintains cell polarity (6, 7). Tight junctional proteins form a trafficking and signalling platform that regulates cell growth, proliferation, differentiation, and dedifferentiation (7). More than 40 different proteins have been located at the tight junctions of epithelial, endothelial and myelinated cells. Two main components of the tight junction filaments have been identified: occludin and claudin. The latter is a protein family with more than 20 members (6).

The expression of claudins is abnormally regulated in several human cancers. In particular, claudin 3 and claudin 4 are frequently overexpressed in several neoplastic conditions, including ovarian, breast, pancreatic, and prostate cancers (8, 9), while claudins 3, 4 and 7 are overexpressed in bladder, thyroid, fallopian tube, stomach, colon and uterus carcinomas

(9). Claudin 4 positivity was associated with a favorable prognosis in triple-negative breast cancer (defined as the absence of estrogen and progesterone receptors and negative HER2 expression). The same study reported that claudin 4 positivity was associated with poorer and claudin 3 positivity with better prognosis in luminal breast cancer (10). Claudins 3 and 4 have diagnostic value in Paget's disease and in differentiating diffuse mesothelioma from metastatic pleural adenocarcinoma (11, 12). Metastatic lower-lip squamous-cell carcinomas had higher claudin 1 expression than nonmetastatic tumors (13). Expression of claudins was lower in diffuse gastric carcinoma when compared to the intestinal type of gastric cancer (14). Immunostaining of claudin 4 and claudin 5 was less marked in one study that included RCC (15), suggesting that these substances might influence renal cancer; however only 9 cases of RCC were included in the study. The same study reported that immunostaining of claudin 3 was occasionally reduced. There are only few studies on the association between the claudins and, the prognosis, development and dissemination of RCC. Two studies have reported that claudin 7 can be used to differentiate between oncocytomas and the chromophobe type of renal cancer in difficult cases (16, 17). Claudin 3 and claudin 4 associated with overall survival based on univariate analyses, but they were not independent predictors of survival (17). Claudin 1 was reported to be an independent prognostic factor and a possible diagnostic marker for papillary renal cell carcinoma (18).

The aim of this study was to investigate expression of claudins 1-5 and 7 in a substantial set of renal cell carcinomas and to compare their expression with the histology and the other known prognostic factors of renal cell cancer.

Materials and Methods

Patients. The study population consisted of 229 cases collected retrospectively (demographics in Table I). A total of 224 patients underwent nephrectomy and there were 5 (2%) autopsy samples, which were excluded from the survival analysis. The operations had been performed between 1985 and 1995 at either the Tampere University Hospital or the Tampere Hospital, Finland. Follow-up was performed for all patients according to clinical practice. Clinical stage was assigned using the TNM 2002 Classification of Malignant tumors (19). Median follow-up was 4 years interquartile range (IQR), (1.27-7.24). Patient history was collected from the records of the two participating hospitals. The ethics committee at the Tampere University Hospital approved the research protocol and the National Authority for Mediolegal Affairs approved the use of tumor samples.

Histopathologic assessment. Archival formalin-fixed, paraffin-embedded RCC material was used. All tissue samples were re-evaluated and classified and graded by one of the authors (PK); a 1 mm core biopsy from the highest grade area of each tumor was transferred to a multi-tissue block for further immunohistochemical analysis. All tumors were graded according to the Fuhrman system and classified according to the Heidelberg classification (20, 21). Histology and grade of renal cell cancers are depicted in Table I.

Table I. *Baseline patients' characteristics*.

Patients (N=229)	135 men (59.0%)
	94 women (41.0 %)
Median age at the time of nephrectomy	65 (IQR 55.9-71.9)
TNM classification	
T1	107 (46.7%)
T2	29 (12.7%)
T3	39 (17.0%)
T4	4 (1.7%)
N+	13 (5.7%)
M1	37 (16.2%)
Stage	
1	104 (45.5%)
2	29 (12.7%)
3	40 (17.4%)
4	56 (24.4%)
Histology	
Clear cell renal cell carcinoma	207 (90.4%)
Papillary renal cell carcinoma	12 (5.2%)
Chromophobe renal cell carcinoma	5 (2.2%)
Sarcomatoid	2 (0.9%)
Unclassified	3 (1.3%)
Gradus	
1-2	23 (10.0%)
3	115 (50.2%)
4	91 (39.7%)

Immunohistochemistry. The primary antibodies used for immunostaining are designed from Zymed Laboratories Inc (South San Fransisco, CA, USA) and were designed for use in formalin-fixed, paraffin-embedded tissues. They were polyclonal rabbit anti-claudin 1 (clone JAY.8), monoclonal mouse anti-claudin 2 antibody (clone 12H12), polyclonal rabbit anti-claudin 3 (clone Z23.JM), monoclonal mouse anti-claudin 4 (clone 3E2C1), monoclonal mouse anti-claudin 5 (clone 4C3C2) and polyclonal rabbit anti-claudin 7 (clone ZMD.241). Before application of the primary antibodies, the sections were heated in a microwave oven in 10 mM citrate buffer, pH 6.0, for 10 minutes. After 60-minutes of incubation with the primary antibody (dilution 1:50 for anti-claudin 1, 2, 3, 4, 5 and 7), a biotinylated secondary anti-rabbit antibody and Histostain-SP kit (Zymed Laboratoris Inc) were used on the sample. In all cases, the colour for immunostaining was developed by diaminobenzidine, after which the sections were lightly counterstained with hematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany). Negative control stainings were made by substituting non-immune rabbit or mouse serum and phosphate buffered saline for the primary antibodies. Immunostaining results were categorized as follows: 0, no immunostaining; 1, weak immunostaining (<50% membrane-bound positivity); 2, moderate immunostaining (50% to 90% membrane-bound positivity); or 3, strong immunostaining (>90% membrane-bound positivity), as shown in Figure 1. Expression of claudins was evaluated only in tumor cells.

Statistical analysis. Statistical analysis was performed using the IBM SPSS Statistic for Windows version 14.0.2. The differences between categorical variables were tested using the Pearson's X^2-test or Fisher's exact test. Survival was analysed by using the

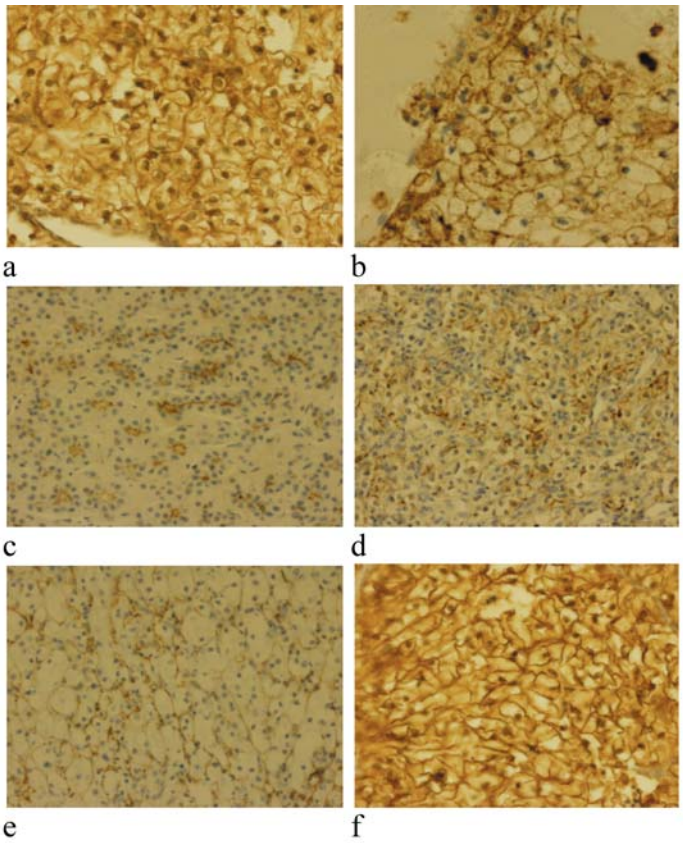

Figure 1. *Immunostaining of claudins 1 (a), 2 (b), 3 (c), 4 (d), 5 (e) and 7 (f) in RCC.*

Kaplan-Meier's survival estimated method. Univariate analysis adjusted for age and gender was performed using the Cox's proportional hazards model.

Results

Median age of the patients was 65 years (IQR 56-72) (Table I). Expression of claudins is described in Table II. Membrane-bound expression for claudins 1, 2, 3, 4, 5 and 7 was detected in 62.2%, 67.4%, 44.6%, 54.7%, 7.4% and 35.4 % of samples, respectively. Strong immunoreactivity was present in claudins 1, 2, 3, 4, 5, 7, 8.5%, 20.2%, 6.2%, 12.0%, 2.1% and 7.3 % of cases, respectively. Claudin 2 was most immunoreactive and was detected in 67.4 % of samples, while claudin 5 was negative in 92.6% of the samples. Many tumors had a rich vascular network and the vessels were strongly positive for claudin 5.

Claudin 2 was positive in all papillary and all chromophobe types of RCC. Papillary RCC was most strongly positive for claudin 2, 3 and 4, with weaker or no staining for other claudins. However, this study represents the most common RCC type, as 90.4% of the tumor samples included clear cell carcinomas.

Table II. *Expression of claudins 1, 2, 3, 4, 5 and 7 in renal cell carcinoma.*

	– n (%)	+ n (%)	++ n (%)	+++ n (%)
Claudin 1	71 (37.8)	54 (28.7)	47 (25.0)	16 (8.5)
Claudin 2	63 (32.6)	50 (26.0)	41 (21.2)	39 (20.2)
Claudin 3	107 (55.4)	46 (23.9)	28 (14.5)	12 (6.2)
Claudin 4	87 (45.3)	54 (28.1)	28 (14.6)	23 (12.0)
Claudin 5	176 (92.6)	8 (4.2)	2 (1.1)	4 (2.1)
Claudin 7	124 (64.6)	26 (13.5)	28 (14.6)	14 (7.3)

–, No immunostaining; +, weak immunoreactivy; ++, moderate immunoreactivy; +++, strong immunoreactivy.

The expression of studied claudins was compared to tumor stage and grade, both being prognostic factors of RCC (Tables III and IV). Cross-tabulation indicated that both claudin 1 ($p<0.001$) and claudin 2 ($p<0.009$) expression was significantly associated with tumor grade (Table IV). Claudin 1 expression was associated with lower-grade tumors and

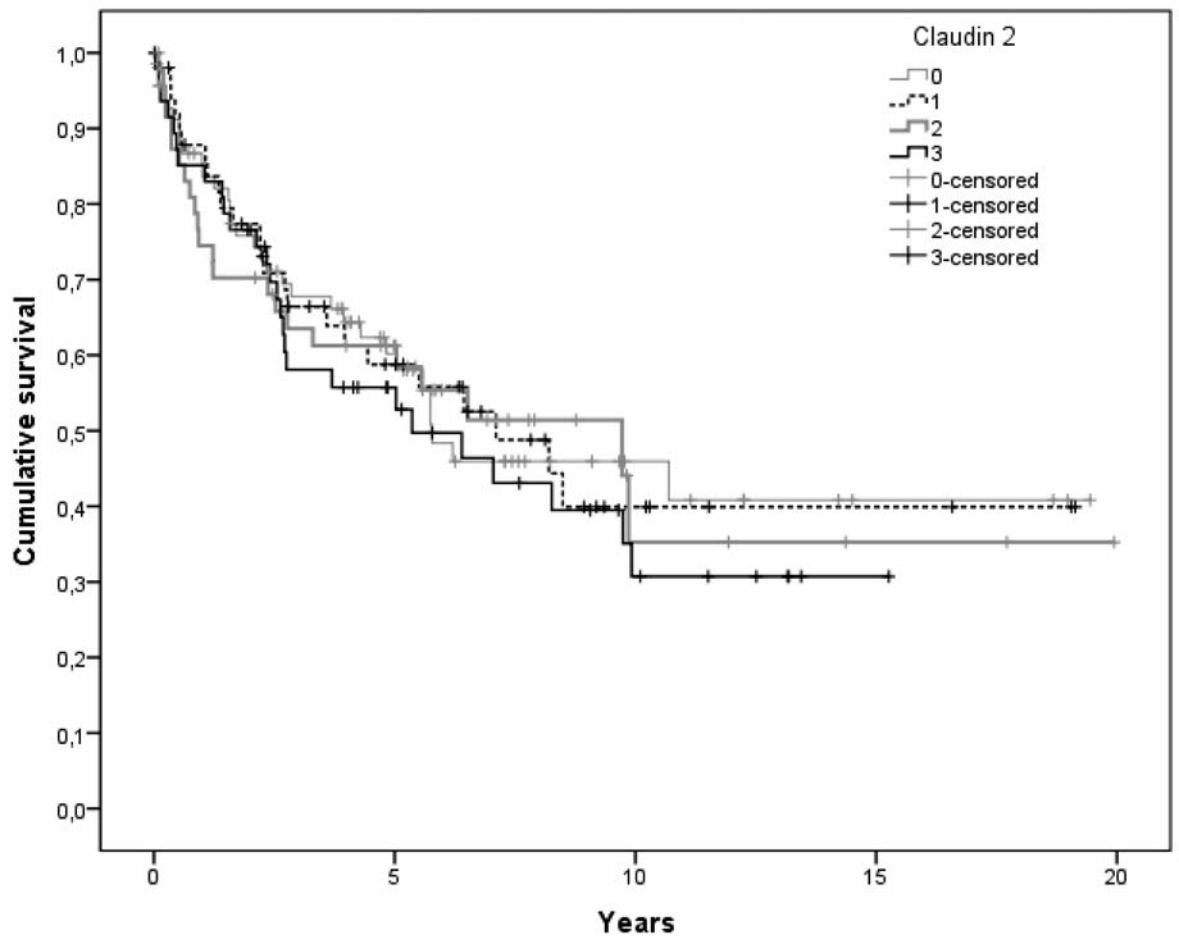

Figure 2. *Kaplan-Meier analysis of claudin 2 association with renal cell carcinoma survival.*

claudin 2 expression with higher-grade tumors. Claudin 4 was nearly statistitically significantly associated with RCC tumor grade (p=0.069). None of the claudins were significantly associated with tumor stage. The most significant results of the univariative analysis are summarized in Table V. Both classical prognostic factors (grade and stage) were statistically significantly associated with survival (Table V). Claudin expression was not independently associated with patient survival (Figure 2 and Table V).

Discussion

The present study was undertaken to evaluate expression of claudins 1-5 and 7 in RCC samples and to study the clinical significance of that expression. Over half of the samples showed positivity for claudins 1, 2 and 4, while claudins 3, 5 and 7 were more poorly expressed. In addition, we evaluated the association between claudin expression, tumor grade and stage, the latter of which reported to be associated with patients' survival in RCC (1-4). Expression of claudins has been studied in several different tumor types. Down-regulation of claudin 4 and overexpression of claudins 2, 3 and 5 have been reported in prostate adenocarcinomas compared with benign prostatic hyperplasia (22). In the same study overexpression of claudin 3 correlated with perineural invasion.

In another study, samples of prostatic adenocarcinoma were studied and decreased expression of claudin 1 and high expression of claudins 3 and 4 in prostatic adenocarcinoma samples correlated with poor prognosis (23). Overexpression of claudins 3 and 4 correlated with myometrial invasion in a study of endometrial tissue variants (24) while in another study overexpression of claudins 3 and 4 was correlated with poorer prognosis in clear cell RCC (25).

There are only few studies on claudin expression in RCC related to clinical data, including a limited study from our Hospital (15). One of those showed that claudin 1 expression was associated with poor survival in renal cell cancer (18).

Table III. *Associations of stage and claudin expression. Shown are Number of cases (n) and percentages are shown. Analysis was performed by Pearson Chi-square test or Fisher's exact test (marked with *).*

	Stage				
	1 n (%)	2 n (%)	3 n (%)	4 n (%)	*p*
Claudin 1					0.331
0	21 (28)	15 (38)	29 (48)	14 (42)	
1	25 (33)	15 (38)	13 (22)	9 (27)	
2	21 (28)	7 (17)	14 (23)	9 (27)	
3	9 (12)	3 (7)	4 (7)	1 (3)	
Claudin 2					0.097
0	31 (40)	6 (15)	25 (41)	10 (29)	
1	21 (27)	11 (27)	13 (21)	7 (21)	
2	14 (18)	10 (24)	12 (20)	11 (32)	
3	12 (15)	14 (34)	11 (18)	6 (18)	
Claudin 3					0.639*
0	42 (55)	19 (45)	36 (80)	21 (62)	
1	17 (22)	13 (31)	15 (25)	6 (18)	
2	13 (17)	8 (19)	6 (10)	3 (9)	
3	5 (6)	2 (5)	3 (5)	4 (12)	
Claudin 4					0.208
0	34 (44)	13 (31)	35 (57)	13 (39)	
1	22 (29)	14 (33)	14 (23)	12 (36)	
2	12 (16)	6 (14)	8 (13)	6 (18)	
3	9 (12)	9 (21)	4 (7)	2 (6)	
Claudin 5					0.533*
0	71 (93)	40 (98)	55 (90)	29 (88)	
1	3 (4)	0 (0)	4 (7)	2 (6)	
2	0 (0)	1 (2)	1 (2)	1 (3)	
3	2 (3)	0 (0)	1 (2)	1 (3)	
Claudin 7					0.416*
0	44 (57)	27 (64)	42 (70)	25 (76)	
1	12 (16)	6 (14)	7 (12)	4 (12)	
2	12 (16)	7 (17)	10 (17)	2 (6)	
3	9 (12)	2 (5)	1 (2)	2 (6)	

Table IV. *Associations of grade to claudins are expressed by number of cases (n) and percentages. Analysis was performed by Pearson Chi-square test or Fisher's exact test (marked with*).*

	Grade			
	1-2 n (%)	3 n (%)	4 n (%)	*p*
Claudin 1				<0.001
0	3 (13)	33 (30)	46 (54)	
1	7 (30)	32 (29)	26 (30)	
2	8 (35)	36 (33)	10 (12)	
3	5 (22)	9 (8)	4 (5)	
Claudin 2				0.009
0	14 (61)	33 (29)	29 (33)	
1	5 (22)	26 (23)	21 (24)	
2	3 (13)	33 (29)	13 (15)	
3	1 (4)	21 (17)	25 (28)	
Claudin 3				0.519
0	11 (48)	58 (51)	53 (62)	
1	8 (35)	29 (25)	18 (21)	
2	2 (9)	20 (18)	9 (10)	
3	2 (9)	7 (6)	6 (7)	
Claudin 4				0.069
0	14 (61)	38 (34)	47 (54)	
1	5 (22)	38 (34)	23 (26)	
2	2 (9)	22 (20)	10 (11)	
3	2 (9)	15 (13)	7 (8)	
Claudin 5				0.953*
0	22 (96)	102 (92)	80 (92)	
1	1 (4)	4 (4)	5 (6)	
2	0 (0)	2 (2)	1 (1)	
3	0 (0)	3 (3)	1 (1)	
Claudin 7				0.218*
0	11 (48)	70 (62)	59 (68)	
1	5 (22)	13 (12)	15 (17)	
2	4 (17)	19 (17)	10 (12)	
3	3 813)	10 (9)	3 (3)	

The present study showed that low expression of claudin 1 is associated with higher tumor grade and also that claudin 4 did not reach statistical significance in association higher tumor grade (p=0.069). It has been previously shown, that moderate-to-strong expression of this claudin is associated with decreased survival in patients with RCC (17).

We have here included 229 RCC samples and found that only 7 % were positive for claudin 5, which is a tight junctional protein. Claudin 4, however, was expressed in 55 % of cases, claudin 3 in 46% and claudin 7 in only 35% of cases. Claudins 1 and 2, again, were more highly expressed (Table II). Median survival time was poor, only 6.5 years but this, however, is in line with our previous larger study where we reported that median overall survival was 5.9 years, 3.4 years and 12 months between obese, normal or underweight patients with RCC, respectively (26). Our data included patients whose RCC has been diagnosed between 1985 and 1995. During this period, computerized tomography and ultrasound were not used as widely as nowadays and thus RCC might have been diagnosed later than nowadays. Our data are part of a larger study which demonstrated that prognosis and diagnosis of RCC has improved by using imaging procedures (27). In addition, targeted-therapies have also improved outcomes in advanced RCC (28).

Table V. *Age- and gender-adjusted univariate and multivariate associations of different staining patterns with other main prognostic parameters were tested using Cox regression models with results given as the hazard ratios (HR) and 95 % confidence intervals (CI) (n=224).*

		Adjusted	Variative
	n	HR	[95% CI]
Grade			
1-2	22	1.00	
3	114	2.84	[1.01-7.99]
4	88	4.76	[1.69-13.4]
Stage			
1	77	1.00	
2	43	2.33	[1.25-4.33]
3	61	2.78	[1.59-4.83]
4	34	9.03	[5.01-16.3]
Claudin 1			
0	79	1.00	
1	64	0.82	[0.51-1.32]
2	53	0.69	[0.42-1.15]
3	18	0.52	[0.22-1.22]
Claudin 2			
0	71	1.00	
1	52	0.90	[0.53-1.55]
2	49	1.00	[0.59-1.72]
3	47	1.09	[0.64-1.84]

There are some differences between claudin expression in RCC when compared to other epithelial cancers. Claudins 3 and 4 are usually strongly expressed in carcinomas of the genitourinary area, such as endometrial and ovarian epithelial tumors and prostate carcinomas. RCC seems to have a decreased claudin expression, at least regarding claudin 4 (15). Claudin 5 has been reported to be specific to endothelial cells, yet immunohistochemical expression of this protein has been found comparatively often in malignant tumors such as ovarian of gastric carcinomas (15). In our series of RCC, claudin 5 expression was low.

The pattern of claudin expression in RCC most likely reflects its expression in the corresponding non-neoplastic tissues of the kidney. Kidney adenocarcinomas originate from tubular epithelial cells. In rabbits, claudins 1, 2 and 4 are expressed in proximal tubule cells, Henle's loop and collecting segments, claudin 3 in the proximal and collecting tubules and claudin 7 in the proximal tubulus, while claudin 5 is absent from tubular cells (25). Interestingly, in our RCC samples, claudins 5 and 7 showed the lowest expression levels suggesting little to no expression of the proteins in tubular cells (25). Most tumors were ordinary kidney clear cell adenocarcinomas. The few papillary and chromophobe types of RCC were universally positive for claudin 2 immunostaining. Claudins 2, 3 and 4 were strongly expressed only in papillary RCC. The papillary-type RCC is less responsive to modern drugs developed in the last decade. Mesenchymal epithelial-transition inhibition alone and in combination with inhibition of epidermal growth factor receptor is a new target being explored for the treatment of papillary type RCC (29). Future experience will show, whether this result is useful for the clinical differential diagnosis between RCC subtypes. Some other tumors are known to show differences in claudin expression by phenotype or histological subtype. In gastric carcinomas, diffuse carcinoma exhibits reduced claudin expression compared to the intestinal subtype and mesotheliomas. In epithelioid mesotheliomas claudin express more strongly than sarcomatoid subtype (15).

Reduced expression of claudin 1 was associated with high-grade tumors. This is consistent with the concept that less differentiated tumors tend to lose their differentiation markers. Dysregulation of claudin expression has been associated with epitheliomesenchymal transition, which could influence the metastatic behaviour of tumors. Abrogated claudin expression could influence cell attachment, decrease cohesion of cancer cells and promote metastatic spread. Observations consistent with this hypothesis have been reported both for breast cancer associated with claudin 7 (30) and for esophageal cancer associated with claudin 3 (31). With respect to RCC, we did not find any association between expression of any claudins and either metastasis or stage. We found that claudins 1 and 2 may have additional prognostic value for patients with RCC. Both claudins were significantly associated with tumor grade.

Conclusion

The tight junctional proteins claudin 1 and 2 were significantly associated with tumor grade. None of the studied claudins were significantly associated with survival in RCC patients. The prognostic value of claudins for patients with RCC merits further investigation.

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VEGFR3 and CD31 as Prognostic Factors in Renal Cell Cancer

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Abstract. *Aim: To evaluate the expression levels of vascular endothelial growth factor receptor-3 (VEGFR3) and CD31 and assess their associations with grade, stage and survival in patients with renal cell cancer (RCC). Patients and Methods: Our study included 224 consecutive patients who received treatment during the years 1985-1995 in Tampere Finland but had not been treated with modern anti-angiogenesis drugs. All tumor samples were re-classified and investigated using immunohistological techniques. Data were collected from patient records and the Finnish Cancer Registry. Results: In total, 54.2% and 98.2% of the tumor samples tested positive for VEGFR3 and CD31 expression, respectively. CD31 expression levels were classified into two groups according to the median level revealing that its high expression was nearly significantly associated with low tumor stage (p=0.069). In an age- and gender-adjusted analysis, low expression of CD31 associated with poorer survival. Grade 3 and grade 4 tumors had significantly higher mortality rates compared to those of grades 1-2 (hazard ratio (HR)=4.91; 95% confidence interval (CI)=1.12-20.4; p=0.029 for grade 3 and HR=9.31; 95% CI=2.23-38.8; p=0.002 for grade 4). In addition, stage 2, 3 and 4 tumors revealed that they possessed significantly higher mortality hazard ratios compared to those of stage 1 tumors (HR=2.62; 95% CI=1.27-5.41; p=0.009 for stage 2, HR=4.37;95% CI=2.29-8.3; p<0.001 for stage 3 and HR 13.8; 95% CI=7.18-26.7; p<0.001 for stage 4). Conclusion: High CD31 expression associated significantly with better survival and VEGFR3 had no association with survival. Both higher tumor grade and stage were associated with a decreased survival time.*

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Key Words: Renal cell cancer (RCC), vascular endothelial growth factor receptor (VEGFR), CD-31, prognostic factor.

Vascular endothelial growth factor (VEGF) is an important regulator of angiogenesis (1). It has also been associated with pathological angiogenesis in tumors and ischemic, inflammatory and pathological intraocular conditions (2-4). There are five mammalian VEGF ligands, each of which occurs as several different variants. These variants bind to vascular endothelial factor receptors (VEGFRs) and induce biological responses (2). The main lymphangiogenic receptor VEGFR3 is widely expressed in blood vessels and it is essential for the development of circulation during early embryogenesis (2, 5). VEGF-C and VEGFR3 signaling are important for lymphangiogenesis and this process is activated in individuals with cancer and inflammation, while it is inactive under normal physiological conditions (5). The *VEGF* genotype +936 has been found to be associated with age-related macular degeneration (6). VEGF expression has been correlated with tumor size and stage and poor survival in renal cell cancer (RCC) patients by univariate analysis (7). Yang *et al*. have found positive VEGF expression in RCC tumor cells but negative expression in normal renal cells (8). The same study showed that positive VEGF expression is correlated with grade, lymph node involvement and vascular invasion. VEGFR-1 has been shown to be up-regulated in endothelial cells in vascular tumors (1).

Anti-angiogenic therapy inhibits the generation of new blood vessels and blocks the growth and metastasis of cancer cells (5). VEGFR3 is a highly interesting therapeutic target because it plays a role in angiogenesis, as well as in lymphatic maintenance (2). Knowledge of RCC biology has improved over the recent years. At least two cellular signaling pathways for molecular-targeted therapy, the VEGF and mammalian target of rapamycin (mTOR) pathways, are known (9). Von Hippel-Lindau (VHL) disease is associated with an increased

Table I. *Clinical and pathological characteristics*.

Patients (n=224)	132 men (58.9 %)
	92 women (41.1 %)
Median age at the time of nephrectomy	65 (IQR 55.9-71.9)
Stage	
1	79 (35.3%)
2	43 (19.2%)
3	61 (27.2%)
4	39 (17.4%)
Histology	
Clear cell renal cell carcinoma	202 (90.2%)
Papillary renal cell carcinoma	12 (5.4%)
Chromophobe renal cell carcinoma	5 (2.2%)
Sarcomatoid	2 (0.9%)
Unclassified	1 (0.4%)
Grade	
1-2	22 (9.8%)
3	114 (50.9%)
4	88 (39.3%)

IQR, Interquartile range.

Table II. *Association of tumor stage with CD31 and VEGFR3 expression according to expression level*.

	Stage				
	1 n (%)	2 n (%)	3 n (%)	4 n (%)	*p*-Value
CD31					0.069
Low	28 (37.8%)	21 (51.2%)	35 (57.6%)	22 (59.5%)	
High	46 (62.2%)	20 (48.8%)	25 (42.4%)	15 (40.5%)	
VEGFR3					0.899
Low	32 (43.8%)	19 (46.3%)	29 (49.2%)	16 (42.1%)	
High	41 (56.2%)	22 (53.7%)	59 (50.8%)	38 (57.9%)	

VEGFR3, Endothelial growth factor receptor-3.

Table III. *Age- and gender-adjusted univariate analysis of VEGFR3 and CD31 with tumor grade and stage by using Cox proportional hazard models*.

	Adjusted			
Grade	n	HR	(95 % CI)	*p*-Value
1-2	22	1		
3	114	4.91	(1.12-20.4)	0.029
4	88	9.31	(2.23-38.8)	0.002
Stage				
1	79	1		
2	44	2.62	(1.27-5.41)	0.009
3	61	4.37	(2.29-8.35)	<0.001
4	39	13.8	(7.18-26.7)	<0.001
VEGFR3 high	115	1		
VEGFR low	97	1.04	(0.69-1.56)	0.087
CD31 high	106	1		
CD31 low	106	1.53	(1.01-2.33)	0.044

VEGFR3, Endothelial growth factor receptor-3; HR, hazard ratio; CI, confidence interval.

risk of RCC. Inactivation of VHL can lead to over-production of VEGF, thereby inducing formation of highly vascular tumors, such as those observed in RCC (10). Mutations in the *VHL* gene have been reported in up to 80% of RCC patients (11). After treatment with multi-targeted tyrosine kinase inhibitor, marked changes in VEGF, VEGFR2 and VEGFR3 plasma levels have been observed in metastatic RCC patients exhibiting objective tumor responses compared to those presenting with stable or progressive disease (12). Another study found that a marked decrease in the soluble VEGF2 concentration in patients with metastatic RCC is correlated with a higher objective response rate and longer progression-free survival (13). Several promising biomarkers for VEGF-targeted therapy have been studied but none fulfilled the criteria for level I evidence (14).

CD31 is a member of an immunoglobulin superfamily that is expressed on the surfaces of circulating platelets, neutrophils, monocytes and naïve B lymphocytes. It plays a major role in tissue regeneration and its expression has been detected in vascular tumors (15). CD31 is a ligand for CD38. One previous study has shown that low CD31 and CD38 expression levels are correlated with better survival in patients with B-cell chronic lymphocytic leukemia (16). Increased CD31 expression has been demonstrated in clear cell RCC (ccRCC) compared to papillary RCC (pRCC). The same study has associated low CD31 expression with higher tumor stage and nuclear grade but has suggested that its expression is not an independent prognostic factor (17). Biswas *et al*. have demonstrated an association between elevated CD31 expression, low tumor grade and improved survival (18).

High tumor stage and grade have been correlated with decreased survival in our larger study of RCC patients treated at the Pirkanmaa Hospital District (19). VEGF is a biomarker that has been independently associated with survival in a previous study of RCC (20). There are few studies evaluating the association of VEGFR3 or CD31 with prognosis in RCC patients. None of the patients evaluated in our study cohort had been treated with the specific angiogenesis inhibitors. The aim of the present study was to evaluate VEGFR3 and CD31 expression levels as prognostic factors in RCC and to assess their associations with tumor stage and grade.

Patients and Methods

RCC. A total of 224 patients with primary RCC were included in this study. The clinical and pathological characteristics of the patients are summarized in Table I. This study included the same patients' materials as our previous study, with the exception of the

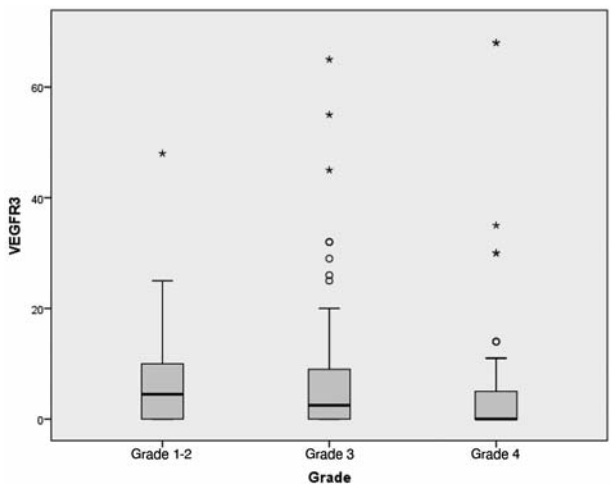

Figure 1. *Differences of vascular endothelial growth factor receptor-3 (VEGFR3) expression between tumor grades. Values are shown by median (black line), interquartile range (box) and range (line bar). Outliers and extreme cases are expressed as dots or stars.*

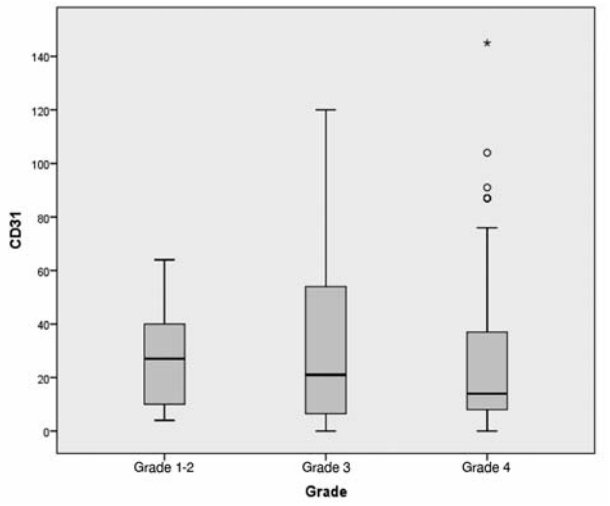

Figure 2. *Differences of CD31 expression between tumour grades. Values are shown by median (black line), interquartile range (box) and range (line bar). Outliers and extreme cases are expressed as dots or stars.*

autopsy samples (21). Patients underwent surgery between 1985 and 1995 at the Tampere University Hospital or Tampere Hospital, Tampere, Finland. RCC was pathologically staged according to the TNM 2002 classifications (22). Patients' data were collected from records at two hospitals and retrospective analysis was performed. The median follow-up time was 5.4 years, with an interquartile range (IQR) of 1.41-11.9. After nephrectomy, patient follow-up and treatment were performed according to standard clinical practice. The research protocol and use of tumor samples were approved by the ethics committee at the Tampere University Hospital and the National Authority for Medicolegal Affairs.

Histopathology. All of the tumors were re-evaluated and re-classified using the Heidelberg classification and Fuhrman grading system (23, 24) by a uropathologist (PK). A multi-tissue block was obtained from the region of each 1-mm biopsied RCC specimen with the highest grade and used for immunohistochemical analysis

Immunohistochemistry. Immunohistochemistry to assess CD31 (1:200, Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) was performed on formalin-fixed, paraffin-embedded tissue sections as part of a tissue microarray (TMA). Briefly, sections were deparaffinized with xylene and rehydrated in graded alcohol, treated in an autoclave in 10 mmol/l sodium citrate (pH 5.0) for 2 min and washed with phosphate-buffered saline. They were then incubated with a primary antibody at 4˚C overnight and antibody binding was detected by a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine (DAB) was used as the chromogen. The slides were counterstained with hematoxylin and eosin and mounted. VEGFR3 was stained with the 9D9 antibody (a mouse monoclonal antibody against the extracellular domain of human VEGFR3; a kind gift from Professor Kari Alitalo, Helsinki, Finland) at a concentration of 10 μg/ml as detailed previously (25).

The mean vessel density (MVD) was quantified as the number of CD31-positive or VEGFR3-positive microvessels per high-powered field at 250× (field of view of 0.407 mm^2, including the entire TMA core) using a Leitz Laborlux 12 bright-field microscope (Leitz GmbH, Wetzlar, Germany. The two fields with the highest vessel densities were counted and an average of the two scores was reported. Scoring was performed in a blinded manner.

Statistical analyses. Statistical analyses were performed using IBM SPSS Statistics for Windows (version 21.0, Armonk, NY, IBM Corp., released 2012). The differences between the categorical variables were tested using the Pearson Chi-square test or Fisher's exact test. Continuous variables were tested by the independent Kruskal-Wallis test due to skewed distribution. Age- and gender-adjusted univariate survival analyses were performed using the Cox proportional hazards models. Survival was illustrated by Kaplan-Meier's survival estimation methods. *p*-Values under 0.05 were considered as statistically significant.

Results

Patients. The median age of the 224 patients was 65 years (IQR, 55.9-71.9) at the time of diagnosis. The most typical tumor type observed in our study was ccRCC (90.2%). Low-grade tumors (grades 1-2) were rare (22 patients, 9.8%) and we classified the tumor grades into three groups as follows: grades 1-2, 3 and 4. Patients' basic characteristics were the same as those reported in our previous study, excluding the five autopsy samples (21) described in Table I.

Expression of VEGFR3. Negative VEGFR3 staining was observed in 97 (45.8%) of the tumors and positive staining (>0 vessels) occurred in 115 (54.2%) Twelve (5.4%) samples had poor immunostaining and were excluded from further analyses. The median number of VEGFR3-positive vessels was 2 (range=0-68). The distribution of VEGFR3 expression according to grade nearly reached statistical

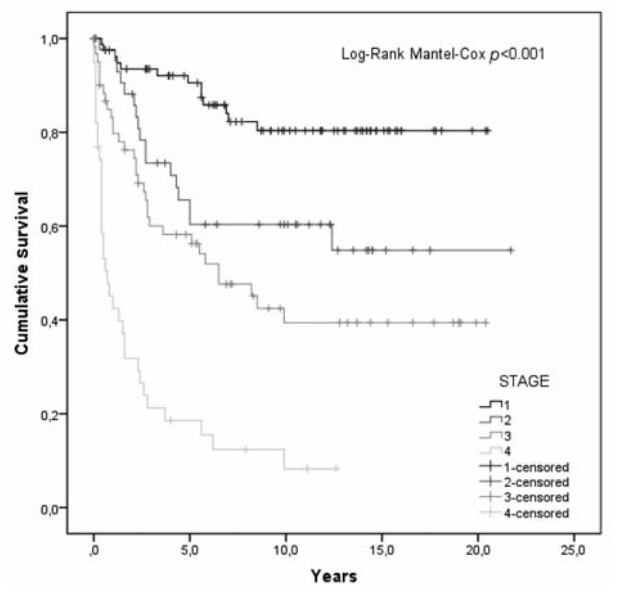

Figure 3. *Kaplan-Meier survival analysis according to tumor stage.*

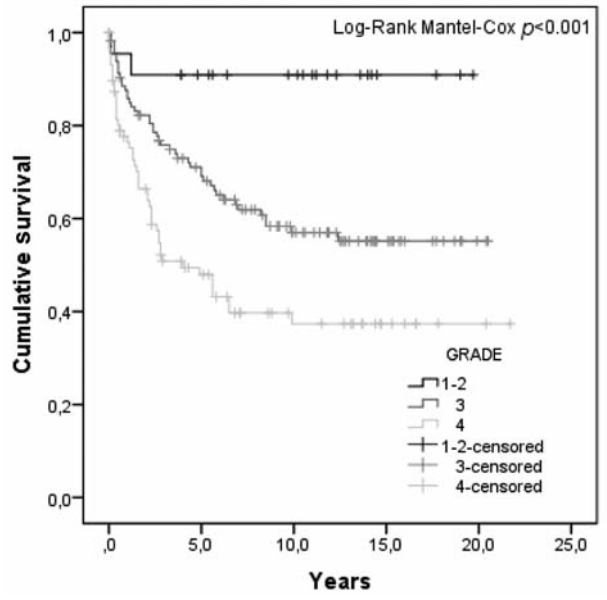

Figure 4. *Kaplan-Meier survival analysis according to tumor grade.*

significance, as shown by the independent Kruskal-Wallis test (p=0.058) but no dependence on stage (p=0.87) was observed. The VEGFR distribution and tumor grade are shown in Figure 1.

VEGFR3 expression and clinicopathological characteristics. We categorized the VEGFR expression levels into low (no positive vessels) and high (>0, positive vessels) groups. Ten (90.9%) pRCC samples showed low expression and it was high in only one (9.1%, p=0.02). Both types of sarcomatoid RCCs exhibited reduced expression and one collecting duct RCC sample showed high expression. Differential VEGFR3 expression was not observed in either the chromophobe RCC or ccRCC samples. Of the grade 1-2 tumors, six (27.3%) showed low expression and 16 (72.7%) had high expression; however, the Pearson Chi-square test showed no statistical significant association (p=0.14). Higher tumor grade did not affect VEGFR3 expression. The expression of this protein was not associated with tumor staging, as revealed by cross-tabulation (p=0.90, n=211) shown in Table II.

Expression of CD31. Negative CD31 staining was observed in only four samples (1.8%). Mean vessel density detected by CD31 expression varied from 0-145. The median expression level was 18. Twelve samples (5.4%) were of poor quality and were excluded from the analysis. The independent Kruskal-Wallis test showed no association between CD31 expression and tumor stage (p=0.31) or grade (p=0.50).

CD31 expression and clinicopathological characteristics.

The CD31 expression values were divided into two groups (low and high) using the median cut-off value of 18. All four (100%) chromophobe RCC samples exhibited low expression. The only collecting duct RCC showed high expression; reduced expression was observed in the only unclassified RCC sample. Ten (83.3%) pRCC samples exhibited low vessel density, while high vessel density was elevated only in two samples (16.7%). A total of 89 (46.6%) ccRCC samples showed a reduction in expression and an increase was observed in 102 (53.4%). A cross-tabulation of the different types of RCC samples *versus* the CD31 expression levels revealed significant differences (p=0.04) according to the Pearson Chi-Square test.

A total of 8 (38.1%) and 13 (61.9%) grade 1-2 tumor samples showed low and high CD31 expression, respectively, in addition to, 52 (48.1%) and 56 (51.9%) grade 3 tumors, and 46 (55.4%) and 37 (44.6%) grade 4 tumors, respectively. The Pearson chi-square test showed no association of low or high CD31 expression with tumor grade (p=0.35) (Figure 2).

The CD31 expression was low in 28 (37.8%) and elevated in 46 (62.2%) stage 1 tumor samples. Furthermore, its expression was low in 22 (59.5%) and high in 15 (40.5%) stage 4 tumor samples. Low CD31 expression showed a nearly statistically significant association with high tumor stage (p=0.069, n=211) shown in Table II.

Survival. The median survival time of the whole patient population was 5.6 years (IQR=1.6-11.9). Both high tumor

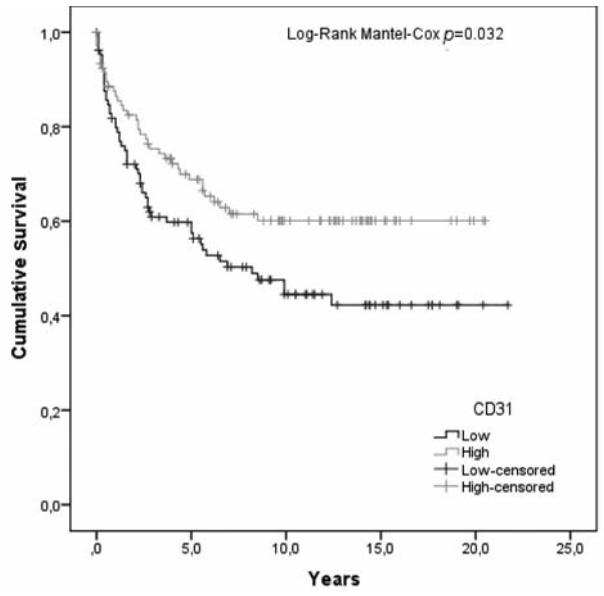

Figure 5. *Kaplan-Meier survival analysis according to CD31 intensity.*

stage and high grade were associated with decreased survival time, as determined by the age- and gender-adjusted Cox regression univariate analysis shown in Table III; (Grade 3: HR 4.91; 95% CI 1.12-20.4; and p=0.029; and grade 4: HR 9.31; 95% CI 2.23-38.8; and p=0.002 (compared to grades 1-2). Stage 2: HR 2.62; 95% CI 1.27-5.41; and p=0.009; stage 3: HR 4.37; 95% CI 2.29-8.35 and p<0.001; and stage 4: HR 13.8; 95% CI 7.18-26.7 and p<0.001, (compared to stage 1).

When the VEGFR3 and CD31 expression values were divided into low (0 and <18, respectively) and high (>0 and ≥18, respectively) groups, Cox regression univariate analysis showed that low CD31 expression associated with longer survival (low CD31 HR 1.53; 95% CI 1.01-2.33; and p=0.044 compared to high CD31, while VEGFR3 expression showed no association (low *versus* elevated VEGFR3 expression HR 1.04; 95% CI 0.69-1.56; and p=0.87), shown in Table III.

With regard to VEGFR3 expression, the RCC-specific survival (RCC-SS) was 44.5% in the low-expression group and 55.5% in the high-expression group (n=212). A total of 44.2% of the patients with RCC-SS and local tumors (stages 1-3, n=173) possessed low VEGFR3 expression and its expression was elevated in 55.8%. The Pearson Chi-square test showed no association of the five-year RCC-SS with VEGFR3 expression for any of the tumor types (p=0.78) or the local tumors (p=0.52). The RCC-SS rates were 43.3% and 56.7% for the patients with low *versus* high CD31 expression, respectively, for all of the tumor types (p=0.037) and it was 43.0% and 57.0%, respectively, for local tumors (p=0.11).

Kaplan-Meier survival analysis. Kaplan-Meier survival analysis was used to assess tumor grade, stage, as well as VEGFR3 and CD31 expression. Higher tumor stage and grade were associated with increased mortality (log-rank Mantel-Cox test p<0.001 and p<0.001, respectively) as shown in Figures 3 and 4. CD31 expression showed statistically significant association with survival (p=0.03), as shown in Figure 5. VEGFR3 expression had no association with survival in Kaplan-Meier analysis (p=0.96.)

Discussion

The aim of the present study was to evaluate VEGFR3 and CD31 expression levels as potential prognostic factors of RCC and to assess their correlations with known prognostic factors in RCC, *e.g*., tumor stage and grade. All tumor samples were re-classified and re-evaluated by one experienced uropathologist (PK). We retrospectively analyzed a series of 224 consecutive patients with RCC tumors. Immunostaining of all tumor samples was performed using TMAs. Our data included patients treated between the years 1985-1995. At that time, no specific anti-angiogenic drugs existed, although twenty-three patients were treated with interferon, which has some antiangiogenic activity.

Our understanding over the molecular mechanisms underlying tumor angiogenesis has recently increased. It has been shown that this process is a result of the interactions of several components of the tumor microenvironment (26). New targeted-therapies, such as those involving VEGF/VEGFR and mTOR pathways, have improved the survival of advanced RCC patients (27). Knowledge with regard to lymphangiogenesis in RCC is limited but some data pertaining to survival and VEGFR3 and CD31 expression in these patients are available (17, 18, 28, 29). Studies have been mainly performed on patients with metastatic renal cell cancer who have been treated with a tyrosine inhibitor or VEGF/VEGFR-blocking agents. Low VEGFR3 expression has been associated with poor survival in patients treated with sunitinib (28). Bieber *et al*. have shown no association of VEGFR3 expression with tumor stage, grade or survival in RCC patients (29). VEGFR3 expression has been found to correlate with histological grade, lymph node status and distant metastasis in one previous study of 82 patients (30). However, it has not been found to be correlated with gender, age, tumor size or TNM staging. Harmon *et al*. have shown that a low baseline plasma level of the soluble form of VEGFR3 is associated with improved progression-free survival but they found that it is not associated with OS in advanced-stage RCC patients treated with sunitinib (31).

Most patients in our study had ccRCC (90.2%), which is the most common form of RCC. A total of 97 (45.8%) of the tumors tested positive for VEGFR3 expression and the majority of the tumors (98.2%) also tested positive for CD31 expression. CD31 is known to be expressed in highly

vascular tumors (15), such as those found in RCC. Most of our tumor samples (80%) exhibited low (0-10) VEGFR3 expression, which may have been because of its general down-regulation in RCC. Immunostaining of VEGFR3 and CD31 failed in twelve cases in each group (5.3%). All tumor sections were evaluated and the most representative area of each patient's tumor sample was selected by two individuals. The samples with failed immunostaining had, presumably, only minor effects on our main results; but they were excluded from our analysis.

In a previous study, CD31 has been shown to be more highly expressed in ccRCC compared to pRCC tumors (17). Similarly, we observed in our materials its reduced expression in pRCC tumors compared to ccRCC tumors. Bieber *et al*. have reported VEGF-C and VEGF-D up-regulation in pRCC compared to ccRCC but no differential VEGFR3 expression (29). In a previous study, expression levels were divided into four groups according to staining intensity (29). Our study showed that classification of VEGFR3 expression into two groups (low and high staining intensities) resulted in the association of low expression with pRCC. Low VEGFR3 and CD31 expression levels were associated with both the chromophobe RCC and pRCC samples. The association between low CD31 expression and high tumor stage was almost statistically significant. High VEGFR3 expression has been shown to be associated with the improved survival of RCC patients after treatment with sunitinib (31). Low CD31 expression in follicular lymphoma patients is significantly correlated with increased OS and progression-free survival (32).

We categorized the VEGFR3 and CD31 expression levels into two groups. High CD31 expression associated with better survival, while VEGFR3 expression showed no association with survival. Two known prognostic factors, tumor grade and stage, were associated with survival in the RCC patients. Patients with stage 4 RCC show poor survival despite recent medical advancements. Therefore, we explored the expression levels of VEGFR3 and CD31 and assessed their correlations with survival in patients with local or metastatic RCC. Our study indicated that local RCC patients with elevated tumor CD31 expression tended to have better RCC-SS rates. However, we found no statistically significant correlation of survival with VEGFR3 expression in local or metastatic RCC patients. Lymphangiogenesis, which is a process involving signaling *via* VEGFR3, plays a role in tumor progression and metastasis (33). Further studies may be performed to assess expression levels of different marker(s), alone or in addition to those of VEGFR3 and/or CD31, to predict survival and to estimate patient responses to novel targeted-therapies. However, detection of low VEGFR3 and CD31 expression may have an additional value in differentiating between chromophobe RCC and PRCC patients.

Conclusion

Low CD31 expression levels associated with poorer survival of the RCC patients and were nearly significantly correlated with high tumor stage. Tumor grade and stage were shown to be powerful prognostic factors. Detection of the expression levels of VEGFR3, CD31 and other lymphangiogenic markers and assessments of their correlations with the survival of RCC patients require further investigation.

Conflicts of Interest

There are no conflicts of interest to be declared.

Acknowledgements

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Combined Angiogenesis and Proliferation Markers' Expressions as Long-Term Prognostic Factors in Renal Cell Cancer

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Abstract

The prognostic role of MIB-1, BCL-2, VEGFR3, and CD31 expression was retrospectively evaluated in 224 renal cell cancer (RCC) patients. The combination of high MIB-1/low BCL-2 was with poor survival compared with low MIB-1/high BCL-2, and the combination of low VEGFR3/low CD31 was also associated with poor survival compared with high VEGFR3/high CD31. These molecular expressions might be valuable in planning the follow-up for RCC patients.

Objectives: The aim of this study was to evaluate the expression of MIB-1, BCL-2, VEGFR3, and CD31 and their associations with long-term survival in patients with renal cell cancer (RCC). **Patients and methods:** This study consisted of 224 RCC patients who underwent radical nephrectomy from 1985 to 1995. Follow-up continued for up to over 20 years. MIB-1 and BCL-2 expression were analyzed alone, and additionally, the expression of MIB-1, BCL-2, VEGFR3, and CD31 were combined in pairs using the following groups: low/low, low/high, high/low, and high/high. **Results:** Low BCL-2 expression (hazard ratio [HR], 2.16; 95% confidence interval [CI], 1.42-3.31; $P < .001$ compared with high BCL-2 in univariate analysis) and high MIB-1 expression (HR, 2.05; 95% CI, 1.32-3.19; $P = .001$ in multivariate analysis) were found to associate for poorer survival in RCC. In multivariate analysis, the combination of high MIB-1/low BCL-2 was associated with poor survival compared with low MIB-1/high BCL-2 (HR, 3.20; 95% CI, 1.66-6.17; $P = .001$), and the combination of low VEGFR3/high CD31 was associated with poor survival (HR, 2.48; 95% CI, 1.29-4.78; $P = .007$) compared with high VEGFR3/high CD31. **Conclusions:** Compared with high BCL-2 expression in combination with low or high MIB-1, VEGFR3, or CD31 expression, low BCL-2 expression in combination with low or high MIB-1, VEGFR3, or CD31 expression has poorer survival in the long-term follow-up of patients with RCC. Analysis of MIB-1, BCL-2, VEGFR3, and CD31 expression might be a useful additional marker to tailor the follow-up of RCC patients.

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Keywords: BCL-2, CD31, MIB-1, RCC, VEGFR3

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Introduction

The most powerful prognostic factors for patients with localized renal cell cancer (RCC) remain tumor stage, tumor grade, and clinical variables such as performance status and presence or absence of symptoms, and serum markers are used to determine the prognosis of patients with metastatic RCC (mRCC).[1-3] Previously, several possible immunochemical markers have been studied to predict the survival of patients with RCC, but none has attained status as an independent prognostic marker.[4-10]

Vascular endothelial growth factor receptor 3 (VEGFR3) is important for lymphangiogenesis in normal situations, and it is also

activated in cancer and inflammation.[11] It maintains both angiogenesis and the lymphatic system, and therefore, it is considered an interesting therapeutic target.[12] Marked decreased VEGFR3 plasma levels have been shown in patients with RCC after treatment with multi-targeted tyrosine kinase inhibitor compared with stable or progressive mRCC.[13] Studies of VEGFR3 expression and its association with tumor stage, grade, and survival in patients with RCC have shown conflicting results.[7,9,14,15]

CD31 is a member of an immunoglobulin superfamily of cell adhesion molecules that are expressed on the surfaces of several blood and endothelial cells.[16,17] Higher expression of CD31 has been associated with a lower tumor grade and better survival in patients with RCC,[5] and our previous study also showed an association between CD31 expression and survival but not tumor grade or stage.[9] Microvessel density has shown to correlate negatively to prognosis in at least RCC studies.[18-20] In addition, CD31 expression is found to associate with undifferentiated microvessels.[21]

MIB-1 is a well-known cell proliferation marker, while BCL-2 is a marker of cell death.[22,23] The expression of these markers has been studied in patients with RCC. Higher MIB-1 expression was found to be independently associated with poorer survival and with a recurrence in these patients.[4,24] Low BCL-2 expression was associated with a higher tumor stage and a poorer prognosis in patients with RCC.[6] On the contrary, 2 studies did not find association with BCL-2 expression and a prognosis in RCC.[8,25]

Although the all markers mentioned above have been studied, none has been identified as an independent prognostic factor. Therefore, the aim of this study was to explore VEGFR3, CD31, MIB-1, and BCL-2 expression separately and in combination and to determine their associations with long-term survival in patients with RCC.

Patients and Methods

Patient Population

This study included patients with RCC who underwent nephrectomy from 1985 to 1995. The surgeries were performed at Tampere University Hospital or at Tampere Hospital, Tampere, Finland. All of the tumor samples were reclassified and re-evaluated using the Heidelberg classification and Fuhrman grading system[26,27] by an experienced uropathologist (P.K.). A total of 202 (90.2%) clear cell renal cell carcinoma (ccRCC), 12 (5.4%) papillary RCC (pRCC), 5 (2.2%) chromophobe RCC, 2 sarcomatoid RCC (0.9%), and 1 (0.4%) unclassified RCC were included in this study. The tumor samples were classified as Fuhrman grade 1 to 2 (22; 9.8%), grade 3 (114; 50.9%), and grade 4 (88; 39.3%). Clinical stage was assessed according to the TNM 2002 Classification of Malignant Tumors,[28] and 79 (35.3%), 43 (19.2%), 61 (27.2%), and 39 (17.4%) of patients with RCC were TNM stages 1, 2, 3, and 4, respectively. The patients' basic characteristics are described in a table in our previous study.[9]

Due to poor immunostaining, 13 (5.8%) patients in the MIB-1 group, 3 (1.3%) in the BCL-2 group, 15 (6.7%) in the MIB-1/BCL-2 group, 21 (9.4%) in the MIB-1/VEGFR3 group, 21 (9.4%) in the MIB-1/CD31 group, 15 (6.7%) in the BCL-2/CD31 group, and 14 (6.3%) in the VEGFR3/CD31 group were excluded from the analyses.

After surgery, the patients' follow-up and treatment were performed according to standard clinical practice at that time. The median follow-up time was 5.4 years, with a range of 0 to 21.7 years. Tampere University Hospital and the National Board of Medicolegal Affairs approved the research protocol and use of the tumor samples.

Immunostainings

Sections were deparaffinized, and antigen retrieval was achieved by heating the sections in a microwave oven for 2 × 7 minutes in 10 mM tris/10 mM ethylenediaminetetraacetic acid (pH 9.0). For acid Ki-67 antigen immunostaining, the monoclonal antibody MIB-1 (IgG1, Immuno-tech S. A., Mareille, France) was used at 1:110 dilution. Counterstaining was accomplished using 0.4% ethyl green in acetate buffer. The staining of MIB-1 was evaluated by visual estimation and by using a computer-assisted image analysis system (CAS-200 Software; Becton Dickinson, Parsippany, NJ, USA). The MIB-1 index was defined as the percent of cells with immunopositivity in the nuclei. We first evaluated patients between 1990 and 1995 by visual estimation; only definitely brown nuclei were recorded as positive, and the same samples were evaluated using a CAS-200. Spearman's correlation between the visual estimation and CAS-200 software was excellent (0.826; $P < .001$). Samples from patients seen between 1985 and 1990 were analyzed by CAS-200, and the results of computer-assisted image analysis were used for statistical analysis.

Monoclonal mouse antihuman BCL-2 oncoprotein clone 124 (Dako, Glostrup, Denmark) was used at 1:60 dilution. Sections were slightly counterstained by hematoxylin, and staining for BCL-2 was analyzed semiquantitatively. Stainings were quantitated by intensity (0-3) and the percent area of expression (0%-100%) and by multiplying these figures to obtain staining scores (0-300).

Immunohistochemistry for CD31 (1:200, Novocastra Laboratories Ltd, Newcastle upon Tyne, UK) was performed on formalin-fixed, paraffin-embedded tissue sections as a part of a tissue microarray. Briefly, the sections were deparaffinized with xylen and rehydrated in graded alcohol, treated in an autoclave in 10 mmol/l sodium citrate (pH 5.0) for 2 minutes, and washed with phosphate buffered saline. Primary antibody was incubated at 4°C overnight, and antibody binding was detected by Vectastain ABC kit reagents (Vector Laboratories, Burlingame, CA, USA). Diaminobenzidine was used as the chromogen. The slides were counterstained with hematoxylin and eosin, and mounted.

VEGFR-3 was stained with the 9D9 antibody (a mouse mAb against the extracellular domain of human VEGFR-3; a kind gift from Professor Kari Alitalo, Helsinki, Finland) at the concentration of 10 μ/L as described in detail previously.[29]

Microvessel density was quantified as the number of CD31-positive or VEGFR-3-positive microvessels per high-power field at ×250 (field of view 0.407 mm^2, including the whole tissue microarray core) using a Leitz Laborlux 12 bright-field microscope (Leitz Wetzlar GmbH, Germany). Two fields with the highest density of vessels were counted, and an average of 2 scores was reported. Scoring was performed in a blinded manner. Specimens were analyzed independently by two investigators.

Statistical Methods

Statistical analyses were performed using IBM SPSS Statistic for Windows version 21.0 (IBM Corp, Amonk, NY, USA; released

2010). The differences between categorical variables were tested using the Pearson χ^2 test. The Cox proportional hazards models were used in age- and gender-adjusted univariate and multivariate survival analyses. In the multivariate model, all dependent variables were entered simultaneously into the model. Furthermore, survival was analyzed using the Kaplan-Meier survival estimation method. All *P*-values under 0.05 were considered statistically significant.

Results

Clinical Characteristics

Our cohort included 132 men (58.9%) and 92 woman (41.1%). The patients' median age at the time of nephrectomy was 65 years (interquartile range, 55.9-71.9 years), and the median survival time was 5.6 years (interquartile range, 1.6-11.9 years).

MIB-1 Expression

Only one sample had negative staining; the remaining samples had positive expression of MIB-1. The median MIB-1 expression value was 1.36, and the highest expression value was 40.9. The MIB-1 expression values were divided into low (< 1.36) and high (≥ 1.36), according to the median staining result. Approximately half of the ccRCC cases (96 samples; 50.3%) showed low expression. Three (30%) papillary RCCs (pRCC) had low and 7 (70%) had high expression. Four (80%) chromophobe RCCs had low and 1 (20%) had high expression. The single collecting duct tumor and the single unclassified RCC showed high expression. One sarcomatoid type of RCC showed low expression, and the other exhibited high expression. There was no association between histologic type of RCC and MIB-1 expression (low or high; $P = .38$). Low expression of MIB-1 was observed in 10 (45.5%) grade 1 to 2 tumors, and high expression was seen in 12 (54.5%); 55 (51.4%) and 52 (48.6%) grade 3 tumors and 39 (47.6%) and 43 (52.4%) grade 4 tumors showed low and high expression of MIB-1, respectively. There was no association between low/high MIB-1 expression and tumor grade ($P = .81$). For tumor stages 1, 2, 3, and 4, the distribution of low/high MIB-1 expression was 43 (57.3%)/32 (42.7%), 23 (54.8%)/19 (45.2%), 23 (40.4%)/34 (59.6%), and 15 (41.7%)/21 (58.3%), respectively ($P = .19$).

BCL-2 Expression

Seventy-five (33.9%) tumor samples had negative staining for BCL-2. The median expression of BCL-2 was 30, and the highest value was 300. BCL-2 staining was divided into low (0-30) and high (31-300) expression groups, based on the median value. Immunostaining of BCL-2 in ccRCC showed no differences; 102 (51.3%) samples had low and 97 (48.7%) had high expression. Ten (83.3%) pRCC had high and 2 (16.7%) had low BCL-2 expression. Three (60%) chromophobe RCCs had low and 2 (40%) had high expression. Both sarcomatoid RCCs had low immunostaining, as did the single unclassified RCC, and the single collecting duct RCC had high immunostaining. Fisher exact test showed $P = .037$ on cross-tabulation between low/high BCL-2 expression and different histological types of RCC. For tumor grades 1 to 2, 3, and 4, the BCL-2 expression was low in 6 (28.6%), 49 (43.8%), and 56 (63.6%) cases and high in 15 (71.4%), 63 (56.3%), and 32 (36.4%) cases, respectively ($P = .002$). Low expression was observed in 30 (39.0%), 19 (43.2%), 37 (60.7%), and 25 (65.8%) tumor samples in tumor stages 1, 2, 3, and 4, respectively, and high expression was observed in 47 (61.0%), 25 (58.8%), 24 (39.3%), and 13 (34.2%) samples, respectively ($P = .011$).

Expressions of VEGFR3 and CD31

The expression of VEGFR3 and CD31 and their distributions with tumor grade and stage were presented in our previous study.[9] Briefly, neither VEGRF3 expressions nor CD31 expressions showed association with tumor stage or grade.

Combinations of MIB-1, BCL-2, VEGFR3, and CD31 Expressions

The expressions of MIB-1, BCL-2, VEGFR3, and CD31 were divided into two groups (low and high). MIB-1, BCL-2, and CD31 were categorized according to their median expression levels of 1.36, 30, and 18, respectively, as follows: MIB-1 low (< 1.35) and high (≥ 1.35); BCL-2 low (0-30) and high (30-300); and CD31 low (≤ 18) and high (> 18). VEGFR3 expression was divided into low (no positive vessels) and high (positive vessels). The cross-tabulation between MIB-1/BCL-2 expression and VEGFR3/CD31 expression is described in Table 1.

Univariate Analysis

Age- and gender-adjusted univariate analysis showed associations between tumor stage, grade, and expressions of MIB-1 and BCL-2 with survival; for stage 4 compared with stage 1 disease: hazard ratio (HR), 13.8; 95% confidence interval (CI), 7.18-26.7; $P < .001$; for stage 3 compared with stage 1 disease: HR, 4.37; 95% CI, 2.29-8.35; $P < .01$; for stage 2 compared with stage 1 disease: HR, 2.62; 95% CI, 1.27-5.41; $P = .007$; for grade 4 compared with grades 1 to 2: HR, 9.31; 95% CI, 2.23-38.8; $P = .002$; for grade 3 compared with grades 1 to 2: HR, 4.91; 95% CI, 1.18-20.4; $P < .001$; for high MIB-1 compared with low MIB-1: HR, 1.76; 95% CI, 1.16-2.68; $P = .008$; for low BCL-2 compared with high BCL-2: HR, 2.16; 95% CI, 1.42-3.31; $P < .001$. Cox regression univariate analysis was also performed for pairs of markers (MIB-1, BCL-2, VEGFR3, and CD31) and all possible variations of their expressions (low/low, low/high, high/low and high/high); the results are summarized in Table 2. Every combination of MIB-1, VEGFR3, or CD31 expression with high BCL-2 expression showed statistically better survival compared with combinations with low BCL-2 expression. All combinations of MIB-1/BCL-2 were significantly associated with survival; for high MIB-1/high

Table 1 Cross-Tabulation of MIB-1/BCL-2 Expressions and VEGFR3/CD31 Expressions

MIB-1/BCL-2	VEGFR3/CD31				Total
	High/High	low/low	High/low	low/High	
	n (%)	n (%)	n (%)	n (%)	n (%)
Low/high	26 (13)	10 (5)	12 (6)	9 (4.5)	57 (28.5)
High/high	18 (9.0)	6 (3.0)	15 (7.5)	4 (2.0)	43 (21.5)
Low/low	12 (6.0)	13 (6.5)	13 (6.5)	7 (3.5)	45 (22.5)
High/low	10 (5.0)	6 (3.0)	19 (9.5)	20 (10.0)	55 (27.5)
Total	66 (33.0)	35 (17.5)	59 (29.5)	40 (20)	200 (100)

The results are indicated in both numbers and percent of cases, $P = .002$ (Pearson χ^2 test)

Table 2 Age- and Gender-Adjusted Univariate Associations of MIB-1, BCL-2, VEGFR3, and CD31 Expressions

	N	Age- and Gender-Adjusted Univariate	
		HR [95% CI]	*P*
MIB-1/BCL-2			
Low/high	58	1.00	
High/high	47	2.13 [1.10-4.12]	.025
Low/low	45	3.02 [1.62-5.62]	.001
High/low	59	3.51 [1.94-6.36]	<.001
MIB-1/VEGFR3			
Low/high	55	1.00	
Low/low	48	1.65 [0.91-3.01]	.10
High/low	46	1.87 [1.02-3.43]	.042
High/high	54	2.36 [1.32-4.22]	.004
MIB-1/CD31			
Low/high	62	1.00	
Low/low	41	1.45 [0.80-2.63]	.22
High/low	59	1.83 [1.07-3.11]	.026
High/high	41	2.14 [1.20-3.82]	.01
BCL-2/VEGFR3			
High/high	60	1.00	
High/low	45	1.10 [0.57-2.13]	.77
Low/low	52	2.37 [1.35-4.15]	.003
Low/high	52	2.71 [1.55-4.76]	<.001
BCL-2/CD31			
High/high	62	1.00	
High/low	44	0.91 [0.46-1.76]	.78
Low/high	41	2.15 [1.21-3.83]	.009
Low/low	62	2.38 [1.41-4.02]	.001
VEGFR3/CD31			
High/high	70	1.00	
High/low	62	1.10 [0.65-1.88]	.75
Low/high	35	2.02 [1.14-3.60]	.017
Low/low	43	2.22 [1.29-3.82]	.004

Cox regression models were used, showing results by hazard ratios (HR) with 95% confidence intervals (CI).

BCL-2: HR, 2.13; 95% CI, 1.10-4.12; $P = .025$; for low MIB-1/low BCL-2: HR, 3.02; 95% CI, 1.62-5.62; $P = .001$; for high MIB-1/low BCL-2: HR, 3.51; 95% CI, 1.94-6.36; $P < .001$, compared with low MIB-1/high BCL-2. The remaining combinations showed no significant association with survival. Univariate analysis of VEGFR3 and CD31 expression with tumor stage and grade showed that low CD31 expression had poorer prognosis in RCC patients but VEGFR3 expression had no association with survival in RCC. The results are described our previous study.[9]

Multivariate Analysis

Multivariate Cox hazard regression analysis was performed including tumor grade, tumor stage, and expressions of MIB-1 and BCL-2 simultaneously into the model with age and gender, described in Table 3. Statistically significant combinations MIB-1/BCL-2 and VEGFR3/CD31 in age- and gender-adjusted univariate analyses were tested with tumor stage and grade in multivariate analysis. Higher tumor stage showed poorer survival, and the combination of MIB-1/BCL-2 almost reached statistically significant association in survival of all combinations; the results are summarized in Table 4.

Kaplan-Meier Analysis

Kaplan-Meier (KM) survival analysis was used to illustrate MIB-1 and BCL-2 expression and the expression of the combinations described above. Increased BCL-2 expression and low MIB-1 expression showed better survival in KM curves compared with low BCL-2 and high MIB-1 expressions (Figures 1A and 1B). Lower BCL-2 expression in combination with low or high MIB-1, VEGFR3, or CD31 expression showed poorer survival compared with any combinations with high BCL-2 expression. The plateau phase, a time after diagnosis when patients have no RCC-related mortality, was estimated by Kaplan-Meier survival curve analysis and was found to be 1.2 years for low grade (1-2) tumors. For higher tumor grades (3 and 4), the plateau occurred almost 10 years later, at 12.4 and 9.9 years, respectively. For stages 1, 2, 3, and 4, the plateau phases were reached within 4, 8.5, 12.4, and 9.9 years, respectively. No RCC-related deaths were observed after 9.9 and 12.4 years in tumors with low and high MIB-1 expression and after 9.9 and 12.4 years in tumors with high and low BCL-2 expression, respectively. Over half of patients having expression combinations of low MIB-1/high BCL-2, low MIB-1/high VEGFR3, high BCL-2 and low/high VEGFR3, or high BCL-2 and low/high CD31 were alive 10 years after nephrectomy. KM curves of the combinations of MIB-1/BCL-2, VEGFR/CD31, and BCL-2/VEGFR3 expression are shown in Figures 2A-C.

Table 3 Age- and Gender-Adjusted Multivariate Associations of Tumor Grade, Tumor Stage, and Expressions of MIB-1 and BCL-2

	N	Age- and Gender-Adjusted Multivariate	
		HR [95% CI]	*P*
Grade			
1-2	22	1.00	
3	114	2.51 [0.59-10.69]	.21
4	88	6.45 [1.50-27.8]	.012
Stage			
1	79	1.00	
2	44	2.96 [1.40-6.24]	.004
3	61	3.58 [1.81-7.08]	<.001
4	39	14.51 [7.13-29.55]	<.001
MIB-1			
Low	104	1.00	
High	107	2.05 [1.32-3.19]	.001
BCL-2			
High	110	1.00	
Low	111	1.40 [0.91-2.21]	.12

Cox regression models were used, showing results by hazard ratios (HR) with 95% confidence intervals (CI).

Table 4 Age- and Gender-Adjusted Multivariate Associations of MIB-1/BCL-2 and VEGFR3/CD31 Expressions With Stage and Grade

	n	Age- and Gender-Adjusted Multivariate	
		HR [95% CI]	*P*
MIB-1/BCL-2			
Low/high	57	1.00	
Low/low	45	1.60 [0.83-3.08]	.16
High/high	42	2.14 [1.07-4.29]	.032
High/low	55	3.20 [1.66-6.17]	.001
VEGFR3/CD31			
High/high	66	1.00	
Low/low	58	0.92 [0.52-1.63]	.79
High/low	40	1.28 [0.70-2.43]	.46
Low/high	35	2.48 [1.29-4.78]	.007
Stage			
1	67	1.00	
2	40	2.60 [1.28-5.25]	.008
3	57	2.58 [1.40-4.73]	.002
4	35	8.85 [4.47-17.5]	<.001
Grade			
1-2	20	1.00	
3	102	1.44 [0.48-4.24]	.51
4	78	3.25 [1.09-9.73]	.035

Cox regression models were used, showing results by hazard ratios (HR) with 95% confidence intervals (CI).

Discussion

In this study, we explored the expression of MIB-1, BCL-2, VEGFR3, and CD31 and their associations with survival in patients with RCC. Our study consisted of 224 patients, for whom all tumor samples were re-evaluated and reclassified by a qualified uropathologist (P.K.). All data were collected directly from the original medical records. The survival data were obtained from the Finnish Cancer Registry; thus were very reliable.

Clinicians today have a better understanding of molecular pathway abnormalities, histological subtypes, and new morphological variants of RCC.[30] The improved knowledge might aid in the discovery of new treatments for patients with RCC.[31,32] The standard follow-up practice for patients with RCC is not clear and has been debated in the literature, and the tendency is toward more individual treatment and management of RCC. We urgently need new molecular markers to plan these patients' individual treatments and follow-up.

In the present study, the expression of the proliferation markers MIB-1 and BCL-2 alone and with the angiogenesis markers VEGFR3 and CD31, and their associations with survival, was explored in patients with RCC. We classified the expression of MIB-1, BCL-2, VEGFR3, and CD31 into low and high expression groups according to their immunostaining scores. High MIB-1 expression was associated with poorer disease-free survival and overall survival in patients with RCC in a previous study.[33] Similarly, our study showed that low MIB-1 expression was independently associated with a better prognosis in these patients. Higher BCL-2 expression in combination with any low or high expression of MIB-1, VEGFR3, or CD31 showed better survival compared with combinations with low BCL-2 expression. Previous studies have also found that increased BCL-2 expression was associated with a better prognosis for patients with RCC.[6] Our univariate analysis showed that a combination of the proliferation marker MIB-1 and an anti-apoptosis marker BCL-2 had a statistically significant association with survival in patients with RCC. Anti-apoptotic proteins such as BCL-2 are often overexpressed in cancer, while apoptotic proteins are deregulated.[34] RCC is, in

Figure 1 (A) Kaplan-Meier Curves for MIB-1 Expression (Low/High). (B) Kaplan-Meier Curves for BCL-2 Expression (Low/High)

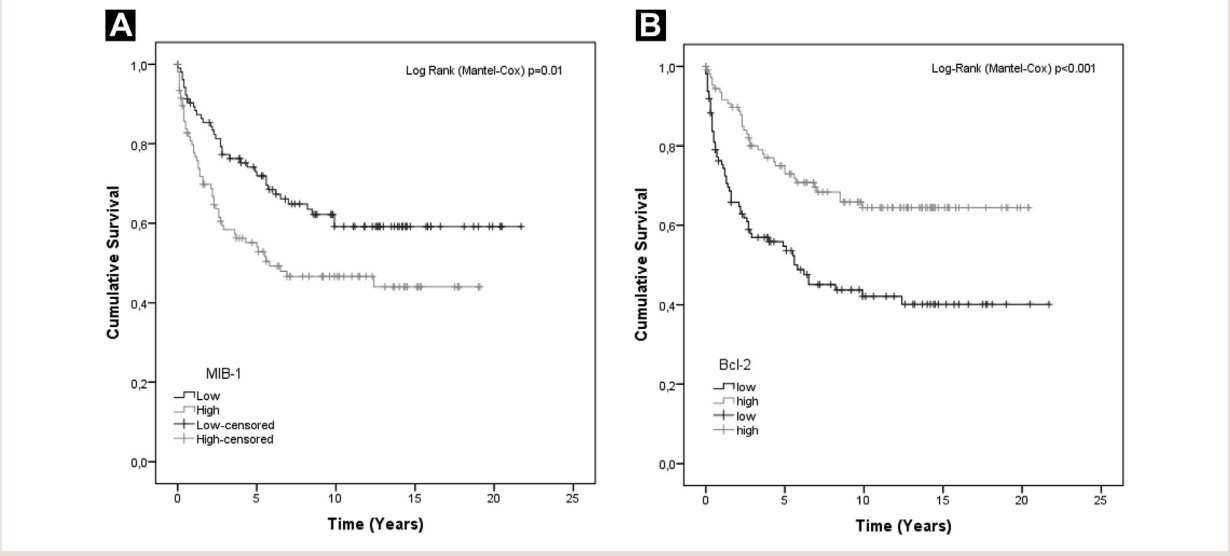
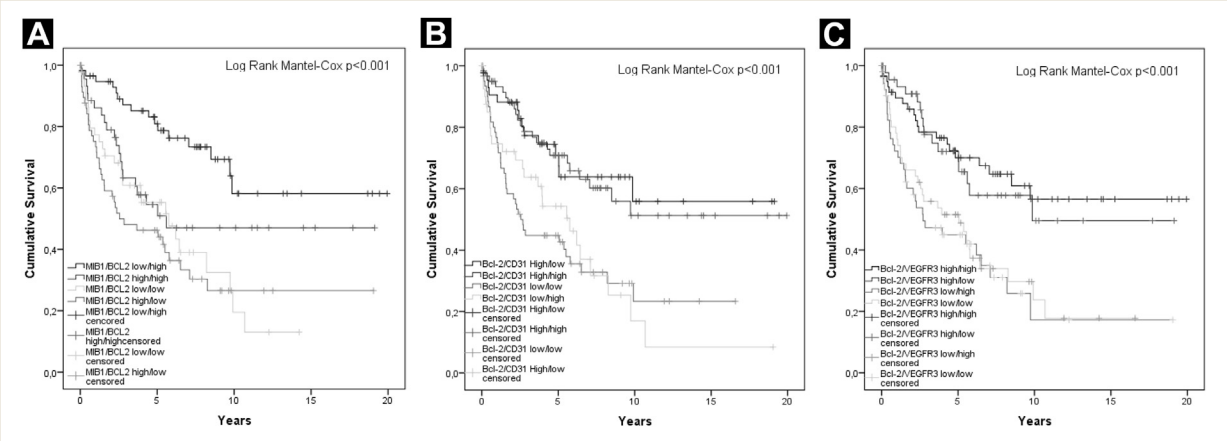


Figure 2 (A) Kaplan-Meier Curves for Combinations of MIB-1 and BCL-2 Expressions (Low/High). (B) Kaplan-Meier Curves for Combinations of BCL-2 and CD31 Expressions (Low/High). (C) Kaplan-Meier Curves for Combinations of BCL-2 and VEGFR3 Expressions (Low/High)



general, a highly vascular tumor, and angiogenesis is very important for tumor growth and spread. Histological tumor necrosis is shown to be associated with poorer survival in RCC.[35] Overexpression of BCL-2 was shown to prevent necrosis,[36] which might be one of the explanatory factors for the improved survival of patients with RCC and higher BCL-2 expression. MIB-1 expression was also significantly associated with survival in multivariate analysis, in which the classical prognostic factors of tumor stage and grade were also included. Our previous study showed that higher CD31 expression was associated with a better prognosis in patients with RCC compared with low CD31 expression.[9] This present study showed that higher CD31 expression had no strong association with survival, whereas high BCL-2 had a strong association with better survival with all combinations of MIB-1, VEGFR3, and CD31 expressions.

VEGFR3 is important for both angiogenesis and lymphatic maintenance,[12] whereas CD31 is involved in tissue regeneration, and its expression has been shown in vascular tumors.[16] This study showed that the combination of high VEGFR3/high CD31 was associated with a better prognosis compared with low VEGFR3/low CD31. This may indicate that patients with better lymphangiogenesis and active tissue regeneration have a better prognosis.

We also examined associations between combinations of MIB-1 and BCL-2 expression, which are related to cell growth and apoptosis, and combinations of VEGFR3 and CD31 expression, which are related to tumor angiogenesis. Cross-tabulation showed a significant association between them, as shown in Table 1. Distributions were in the same line as survival analysis showed; however, 4.5% of cases with low MIB-1/high BCL-2, which was associated with better survival, had low VEGFR3/high CD31, which was associated with poorer survival; in addition, 5% of cases with high MIB-1/low BCL-2, which was associated with a poorer prognosis, had high VEGFR3/high CD31, which was associated with a better prognosis. Based on these results, we cannot clearly indicate clear associations between the expressions of these combinations of factors; perhaps there are other factors of which we are not yet aware that affect tumor growth, death, and tumor angiogenesis.

This present study included 224 consecutive patients whose RCC was diagnosed from 1985 to 1995. During that time, radiological examinations were performed less frequently than they are currently. Over 50% of RCCs are currently detected incidentally.[30] This might be the reason that our study population consisted of only 22 (9.8%) patients with grade 1 or 2 tumor and 79 (35.3%) patients with stage 1 tumor. Our study showed an association between higher Bcl-2 expression and pRCC, but no association between MIB-1 expression and histological type of RCC. This might be explained by the fact that the majority histological type in our study was ccRCC (90.2%) and this represents a higher proportion than is generally found in RCC.[26,37,38] Due to inadequate immunostaining, we excluded 3 to 21 patients (1.3%-9.4%) from the analyses. The excluded portion was, however, minor and therefore had an insignificant effect on the results.

Our study showed that over 50% of patients with low MIB-1 or high Bcl-2 expression were alive at the end of long-term follow-up. The survival plateau was reached already after 1.2 years in patients with grade 1 to 2 tumors, whereas for other grades and stages, the MIB-1 and Bcl-2 expression groups' plateaus were achieved between 8.5 and 12.4 years. Additionally, 50% of patients with low MIB/high BCL-2, low MIB-1/high VEGFR3, high BCL-2 and low/high VEGFR3, or high BCL-2 and low/high CD31expression survived 10 years after the diagnosis of RCC. These data showed that all patients with RCC who were alive 10 years after nephrectomy had an excellent prognosis. The follow-up guidelines of RCC have been recently discussed in the literature.[32] Smith et al. showed that nearly one-third of RCC recurrences were missed when patients were followed according to the American Urological Association or National Comprehensive Cancer Network guidelines.[39] New molecular markers or their combinations might be needed to improve the assessment of recurrence risk and to tailor treatment and follow-up for patients with RCC. MIB-1, BCL-2, VEGFR3,

and CD31 might be useful for individual follow-up of patients with RCC and should be tested in prospective trials.

Conclusion

These data showed that low BCL-2 alone or in any combination with low or high MIB-1, VEGFR3, or CD31 expression was associated with poorer survival in patients with RCC. Low MIB-1 expression was an independent predictor of better prognosis in patients with RCC. Low VEGFR3/low CD31 expression was associated with poor survival compared with high VEGFR3/high CD31 expression. These markers might be useful for planning the follow-up of patients with RCC.

Clinical Practice Points

- There are no molecular markers to predict survival in patients with RCC. We evaluated the expressions of MIB-1, BCL-2, VEGFR3, and CD31 and their role in the prognosis of RCC.
- This study showed that low MIB-1 and high BCL-2 expressions were associated with improved survival in RCC.
- High VEGFR3/high CD31 expression showed better survival compared with low VEGFR3/high CD31 expression.
- Molecular markers might be useful for planning patients' follow-up.

Disclosure

The authors have stated that they have no conflicts of interest.

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