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PROGNOSTIC MARKERS AND PROLONGED INTERFERON-α THERAPY IN RENAL CELL CARCINOMA

by

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To my family, especially to my daughter Aurora

Now that almost anything seems technically possible, the key issue for the twenty-first century biologist is to identify the right questions to ask.

L. Franks and M. Knowles

ABSTRACT

Minna Kankuri-Tammilehto

Prognostic Markers and Prolonged Interferon-α Therapy in Renal Cell Carcinoma

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The five-year survival for renal cell carcinoma (RCC) is ~50%. According to previous studies, for metastatic RCC (mRCC) with interferon-α (IFN-α) based therapies the five-year survival is 3-16%. Typically, IFN-α treatment duration has been <6 months. Current questions in the use of IFN-α alone or with targeted therapies are the optimal dose and schedule.

The main aims were to investigate 1) the efficacy and tolerance of IFN-α with prolonged and intermittent administration in mRCC, and 2) the prognostic significance of p53, Ki-67 and COX-2 protein expressions in RCC. For 117 mRCC patients, IFN-α_{2a} (Roferon-A®) was planned to be continued at the maximal tolerated dose with a one-week pause each month up to 24 months or progression or intolerable toxicity. Protein expressions were analyzed immunohistochemically from nephrectomized and paraffin-embedded tissues from three different groups: primary metastases (n=29), later metastases (n=37), and no metastases (n=51).

Mean duration of IFN-α therapy was 11 months [0.5-32 months]. The rate of objective responses was 17%, stable disease 42%, and late responses (after 12 months' therapy) 3%. Progression-free survival and median overall survival were 8 and 19.1 months, respectively. Five-year survival was 16%; patients with lung metastases had higher five-year survival. No life-threatening side-effects were observed. Prolonged therapy (>12 months) is recommended for stable and responding patients.

Double positivity for p53 and Ki-67 expression indicates high metastases probability. Positive COX-2 expression indicates slow metastases development. For mRCC, positive p53 and Ki-67 expressions indicate poor prognosis, positive COX-2 expression indicates favorable prognosis. COX-2 positivity in Ki-67 negative tumors strongly indicates improved survival in mRCC.

Key words: COX-2, interferon-α, Ki-67, p53, renal cell carcinoma, response, long-term survival

TIIVISTELMÄ

Minna Kankuri-Tammilehto

Molekyylimarkkerit ja pitkäaikainen alfainterferonihoito munuaissyövässä

Onkologia ja sädehoito ja Patologia

Turun Yliopisto, Turun Yliopistollinen Keskussairaala

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Munuaissyöpäpotilaiden viiden vuoden elossaololuku on noin 50 %. Aikaisempien tutkimuksien mukaan viiden vuoden elossaololuku metastasoituneessa munuaissyövässä on 3-16 %, kun käytettiin alfainterferonia sisältävää hoitoa. Tyypillisesti alfainterferonia on käytetty vähemmäin kuin 6 kuukautta. Avoimia kysymyksiä ovat alfainterferonin optimaalinen hoitoannos ja hoidon kesto yksin tai yhdessä uusien täsmähoitojen kanssa.

Tärkeimmät tavoitteet olivat tutkia 1) jaksotetun pitkäaikaisen alfainterferonihoidon tehoa ja siedettävyyttä metastasoituneessa munuaissyövässä ja 2) p53-, Ki-67- ja COX-2-proteiinituotannon ennusteellista merkitystä munuaissyövässä. Tutkimuksessa 117 metastasoituneelle munuaissyöpää sairastaneelle potilaalle etsittiin yksilöllinen hänen sietämänsä maksimaalinen hoitoannos rekombinanttia alfa$_{2a}$-interferonia (Roferon-A®). Hoitoa pyrittiin jatkamaan 24 kuukauden ajan. Kolmen hoitoviikon jälkeen pidettiin yhden viikon tauko. Hoito lopetettiin, jos ilmaantui vakavia haittavaikutuksia tai tauti eteni. Toisessa tutkimuksessa proteiinituotanto analysoitiin immunohistokemiallisesti munuaissyöpäpotilaiden kasvainnäytteistä, joita oli säilytetty parafiinissa. Kasvainnäytteet oli otettu talteen munuaisen poistoleikkauksen yhteydessä. Nämä potilaat jaettiin kolmeen eri ryhmään: metastasointi primaarivaiheessa (n=29), metastasointi myöhemmin (n=37) ja ei metastasointia (n=51).

Keskimääräinen alfainterferonihoidon kesto oli 11 kuukautta (kk) [0,5 – 32 kk]. Objektiivinen hoitovaste todettiin 17 %:lla, tautitilanne pysyi ennallaan 42 %:lla ja myöhäinen vaste (yli 12 kk:tta hoidon aloittamisesta) todettiin 3 %:lla. Aika vasteen saavuttamisesta taudin etenemiseen oli keskimäärin 8 kk ja elinaika 19,1 kk. Viiden vuoden elossaololuku oli 16 %. Jos metastasoituneella munuaissyöpäpotilaalla oli keuhkometastasointi, hän selvisi todennäköisemmin viisi vuotta kuin muut potilaat. Henkeä uhkaavia sivuvaikutuksia ei todettu. Yli 12 kk:n ajan kestävä alfainterferonihoito on hyödyllistä niille potilaille, jotka ovat saaneet objektiivisen hoitovasteen tai tautitilanne on pysynyt ennallaan.

Positiivinen p53- ja Ki-67-ekspressio yhdessä viittaavat suureen metastasoinnin todennäköisyyteen. Positiivinen COX-2-ekspressio viittaa viivästyneeseen metastaasien ilmaantumiseen. Metastasoituneilla potilailla positiiviset p53- ja Ki-67-ekspressiot viittaavat huonoon ennusteeseen, mutta positiivinen COX-2 ekspressio viittaa suotuisaan ennusteeseen. Positiivinen COX-2- ja negatiivinen Ki-67-ekspressio yhdessä viittaavat parantuneeseen ennusteeseen metastasoituneessa munuaissyövässä.

Avainsanat: alfainterferoni, COX-2, elinaika, Ki-67, munuaissyöpä, p53, vaste

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ABBREVIATIONS

A13CRA	13-cis-retinoid acid
ACKD	acquired cystic kidney disease
ADCC	antibody-dependent cell-mediated cytotoxicity
AJCC	the American Joint Committee on Cancer
ASR	age- standardized incidence rate
BHD	Birt-Hogg-Dubé syndrome
BHD	Birt-Hogg-Dubé gene
BMI	body mass index
CA9	carbonic anhydrase 9
c-erbB2	Her-2
CI	confidence interval
CISH	chromogenic *in situ* hybridzation
c-met	see MET
CNS	central nervous system
COR	cumulative odds ratios
COX-2	cyclooxygenase 2
CR	complete response
CR1	complement receptor 1
CR3	complement receptor 3
CRP	c-reactive protein
CT	computerized tomography
DNA	deoxyribonucleic acid
DSS	disease-specific survival
EAU	European Association of Urology
ECOG	the Eastern Cooperative Oncology Group
ECOG-PS	ECOG performance status
EGFR	epidermal growth factor receptor
EPO	erythropoietin
ESMO	European Society for Medical Oncology
EU	the European Union
FACT-G	the functional assessment of cancer therapy general scale
FCR	Finnish cancer registry
FcγRI	phagocyte receptor expression I
FcγRII	phagocyte receptor expression II
FcγRIII	phagocyte receptor expression III
FH	fumarate hydratase gene
FHIT	fragile histidine triad gene
FISH	fluorescent *in situ* hybridization
FKSI	functional assessment of cancer therapy-kidney symptom index
FLCN	folliculin gene
FPTC-PRN	familial papillary thyroid carcinoma-papillary renal neoplasia
G250	carbonic anhydrase IX monoclonal antibody
Gy	Gray
HD-IL-2	High dose interleukin-2
Her-2	Human Epidermal growth factor Receptor 2
Her-2	Her-2 proto-oncogene
HIF	hypoxia-inducible factor
HIF-1α	hypoxia inducible factor - 1 alpha
HLRCC	hereditary leiomyomatosis and RCC
HPT-JT	hyperparathyroidism-jaw tumor
HR	hazard ratio
HRPT2	hyperparathyroidism 2 gene
IFN	interferon
IFN-α	leucocyte interferon-alpha
IFN-β	fibroblast interferon-beta
IFN-γ	immune interferon-gamma
IGF-I	insulin-like growth factor-I
IL	interleukin
IL-2	interleukin-2
IVC	inferior vena cava
Ki-67	proliferation biomarker
LD-IL-2	low-dose interleukin-2
lm	later metastases (after nephrectomy), metachronous metastases
LOH	loss of heterozygosity
mAb	monoclonal antibody
MET	hepatocyte growth factor receptor
MET	MET proto-oncogene
MHC	major histocompatibility complex
MNV	median nuclear volume
mos	months
MPA	medroxyprogesterone acetate
mRCC	metastatic renal cell cancer
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MSKCC	Memorial Sloan Kettering Cancer center
mTOR	mammalian target of rapamycin
MU	million units
NCCN	National Comprehensive Cancer Network
NK	natural killer cell
nm	no metastases during 7.5 years' follow-up
NSS	nephron-sparing surgery
OR	odds ratios
OS	overall survival
p53	protein p53, biomarker of cell cycle check point
PCR	polymerase chain reaction
PD	progressive disease
PEG-IFN-α	pegylated interferon-α
PET	positron emission tomography
pm	primary metastases (at nephrectomy), synchronous metastases
PN	partial nephrectomy
PR	partial response
PRCC	papillary renal cell carcinoma
pTNM	pathological TNM
pVHL	von Hippel-Lindau protein
QoL	Quality of Life
RCA	renal cell adenoma

RCC	renal cell carcinoma
RhuMAb	recombinant humanized monoclonal antibody
RNA	ribonucleic acid
RPLND	that retroperitoneal lymph node dissection
SD	stable disease
TCS2	tuberous sclerosis 2 gene
tiw	three times a week
TNF	tumor necrosis factor
TNM	T=tumor, N=node, M=metastases
TP53	Tumor protein p53 gene
TSC1	tuberous sclerosis 1 gene
UCLA	University of California
UICC	Union Internationale Contre le Cancer (The International Union Against Cancer)
US	Ultrasonography
VEGF	vascular endothelial growth factor
VEGFR-TKI	vascular endothelial growth factor receptor tyrosine kinases
VHL	von Hippel-Lindau disease
VHL	von Hippel-Lindau gene
WHO	World Health Organization
YCC	Yale Comprehensive Cancer Center

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals (I-VI).

I
Kankuri M, Pelliniemi T-T, Pyrhönen S, Nikkanen V, Helenius H, Salminen E (2001): Feasibility of Prolonged Use of Interferon-α in Metastatic Kidney Carcinoma. A Phase II Study. Cancer 92 (4): 761-767.

II
Kankuri-Tammilehto M, Söderström K-O, Pelliniemi T-T, Hinkka-Yli-Salomäki S, Pyrhönen S, Salminen E (2009): Long-Term Outcome of Metastatic Renal Cell Carcinoma with Prolonged Use of Interferon-α Administered Intermittently. A Phase II Study. Submitted.

III
Salminen E, **Kankuri M**, Nuutila J, Lilius E-M, Pelliniemi T-T (2001): Modulation of IgG and Complement Receptor Expression of Phagocytes in Kidney Cancer Patients during Treatment with Inteferon-α. Anticancer Research 21 (3B): 2049-2055.

IV
Kankuri M, Söderström K-O, Pelliniemi T-T, Vahlberg T, Pyrhönen S, Salminen E (2006): The Association of Immunoreactive p53 and Ki-67 with T-stage, Grade, Occurrence of Metastases and Survival in Renal Cell Carcinoma. Anticancer Res 26 (5B): 3825-3833.

V
Kankuri-Tammilehto M, Söderström K-O, Pelliniemi T-T, Vahlberg T, Pyrhönen S, Salminen E (2009): Prognostic Evaluation of COX-2 Expression in Renal Cell Carcinoma. Submitted.

VI
Kankuri-Tammilehto M. Chapter IV - Association of p53, Ki-67, COX-2, and Her-2 Expressions, as well as T-stage and Histopathological Grade, with Occurrence of Metastases and Survival in Renal Cell Carcinoma. Review. In: Watanabe A, ed. Cancer Metastases Research. New York: Nova Science Publishers, Inc., 2008, pp.109-132.

1 INTRODUCTION

Renal cell carcinomas (RCCs) are renal epithelial neoplasms in renal parenchyma, also called renal adenocarcinomas. The majority of RCCs arise from the cells of renal proximal tubules of nephrons, but 5% of cases arise from the cells of the collecting ducts (Chao *et al.* 2002, Kovacs *et al.* 1997, Störkel *et al.* 1997). RCCs are typically highly vascularized solid tumors (Kovacs *et al.* 1997), and they have the ability to spread via multiple routes, such as directly into the perinephric tissues, or hematogeneously through the renal vein and inferior vena cava and lymphatic tissues. Renal tumors are members of a complex family with unique histology, cytogenetic defects and variable metastatic potential (Linehan *et al.* 2003, Thoenes *et al.* 1986). Of all RCCs, 70-80% are of conventional type, also known as clear cell RCCs. Of these, approximately 75% have a mutation in the von Hippel-Lindau tumor suppressor gene (*VHL)* in the short arm of chromosome 3 (Maxwell *et al.* 1999, Gnarra *et al.* 1994).

Macroscopic hematuria, pain and palpable tumor, together called the classic triad, indicate metastatic disease (Cunningham 1938). Nowadays, 30-60% of RCC tumors are found incidentally in abdominal imaging performed for some other reason than suspected renal tumor, such as the evaluation of non-specific abdominal or musculoskeletal complaints (Jayson and Sanders 1998).

The expected five-year survival rate for all RCC stages is approximately 50%. For those patients with performance status enabling current treatments the rate is slightly higher than 60% (Parkin *et al.* 2003). According to a few previous studies on long-term outcome for metastatic RCC (mRCC), the five-year survival is from 3% to 16% (Atzpodien *et al.* 2002, Motzer *et al.* 2000a, Minasian *et al.* 1993) if metastasectomy is not a possible treatment. For localized RCC, nephrectomy is the only curative treatment (Robson *et al.* 2002), and there is no effective adjuvant therapy. Possible treatments for mRCC, in addition to cytoreductive nephrectomy (Flanigan *et al.* 2001, Mickisch *et al.* 2001), are immunomodulators, such as interferon-α (IFN-α) (Pyrhönen *et al.* 1999), interleukin-2 (IL-2) (Négrier *et al.* 2007, Spanknebel *et al.* 2005), and more recently tyrosine kinase inhibitors, such as sunitinib (Motzer *et al.* 2007), sorafenib (Escudier *et al.* 2007a), and mTOR inhibitor temsirolimus (Hudes *et al.* 2007). Everolimus, another mTOR inhibitor, has an encouraging antitumor activity against mRCC (Motzer 2008a). The efficacy of bevacizumab, an antiangiogenesis monoclonal antibody, has also been shown (Yang *et al.* 2003a). Interferon-alpha (IFN-α) has been considered the standard comparator when investigating novel targeted therapies in mRCC.

Compared to other biological response modifiers, chemotherapy and hormonal therapy, IFN-α is associated with survival benefits (MRCRCC *et al.* 1999, Pyrhönen *et al.* 1999). High dose

interleukin-2 therapy (HD-IL-2) may improve survival in patients with the poorest prognosis (McDermott *et al.* 2005). The current questions concerning the use of immunomodulators, such as IFN-α, are timing (Motzer *et al.* 2007, Stadler *et al.* 2007) and optimal dosage as monotherapy or in combination with targeted therapies (Bracarda *et al.* 2007).

RCC is a heterogeneous disease: the prognosis differs at the same stage and grade (Tsui *et al.* 2000a). Currently, T-stage is the best known prognostic factor for locally confined RCC. The most often represented factors in mRCC are performance status, time to metastases, number of metastatic sites, and prior nephrectomy. All the molecular mechanisms that affect the development, progression and clinical behavior of RCC are not known. A better understanding of these and the factors affecting the response to IFN-α enables us to target treatment more selectively in mRCC. Molecular biomarkers, such as p53, Ki-67 and COX-2, are potential candidates for staging, assessing prognosis, and guiding targeted therapies (Masters 2007).

2 REVIEW OF THE LITERATURE

2.1 CLINICAL BEHAVIOR OF RENAL CELL CARCINOMA (RCC)

2.1.1 EPIDEMIOLOGY

Kidney cancer* represents 2-3% of all diagnosed malignancies worldwide (Parkin *et al.* 2003). In some Northern and Central European countries the incidence is higher, e.g. in Iceland 4.1%, and in the Czech Republic 5.4% (Parkin *et al.* 2003). In Finland, kidney cancer represents 2-3% of all malignancies, and the age-standardized incidence rate (ASR) is 8.8/100 000 for men and 5.4/100 000 for women (Finnish Cancer Registry (FCR) 2007). Kidney cancer is more common among urban than among rural residents. In the European Union, the estimated annual number of new kidney cancers is 46 000 (Ferlay *et al.* 2001). In Finland, 735 new kidney cancers were diagnosed in 2007, and the number of kidney cancer patients alive on the 1st of January 2008 was 6 017. In 2007, altogether 346 patients succumbed to kidney cancer in Finland (FCR 2007).

Since the 1970s, the annual increase in RCC incidence has been 2-4% (Mathew *et al.* 2002). This has been attributed to the use of radiological imaging which is able to find presymptomatic RCC lesions (Jayson and Sanders 1998), as well as the increased prevalence of etiologic risk factors, such as cigarette smoking (Hunt *et al.* 2005) and obesity (Chow *et al.* 2000, Yuan *et al.* 1998). The rise in incidence has been greater in women than in men (Chow *et al.* 2000). The increase has been highest in localized disease, especially in tumors that are less than 4 cm in diameter (Hollingsworth *et al.* 2006). In Scandinavia, a decrease in incidence has been observed since the 1980's in Sweden and Denmark, and since the 1990's in Finland, in contrast to Norway (ANCR 2009). The reason for this phenomenon is unclear, but one explanation may be variations in cigarette smoking (Mathew *et al.* 2002) and tobacco legislation. In Danish men the incidence started to increase again in the 2000's.

Of kidney cancer cases, 84% are diagnosed between 50 and 84 years of age (FCR 2007, Parkin *et al.* 2003), and the median age of diagnosis is around 64 years for Caucasians and 58 years for African-Americans (FCR 2007, Parkin *et al.* 2003). RCC has been observed in as young as six-month-old children, possibly as a hereditary disease (Sanchez-Ortiz *et al.* 2004, Renshaw *et al.*

* In epidemiological statistics, RCC and renal pelvis cancer are usually not reported separately, but combined under the heading of kidney cancer. Eighty per cent of adult kidney tumors are RCCs; transitional-cell carcinomas of the renal pelvis account for approximately 15%, and the remaining (< 5%) kidney tumors include a variety of rare lesions such as embryonic renal neoplasms, renal sarcomas and tumors of primarily mesenchymal origin (Parkin *et al.* 2003).

1999). Young patients are more likely to have unfavorable histopathological features and lymph node metastases than older patients (Sanchez-Ortiz *et al.* 2004, Renshaw *et al.* 1999).

A male-to-female ratio in RCC incidence is from 1.3:1 to 2.5:1 (FCR 2007, Motzer *et al.* 1999). At primary diagnosis, men more often than women have stage III-IV disease (Thrasher and Paulson 1993).

In the USA, mortality caused by kidney cancer rose 1.3% annually from 1975 to 1992 (annual percentage change), but decreased by 0.5% annually from 1992 to 2002 (Edwards *et al.* 2005). In the EU, the fall in kidney cancer mortality between 1994 and 1999 was over 10%, being most marked in those Northern and Central European countries where mortality from kidney cancer had been observed as very high compared to other European countries (Levi *et al.* 2004). It is estimated that kidney cancer is responsible for 102,000 deaths per year worldwide (Parkin *et al.* 2003). In Finland, 345 patients die annually of RCC (FCR 2007)

2.1.2 ETIOLOGY AND RISK FACTORS

The etiology of RCC is still largely undefined. The most consistently established causal risk factors for RCC are cigarette smoking and obesity, in addition to acquired cystic kidney disease and inherited susceptibility (Table 1), but the significance of many other potential risk factors is being investigated.

Cigarette smoking has been found to be a definitive risk factor for RCC (Hunt *et al.* 2005, McLaughlin *et al.* 1984). The strong dose-response relationship of cigarette smoking and RCC and the decrease in risk of RCC following cessation of cigarette smoking (15-30% after 10 to 15 years since cessation) (Hunt *et al.* 2005, McLaughlin *et al.* 1984) support a causal interpretation of the association. The relative risk is 1.54 for male and 1.22 for female smokers (Hunt *et al.* 2005). It has been estimated that up to 30% of RCC in men, and up to 20% in women may be directly due to cigarette smoking (McLaughlin *et al.* 1984).

The carcinogenic agent in cigarette smoke has not been clearly identified (Hunt *et al.* 2005, McLaughlin *et al.* 1984). Most of the constituents in cigarette smoke are metabolized or excreted through the urinary tract. Nitrous compounds, especially N-nitrosodimethylamine, found in cigarette smoke, has been observed to cause epithelial renal tumors in animal models (IARCC 2004), and *VHL* gene mutation has been associated with N-nitrosodimethylamine (Shiao *et al.* 1998). Metabolic gene polymorphisms have been suggested as risk factors for RCC. These polymorphisms are involved in the activation or detoxification of carcinogens in cigarette smoke.

Table 1. Risk factors of RCC.

Risk factors for RCC	Evidence of risk factor	References
Self-inflicted		
Cigarette smoking	Well-established	Hunt *et al.* 2005, McLaughlin *et al.* 1984
Obesity	Well-established	Samanic *et al.* 2006, Bergström *et al.* 2001, Chow *et al.* 2000
Low recreational activity	Potential	Menezes *et al.* 2003
Illness		
Acquired cystic kidney disease	Well-established	Schwarz *et al.* 2007, Maisonneuve *et al.* 1999
Hypertension	Highly potential	Chow *et al.* 2000, Shapiro *et al.* 1999, Yuan *et al.* 1998, McLaughlin *et al.* 1995
Diabetes mellitus	Potential	Washio *et al.* 2007, Lindblad *et al.* 1999
Medication		
Diuretics	Potential	Yuan *et al.* 1998, McLaughlin *et al.* 1995
Oral contraceptives	Potential	Lindblad *et al.* 1995
Reproductive hormone factors		
Prior hysterectomy and oophorectomy	Potential	Lindblad *et al.* 1995
Occupational		
Thrichloroethene	Highly potential	Brüning *et al.* 2003, Brüning *et al.* 1997
Asbestos	Potential	Sali and Boffetta 2000
Sedentary work	Potential	Bergström *et al.* 1999
Arsenic	Potential	Marshall *et al.* 2007
Cadmium	Potential	Pesch *et al.* 2000
Nutrition		
Fried meats	Potential	Yuan *et al.* 1998
Low consumption of orange or green vegetables	Potential	Yuan *et al.* 1998
Low intake of D-vitamin	Potential	Karami *et al.* 2008
Low intake of E-vitamin	Potential	Zhang *et al.* 1997
Low intake of magnesium	Potential	Wolk *et al.* 1996
Hereditary	Well-established	Table 2

Obesity, increased body mass index (BMI), has been established as a risk factor for RCC in both men and women (Bergström *et al.* 2001). It has been estimated that more than 30% of RCC may be due to obesity (Bergström *et al.* 2001). The mechanisms by which obesity influences renal carcinogenesis are unclear, although the risk is suggested to be mediated via, e.g. sex steroid hormones and increased levels of insulin-like growth factor-I (IGF-I) (Bergström *et al.* 2001, Chow *et al.* 2000).

Hypertension seems to be an independent risk factor for RCC (Yuan *et al.* 1998), with the association being stronger for women (Chow *et al.* 2000, Shapiro *et al.* 1999). However, it has been difficult to distinguish the effect of therapy from its indication (McLaughlin *et al.* 1995).

Acquired cystic kidney disease (ACKD) causes an increased risk of RCC (Maisonneuve *et al.* 1999). ACKD occurs in end-stage renal disease and may develop in 20% of long-term dialysis patients (Schwarz *et al.* 2007, Maisonneuve *et al.* 1999), or due to kidney transplantation (Schwarz *et al.* 2007). The incidence of RCC in patients with end-stage renal disease is approximately 40 to 100 times (Maisonneuve *et al.* 1999), and in chronic renal failure, nine times higher than in the general population (Takahashi *et al.* 1993).

Thrichloroethene is a carcinogen that causes renal adenomas and carcinomas in animal models (Mensing *et al.* 2002), and its toxic metabolites have been associated with the *VHL* gene mutation (Brüning *et al.* 1997). Thrichloroethene exposure has been found in the urine of workers at a cardboard manufacturing plant. An association between a long period of high exposure to thrichloroethene and RCC was observed (Brüning *et al.* 2003).

Hereditary RCC accounts for less than 4% of all RCCs (Eble *et al.* 2004) (Table 2). Hereditary RCC is often characterized by an early age at onset (approximately 45 years), and frequently the bilaterality and multicentricity of the primary tumor (Gnarra *et al.* 1994), although, e.g. in HLRCC (Hereditary leiomyomatosis and RCC) and familial non von Hippel-Lindau (VHL) RCC syndromes, primary tumors are typically unilateral and solitary. The aggressiveness of hereditary RCCs varies depending on the syndrome and mutation type. If conventional RCC is of VHL disease origin, tumors of less than 3 cm have a low rate of metastatic potential and minimal invasive surgery is recommended (Zbar *et al.* 1999). However, if HLRCC is detected, radical nephrectomy is recommended due to the aggressiveness of RCC in this syndrome (Lehtonen *et al.* 2006). Li-Fraumeni syndrome, a rare autosomal dominant disorder, causes typically breast cancer, sarcoma, and leukemia, but in rare cases also RCC. In 70% of Li-Fraumeni syndrome germline mutations have been found in the *TP53* tumor suppressor gene on 17p13.1 (Ruijs *et al.* 2006). Identification of family members at risk of hereditary RCC is important for organizing follow-up and surveillance programs with regular imaging of the kidneys. The identification of the genes that are responsible for familial renal tumors will result in better understanding of renal tumorigenesis, even in non-hereditary renal tumors.

Table 2. Hereditary syndromes that predispose to RCC.

	von Hippel-Lindau (VHL)	**Hereditary PRCC**	**Hereditary leiomyomatosis and RCC (HLRCC)**	**Birt-Hogg-Dubé (BHD)**	**Tuberous sclerosis**	**Constitutional chromosome 3 translocation**	**Familial non-syndromic RCC**	**Familial papillary thyroid carcinoma-papillary renal neoplasia (FPTC-PRN)**	**Hyperpara-thyroidism-jaw tumor (HPT-JT)**
RCC subtype	Conventional	Papillary type 1	Papillary type 2, collecting duct	Conventional, papillary, chromophobe	Conventional	Conventional	Conventional	Papillary type 1	Papillary type 1
Risk for RCC	70-80%		20-30%	15-40%	1-3%				
Gene	*VHL (3p25)*	*MET*	*FH*	*FLCN (BHD)*	*TSC1, TSC2*	*FHIT,* unknown	Unknown	Unknown	*HRPT2*
Chromosome	3p25-26	7q31-34	1q42-q44	e.g. 17p12-q11.2	9q34, 16p13.3	3p14, 3q13.3, 3q21	Unknown	1q21	1q21-q32
Characteristic of gene	Tumor suppressor gene	Proto-oncogene	Tumor suppressor gene	Tumor suppressor gene?	Tumor suppressor genes		Unknown		Tumor suppressor gene
Type of hereditary	Autosomal dominant	Autosomal dominant	Autosomal dominant	Autosomal dominant	Autosomal dominant	Initially balanced translocation between chromosome 3 and chromosome 6 or 8, then somatic lost of chromosome 3 and *VHL* mutations	Mostly autosomal dominant, also autosomal recessive		
Incidence of syndrome	1:36 000	1:10000000			1:6000	Some known families	Some known families		
Other signs of the syndrome than RCC	Retinal haemangioma, cerebellar or spinal haemangioblastoma, pheochromocytoma, renal or pancreatic cyst, endocrine pancreatic tumor	Breast cancer, pancreatic cancer, biliary tract cancer, lung cancer, malignant melanoma	Cutaneous and uterine leiomyomatosis, leiomyosarcoma, breast cancer, prostate cancer, bladder cancer	Cutaneous hair follicle bening tumor, pulmonary cyst, pneumothorax	Hamartoma, fibroma, renal angiomyolipoma, rhabdomyoma, hypopigmentation, epilepsy, mental retardation	Thyroid cancer, bladder cancer, pancreatic cancer, gastric cancer		Papillary thyroid carcinoma, papillary renal adenoma, renal oncocytoma	Parathyroid adenoma/carcinoma, ossifying jaw tumor, renal cyst, renal hamartoma, adult Wilms tumor
References	Zbar *et al.* 1999, Zbar *et al.* 1996	Schmidt *et al.* 1997	Koski *et al.* 2009, Lehtonen *et al.* 2006	Schmidt *et al.* 2001	Lendvay and Marshall 2003	Bonné *et al.* 2004, van Kessel *et al.* 1999	Woodward *et al.* 2008, Hemminki and Li 2004	Malchoff *et al.* 2000	Haven *et al.* 2000

2.1.3 SIGNS AND SYMPTOMS

Due to the location of the kidney deep in the retroperitoneum, RCC may progress unnoticed for a long period: the early stage of RCC is often asymptomatic. Symptoms from the tumor are noticeable when it invades adjacent structures or the kidney's collecting system.

Typical signs of RCC are microscopic or macroscopic hematuria (40-60% of patients), pain (40%) and palpable tumor (30%). The phenomenon of macroscopic hematuria, flank pain and palpable abdominal mass existing at the same time is called *the classic triad*. The classic triad is observed in mRCC (Cunningham 1938), and nowadays it occurs in 10% of patients (Jayson and Sanders 1998). *An inflammatory syndrome* with fever, anemia, erythrocytosis, thrombocytosis, increased serum acute-phase protein levels and hypoalbuminemia (Boxer *et al.* 1978) is frequently associated with RCC. Approximately one-third of RCC patients develop a *paraneoplastic syndrome*, which is caused by a polypeptide hormone production of the RCC or normal cells, or antibody produced in response to the tumor. The signs of paraneoplastic syndrome are, e.g. cachexia, hypertension, humoral hypercalcemia, hyperglycemia, increased erythropoietin (EPO), anemia, c-reactive protein (CRP) elevation and erythrocytosis (Kim *et al.* 2003, Blay *et al.* 1997, Gucalp 1992). Stauffer's syndrome, i.e. paraneoplastic cholestasis, (3-20%) is a non-metastatic hepatic dysfunction causing hepatosplenomegaly, fever, fatigue and weight loss (Blay *et al.* 1997). The etiology of Stauffer's syndrome is unknown. In addition, glomerulonephropathias (30%) and amyloidosis (4%) are observed in RCC patients (Rosenblum 1987).

2.1.4 NATURAL COURSE

Metastatic disease is seen in 20-30% of RCC patients at diagnosis (Janzen *et al.* 2003, Mc Nichols *et al.* 1981). Half of the patients diagnosed with local RCC will later have a recurrence of their cancer: two thirds within the first year (Janzen *et al.* 2003), and the majority within five years (Lam *et al.* 2005, McNichols *et al.* 1981). The risk for late recurrence, at over 10 years from nephrectomy, is at least 10% (McNichols *et al.* 1981). Typical sites of metastases in decreasing order of frequency are lungs, lymph nodes, bone, liver, adrenal gland, opposite kidney, and brain (Saitoh *et al.* 1982b), but metastases can be found in almost any organ, e.g. in thyroid gland, ureter and pancreas (Saitoh *et al.* 1982a).

Spontaneous regression has been suggested in approximately 1% of RCC patients, although some studies have reported higher percentages, from 4.4% to 6.6%, for selected patient groups (Gleave *et al.* 1998, Marcus *et al.* 1993). In a Cochrane meta-analysis, complete spontaneous remissions were observed in 3.3% of the assessed population (Coppin *et al.* 2007). Spontaneous regression has been

seen in both nephrectomized and in non-nephrectomized patients. It has most often occurred in RCC with pulmonary metastases, but also with extrapulmonary lesions, such as bone, liver, and central nervous system (CNS) lesions (Gleave *et al.* 1998, Lokich 1997). Additionally, non-progression of RCC at 12 months after nephrectomy has been observed in approximately 7% of patients (Oliver *et al.* 1989). Spontaneous regression has also been verified by biopsy-proven histological and radiological imaging of regressed lesions (Christophersen *et al.* 2006, Nakajima *et al.* 2006).

RCC is able to manipulate and suppress the body's natural immunity. The phenomenon of spontaneous regression reflects the ability of the host immune system to control the RCC tumor by inducing an immune response and enhancing antitumor immunity against the tumor. In previously untreated patients, lymphocytic infiltration in RCC tumors has been found. It is speculated that the rich supply of macrophages, lymphocytes, and immunoglobulin in the lung might suppress the metastases through host immune mechanisms (Freed *et al.* 1977). However, no enhancement of natural killer (NK) cells, lymphokine-activated killer cells or lymphocyte proliferative response has been observed (Abubakr *et al.* 1994), and the exact mechanisms are unclear.

2.1.5 IMAGING

Ultrasonography (US), computerized tomography (CT) and nuclear magnetic resonance imaging (MRI) are in routine clinical use for imaging renal tumors. The ability of ultrasonography to differentiate renal masses from renal cysts was realised in the 1970's. US has become the primary examination in patients with acute flank pain and hematuria, and to differentiate cystic from solid renal mass, but it is not reliable enough to screen multiple cysts in hereditary RCC (Tosaka *et al.* 1990). In the 1980's, CT was shown to accurately image renal masses and cysts (Gillenwater and Howards 1981), and proved to be a cost-effective and a less invasive technique than angiography. Nowadays, CT is the most sensitive imaging method for RCC. MRI became popular during the 1990's, and many advantages of MRI compared to CT have been observed. MRI has a multiplanar imaging capability, particularly in studying the vasculature and lymph node metastases, and it may detect subcentimeter renal lesions (Ergen *et al.* 2004, Hricak *et al.* 1988). Radiation exposure is avoided by using MRI, and it is a safe method for patients with renal insufficiency (Rofsky *et al.* 1991). Bone scintigraphy detects bone metastases about six months earlier than conventional x-rays (Ghanema *et al.* 2005).

Novel potential imaging techniques, such as positron emission tomography (PET) with iodine-124-labeled antibody chimeric G250 (Divgi *et al.* 2008) and immunoscintography with a monoclonal antibody G250 linked to 99m-technetium are being investigated (Oosterwijk and Debruyne 1995).

The frequent use of US, CT and MRI has increased incidental findings in approximately 10-60% of all RCC cases (Homma *et al.* 1995), and has improved the survival of RCC patients since the 1970's (Brenner and Hakulinen 2001). This and the use of immomodulators have increased the five-year survival of RCC patients in twenty years from 52% (1974-76) to 60% (1989-95) (Greenlee *et al.* 2000). Therefore, the development of a protocol for RCC routine screening in the general population has been proposed (Tsui *et al.* 2000b, Tosaka *et al.* 1990).

2.2 STAGING AND PROGNOSTIC FACTORS IN RCC

2.2.1 WHO AND HEIDELBERG CLASSIFICATIONS FOR TYPING OF RENAL TUMORS

The classification of renal tumors evolved in the 1980's. In 1981, the World Health Organization (WHO) classified epithelial renal tumors as renal cell adenoma and carcinoma (Mostofi 1981). Thoenes *et al.* classified renal tumors by tumor cell type, growth pattern, and grading of malignancy (Thoenes *et al.* 1986). In Heidelberg, in October 1996, the morphology was combined with genetic findings for a new classification, called the Heidelberg classification of renal tumors, in a workshop organized by the Union Internationale Contre le Cancer (UICC) and the American Joint Committee on Cancer (AJCC) (Kovacs *et al.* 1997, Störkel *et al.* 1997) (Table 3).

Table 3. The Heidelberg classification of epithelial renal tumors (According to Cheville *et al.* 2003, Amin *et al.* 2002, Zambrano *et al.* 1999, Kovacs *et al.* 1997, and Störkel *et al.* 1997).

Subtype according to Heidelberg classification	Incidence among all RCCs	Origin of the tumor type	Cell / tissue characteristics	Growth pattern	Five-year DSS for locally confined RCC
RCA, metanephric					
RCA, papillary		Proximal tubules			
RCA, oncocytic		Intercalated cells of collecting ducts	Round and vesicular cells with granular eosinophilic cytoplasm	Solid	
RCC, conventional / clear cell	70-80%	Proximal tubules	Clear cytoplasm; foci with eosinophilic cytoplasm are common	Solid, trabecular, tubular, cystic, rare papillary	69-76%
RCC, papillary*	10-15%	Proximal tubules	Small cells with scanty cytoplasm to large cells with abundant cytoplasm; basophilic, eosinophilic or pale staining	Papillary, tubulopapillary or solid growth pattern	86-87%
RCC, papillary type 1			Type1: Basophilic, smaller cells with less cytoplasm		
RCC, papillary type 2			Type2: Eosinophilic, larger cells with more abundant cytoplasm		
RCC, chromophobe	3-5%	Intercalated cells of collecting ducts	Pale or eosinophilic granular cytoplasm, that stains blue with Hale's colloidal iron stain	Large solid sheets	87-100%
RCC, collecting duct (includes medullary RCC)	Very rare	Medulla of collecting ducts	Surrounded by demoplastic stroma	Irregular, channels-like structures	
RCC, unclassified	4-5%	Unclassified			24%

RCA=renal cell adenoma, RCC= renal cell carcinoma, DSS=disease-specific survival
*According to Delahunt and Eble (1997), papillary RCC should be divided into two subgroups: papillary RCC 1 and papillary RCC 2.

Morphological differences exist between the 1998 WHO re-classification and the Heidelberg classification (Mostofi *et al.* 1998, Kovacs *et al.* 1997, Störkel *et al.* 1997). The former included granular cell and spindle cell carcinomas, the latter does not as the granular phenotype is a component of several RCC tumor types (Störkel *et al.* 1997), and the spindle cell type can develop in all types of RCCs as a result of the dedifferentiation process (Störkel *et al.* 1997). In 2004, WHO published the reassessed classification which is now based on both genetic and pathological abnormalities (Eble *et al.* 2004) (Table 4).

Table 4. The WHO 2004 histological classification of renal cell tumors (According to Lopez-Beltran *et al.* 2006).

RCC subtype according to the 2004 WHO classification	Incidence among all RCCs	Cell / tissue characteristics	Growth pattern	Genetic
Clear cell RCC	75%	Clear cytoplasm; cells with eosinophilic cytoplasm occasinaly	Solid, tubular, cystic, rare papillae	-3p, +5q22, -6q, -8p, -9p, -14q
Multilocular clear cell RCC	Rare	Clear cytoplasm, small dark nuclei	Cystic, no solid component	VHL gene mutation
Papillary RCC	10%	Type1 (basophilic) or type 2 (eosinophilic)	Tubulo-papillary, solid	-Y , +3q, +7, +8, +12, +16, +17, +20
Chromophobe RCC	5%	Pale or eosinophilic granular cytoplasm	Solid	-1, -2, -6, -10, -17, -21
Carcinoma of the collecting ducts of Bellini	1%	Eosinophilic cytoplasm	Irregular channels	-1q, -6p, -8p, -13q, -21q, -3p
Renal medullary carcinoma	Rare	Eosinophilic cytoplasm	Reticular pattern	Unknown
Xp11 translocation carcinomas	Rare	Clear and eosinophilic cytoplasm	Tubulo-papillary	t (X; 1) (p11.2; q21), t (X; 17) (p11.2; q25), Other
Carcinoma associated with neuroblastoma	Rare	Eosinophilic cells with oncocytoid features	Solid	Allelic imbalance at 20q13
Mucinous tubular and spindle cell carcinoma	Rare	Tubules, extracellular mucin and spindle cells	Solid	-1, -4, -6, +7, -8, +11, -13, -14, +16, +17
RCC, unclassified	4-6%	Variable, sarcomatoid	Solid	Unknown

The Heidelberg classification is the basis of future classification systems in which the genetic and molecular properties, reflecting biological behavior rather than descriptive appearance, are the main criteria for classifying renal tumors (Moch 2000, Kovacs *et al.* 1997, Störkel *et al.* 1997). Each pathomorphological epithelial renal tumor entity should express specific chromosomal aberrations that completely reflect the pathomorphological phenotypes (Argani *et al.* 2007, Brunelli *et al.* 2003). Immunohistochemistry can be helpful in the differential diagnosis of renal neoplasms by characterizing the tumor entity according to a special antigen pattern (Magyarlaki *et al.* 2001). The Heidelberg classification identifies, for conventional, papillary, chromophobe and collecting duct RCCs, a strict correlation between the morphological phenotype and the complement of alterations evidenced by cytogenetic analysis of the neoplastic karyotype (Antonelli *et al.* 2003, Bodmer *et al.* 2002) (Figure 1). Progress in our knowledge of genetic alterations leads to new suggestions for RCC entities (Eble 2003). For instance, Gobbo *et al.* (2008) suggested that clear cell papillary RCC should be identified as an entity of its own. Different subgroups have different responses to therapy and outcome. With the progress of research, the Heidelberg classification may lead to more specific treatments in different subgroups of RCC patients.

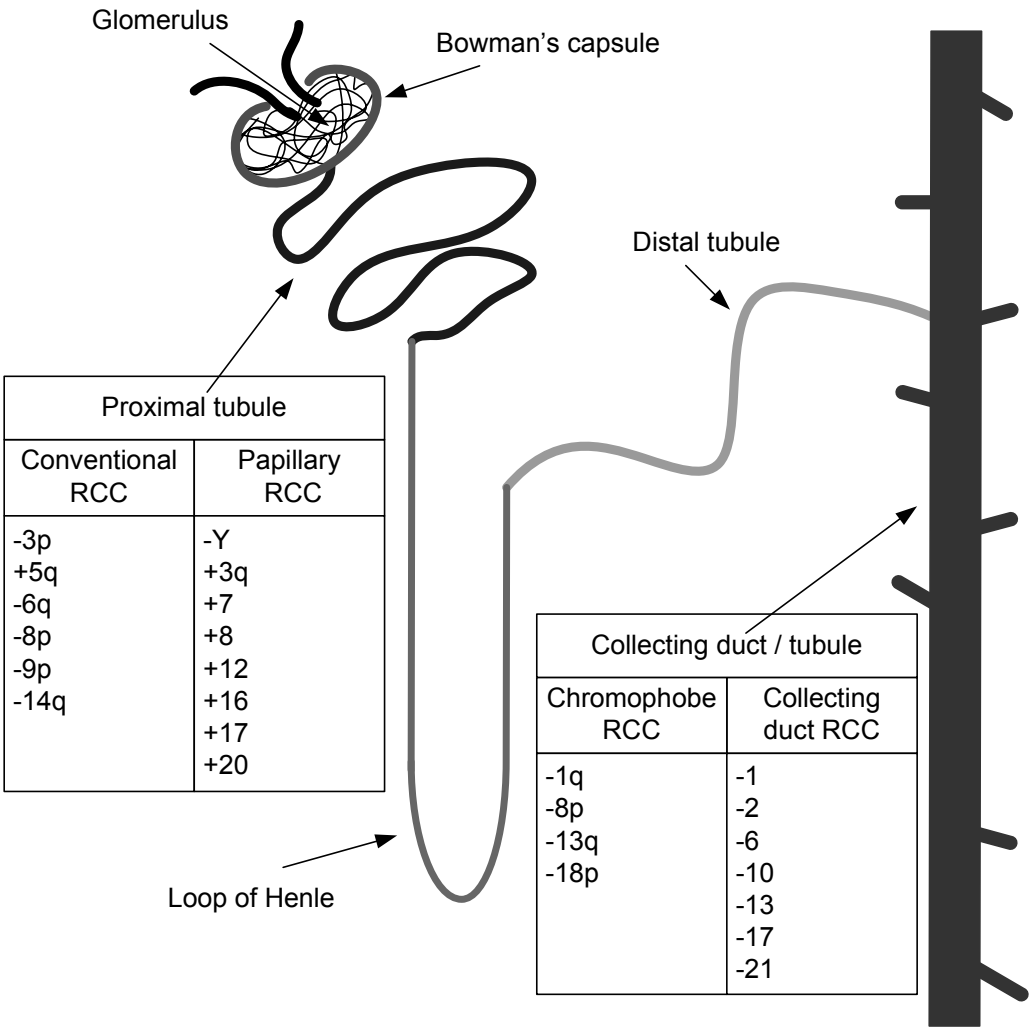

Figure 1. The genetic changes that characterize the different RCC subtypes according to the Heidelberg classification (Modified from Bodmer *et al.* 2002).
The number reflects the chromosome in which its genetic aberration is located.
- means loss of function.
+ means gain of function.
p is the short arm of the chromosome.
q is the long arm of the chromosome.

Conventional RCC represents 70-80% of all RCCs. Approximately 75% of sporadic conventional RCCs contain mutations in VHL, the tumor suppressor gene in the short arm of chromosome 3 (Maxwell *et al.* 1999, Gnarra *et al.* 1994), of which 50% show loss of heterozygosity (LOH) (Kovacs *et al.* 1997, Gnarra *et al.* 1994) and 10-20% silencing of the wild-type allele by promoter hypermethylation (Herman *et al.* 1994). Conventional RCC is genetically more homogeneous than the other types of RCC (Alimov *et al.* 2004). These tumors tend to be more symptomatic and have metastases more commonly than papillary and chromophobe RCCs (Amin *et al.* 2002).

Conventional RCC is associated with the best response to and most favorable outcome from IFN-α therapy compared to the other RCC subgroups (Motzer *et al.* 2002a). Cystic conventional RCC is often both low grade and low stage with a generally better prognosis to other conventional RCCs (Han *et al.* 2004).

Papillary RCC consists of two subtypes which are biologically and clinically distinct (Yang *et al.* 2005, Delahunt and Eble 1997).

Collecting duct RCC, a rare entity, is highly aggressive and the prognosis in metastatic disease is from some weeks to some months (Antonelli *et al.* 2003). Patients with collecting duct RCC are also at risk of transitional cell carcinoma / bladder cancer (Antonelli *et al.* 2003), which may reflect the common embryologic origin of collecting duct and urothelial cells. The positivity for *Ulex europaeus* lectin staining is characteristic of collecting duct RCCs (Delahunt and Eble 1997). A High incidence of Human Epidermal Growth Factor Receptor 2 (Her-2, c-erbB2) oncogene amplification and strong MET-protein expression has been found in collecting duct RCCs (Choi *et al.* 2006, Matei *et al.* 2005).

The *sarcomatoid component* may occur in all histological subtypes of RCCs and it associates with synchronous distant metastases and very short median survival of 6.6 to 8.0 months for stage II-IV RCC (Cheville *et al.* 2003, Cangiano *et al.* 1999)

Unclassified RCC includes those tumors that do not fit into other classes according to the Heidelberg classification. Unclassified RCCs are usually highly aggressive tumors (Zisman *et al.* 2002a).

The prognostic power of the Heidelberg classification has been investigated. It does not have independent prognostic ability, and thus it should not be considered as a major prognostic variable comparable to T-stage and histopathological tumor grade (Patard *et al.* 2005).

2.2.2 TNM CLASSIFICATION SYSTEM FOR PATHOLOGICAL TUMOR STAGING

The first documented staging system for RCC, based on physical characteristics and tumor spread was published in 1958 (Flocks and Kadesky 1958). In the 1960's, it was modified by Robson *et al*, with the addition of tumor venous invasion (Table 5) (Robson *et al.* 2002). The poor correlation between the different Robson stages and survival, e.g. Robson stage II and III tumors had equal survival, led to the recommendation to use the TNM (tumor, node, metastases) system as a staging system for the extent of the tumor spread. In the Union International Contre Le Cancer's (UICC) TNM system, T means tumor, N node, and M metastasis. Since 1978, the TNM classification

system has integrated characteristics such as tumor size, vascular involvement, nodal spread and distant metastases (Bassil *et al.* 1985, Harmen 1978). In 1983, the pathological TNM (pTNM) classification system for renal carcinomas divided Robson stage III into subgroups. In 1992, Dinney *et al.* reported that after 10-15 years' follow-up, survival rates did not differ significantly between RCC tumors classified as T1-3b N0 M0 at the time of initial diagnosis.

Table 5. The Robson staging system for RCC in the 60's.

Stage grouping	
Stage I	Tumor confined to kidney
Stage II	Tumor invades perinephric fat but confined to Gerota's fascia or adrenal
Stage IIIa	Tumor invades renal vein or inferior vena cava
Stage IIIb	Tumor involves regional lymph nodes
Stage IIIc	Tumor involves both local vessels and lymph nodes
Stage IVa	Tumor involves local organs (i.e. colon, pancreas)
Stage IVb	Distant metastases

The pTNM classification system was updated by the UICC and the AJCC in 1997 (Table 6, Table 7). The cut-off between T1 and T2 tumors was increased from 2.5 cm to 7 cm, in order to increase the difference in survival from these two tumor types. Also the definitions of tumor thrombus involvement and nodal spread were changed (Guinan *et al.* 1997). Analysis of outcome in nephrectomized patients has shown that the 1997 TNM-system cut-off point between T1 and T2 tumors is too high, and a cut-off point of 4.5 – 5.0 cm has been suggested (Elmore *et al.* 2003, Zisman *et al.* 2001a). In 2002, the pTNM classification system was revised: T1 was divided into T1a and T1b by a cut-off point of 4 cm, according to the suitability for partial nephrectomy, and prognostication (Sobin and Wittekind 2002, Guinan *et al.* 1997). The cut-off point between T1 and T2 was not changed. A uniform staging classification, the TNM staging system, has improved the division of patients into radical or partial nephrectomy candidates (Janzen *et al.* 2003, Javidan *et al.* 1999).

Table 6. UICC stage grouping according to the 2002 TNM classification.

Stage grouping			
Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage III	T3	N0	M0
	T1, T2, T3	N1	M0
Stage IV	T4	N0, N1	M0
	Any T	N2	M0
	Any T	Any N	M1

Table 7. The UICC TNM classification for staging RCC tumors (ICD-O C64). Comparison of different modifications. (According to UICC TNM classification of malignant tumors, 4th, 5th, and 6th ed.) (Hermanek and Sobin 1987, Sobin and Fleming 1997, Sobin and Wittekind 2002)

	1987 TNM classification	1997 TNM classification	2002 TNM classification
Primary tumor (T)			
TX	Can not be assessed		
T0	No evidence		
T1	≤ 2.5 cm and confined to the kidney	≤ 7.0 cm in greatest dimension, limited to the kidney	
T1a	Not used		≤ 4 cm
T1b	Not used		> 4 cm but ≤ 7 cm
T2	> 2.5 cm and confined to the kidney	> 7.0 cm in greatest dimension, limited to the kidney	
T3	Extends into adrenal gland, perinephric fat, major vessels	Extends into adrenal gland, perinephric tissues, major veins	
T3a	Extends into adrenal gland, perinephric fat	Extends into adrenal gland, perinephric tissue	Extends into adrenal gland, perinephric tissues[1]
T3b	Extends into inferior vena cava	Extends into renal vein(s), vena cava	Extends into renal vein(s)[2], vena cava or its wall below diaphragm
T3c	Extends into vena cava above the diaphragm		Extends into vena cava or its wall above diaphragm
T4	Directly extends beyond Gerota's fascia		
Regional lymph nodes (N)			
NX	Can not be assessed		
N0	No involvement		
N1	Metastasis into a single lymph node < 2 cm	Metastasis into a single regional lymph node	
N2	Metastasis into a single lymph node 2 - 5 cm, multiple nodes < 5 cm	Metastasis into more than one regional lymph node	
N3	Metastasis into lymph nodes > 5 cm	Not used	
Distant metastases (M)			
MX	Cannot be assessed		
M0	No		
M1	Yes		

[1] Includes renal sinus (peripelvic) fat.
[2]Includes segmental (muscle-containing) branches.

Pathological tumor stage (T-stage) has been observed to be the most important factor in predicting the survival of patients who have undergone nephrectomy (Delahunt *et al.* 2002, Robson *et al.* 2002, Tsui *et al.* 2000a). The observed five-year survival is 83% for stage T1, 57% for T2, 42% for T3, and 28% for T4 (Tsui *et al.* 2000a). For patients with stage I disease (tumor confined to the kidney) the five-year survival is approximately 90%, and for those with stage I and chromophobe RCC it is almost 100% (Zisman *et al.* 2001b). The five-year survival rate for stage III disease is approximately 50% (Zisman *et al.* 2001b). There is an 80% difference in survival rates between patients with local disease compared to those with advanced disease and distant metastases (ACS 2004). Additionally, in a retrospective review of 2 473 RCC patients from 1975 - 1985, regardless of T-stage, tumor size was observed to have an inverse association with survival (Guinan *et al.*

1995). However, this kind of association has not been found in all studies (Lopez Hänninen *et al.* 1996).

T-stage can be used in estimating the correct duration and frequency of follow-up of RCC patients after nephrectomy. RCC with a diameter of less than 3.0 cm grows slowly; only 2.5% have metastases during the first three years (Bosniak *et al.* 1995). Therefore, in the treatment of those in whom surgery is contraindicated, careful monitoring (watchful waiting) by computed tomography (CT scan) may be used (Roberts *et al.* 2005, Bosniak *et al.* 1995). Additionally, a high T-stage has been used as an inclusion criterion for adjuvant treatments in trials (Atzpodien *et al.* 2005, Repmann *et al.* 2003). The updated TNM system is a useful tool correlating with survival and disease-free periods, although modifications in the TNM system may cause difficulty in comparing outcome data in different studies (Belldegrun *et al.* 1999, Störkel *et al.* 1989).

Several studies have been published on the prognostic power of the 2002 TNM classification in RCC patients (Ficarra *et al.* 2007, Gilbert *et al.* 2006). The TNM 2002 classification is useful, but some adjustments have been proposed, particularly concerning the tumor size cut-off (Bonsib 2005, Ficarra *et al.* 2005, Wunderlich *et al.* 2004, Zisman *et al.* 2001b), assessment of the invasion of the renal sinus fat tissue (Bonsib 2005), assessment of renal vein or vena cava inferior invasion (Kim *et al.* 2004a), and invasion of the ipsilateral adrenal gland (Kirkali *et al.* 2007a).

Bonsib (2005) observed that the incidence of renal sinus fat tissue invasion is higher when tumors exceed 4 cm (Bonsib 2005), and that T1b-T2 conventional RCC cases are rare if careful evaluation of sinus invasion is performed (Bonsib 2005). According to the study of Ficarra *et al.* (2004), the 2002 TNM staging system does not seem to be able to differentiate cancer-specific survival between pT1b and pT2 RCC (Ficarra *et al.* 2004). Ficarra *et al.* proposed reclassification of pT3-4 RCC. Similar outcomes in patients with stage T3a tumors and patients with tumors confined to the renal capsule (T1-2) have been reported (Gilbert *et al.* 2006, Roberts *et al.* 2005). RCC patients with microscopic vascular invasion have been associated with poorer prognosis (Kirkali and van Poppel 2007b, Van Poppel *et al.* 1997). Approximately 25% of RCCs extend into the renal vein or the inferior vena cava (Hatcher *et al.* 1991). A thrombus in the inferior vena cava is a prognosticator for cancer recurrence (Kirkali and van Poppel 2007b). A difference in five-year survival has been observed between RCC with a free-floating tumor thrombus in the inferior vena cava (69%) and RCC that has invaded the inferior vena cava wall (25%) (Hatcher *et al.*1991). A complete inferior vena cava (IVC) thrombectomy, even in a metastatic setting, may result in improved survival, and it also leads to better quality of life (Kirkali and van Poppel 2007b). Renal vein extension may be a factor related to concomitant positive lymph node status or distant metastases (Ljungberg *et al.* 1995, Selli *et al.* 1983, Skinner *et al.* 1971). However, the therapeutic

value of lymph node dissection remains unproven (Mickish 1999). Renal pelvis invasion seems to associate with higher stage tumors with their attendant prognosis (McNichols *et al.* 1981). Collecting system invasion has been associated with decreased survival in low stage tumors (Palapattu *et al.* 2003).

2.2.3 WHO CLASSIFICATION FOR HISTOPATHOLOGICAL TUMOR GRADING

The first grading system was reported in 1932 (Hand and Broders 1932). Later, many grading systems were created by, e.g. WHO (Table 8) with revision in 2004 (Eble *et al.* 2004), Fuhrman *et al.* in 1982 (Table 9), and Thoenes *et al.* in 1986. None of the tumor garding systems has gained universal acceptance (Kanamaru *et al.* 2001, Medeiros *et al.* 1997).

In grading systems, the major criteria are nuclear and nucleolar appearances, while in some systems, tumor architecture and cell type is also included (Mostofi *et al.* 1998, Goldstein 1997, Fuhrman *et al.* 1982, Syrjänen and Hjelt 1978, Skinner *et al.* 1971). The WHO grading system is based on the size and prominence of nucleoli (Eble *et al.* 2004, Mostofi *et al.* 1998) (Table 8), while the Fuhrman grading system is based on nuclear size, shape, and presence or absence of nucleoli (Fuhrman *et al.* 1982). The WHO grading system contains three grades, whereas the Fuhrman contains four.

Table 8. The WHO classification 1998 / 2004 for histopathological tumor grading.

Grade	WHO
1	Anaplasi and nuclei are smaller than in non-neoplastic tubule
2	Anaplasi and nuclei are same size as in non-neoplastic tubule
3	Anaplasi and nuclei are larger than in non-neoplastic tubule

Additionally, the percentage of sarcomatoid lesions is analyzed.

Table 9. The Fuhrman classification for histopathological tumor grading.

Grade	Fuhrman
1	Nuclei are small, round and uniform (10um) with inconspicuous or absent nucleoli.
2	Nuclei are slightly irregular (15 um) with small nucleoli
3	Nuclei are very irregular (20um) with large and prominent nucleoli
4	Nuclei are large and pleomorphic often poly-lobed and bizarre (>20um)

Several studies have failed to demonstrate any statistically significant differences in the survival of patients with different grades, when all three or four grades are analyzed separately (Uchida *et al.* 2002, Kanamaru *et al.* 2001, Rioux-Leclercq *et al.* 2000, Usubutyn *et al.* 1998, Selli *et al.* 1983). In one study, the median five-year disease-specific survival (DSS) was 94% for G1, 86% for G2, 59% for G3, and 31% for G4 (Ficarra *et al.* 2001). In a Scandinavian study, the five-year DSS rate was 87% for G1, 71% for G2, 46% for G3, and 15% for G4, when the study included consecutive patients from 1971 to 2000 (Gudbjartsson *et al.* 2005).

Currently, different grading systems are utilized at different institutions. Tumor-grading systems have been criticized because of their subjectivity in tumor evaluations (Lanigan *et al.* 1994). Therefore, comparison of different patient cases with respect to histopathological grade is difficult. More quantitative measures which describe the size or the shape of the nuclei have been requested by pathologists. Nuclear roundness (Delahunt *et al.* 1994) and a measure of the median nuclear volume (MNV) (Soda *et al.* 1999) have been found to be independent prognostic factors in a few studies. In 1997, an international consensus conference on RCC by UICC and AJCC outlined recommendations for the grading of RCC (Goldstein 1997): the grading system should be based on standardized and reproducible criteria that reflect the heterogeneity of nuclear and nucleolar features within a tumor, and each grade should result in significant differences in patient outcome. Recently again, a joint group of urologists and pathologists has published a proposal that the criteria for nuclear grading should be different for the subtypes of RCC according to the Heidelberg classification (Paner *et al.* 2006). The chromophobe type typically consists of irregular nuclei and prominent nucleoli, and therefore, a new classification model for chromophobe RCC has been proposed (Paner *et al.* 2006). Additionally, reducing the grades in the Fuhrman system has been proposed, for better outcome stratification (Rioux-Leclercq *et al.* 2007, Lohse *et al.* 2002, Bretheau *et al.* 1995).

2.2.4 PROGNOSTIC MODELS

RCC is a heterogeneous disease: the prognosis differs even within the same stage and grade. Prognostic factors aim to specify the diagnosis, staging and prognosis, and they may help to determine the follow-up. Additionally, prognostic factors are used in clinical trial design, in interpretation, and they may guide targeted cancer therapies. Prognostic models, anagrams and nomograms, have been developed to find those nephrectomized RCC patients who potentially have a long-term recurrence-free interval and survival, as well as those mRCC patients who have long-term survival.

2.2.4.1 PROGNOSTIC MODELS IN LOCALIZED DISEASE

Tumor size and grade are the most important prognostic factors for the survival of RCC patients with a locally confined disease. Table 10 shows the prognostic algorithms and nomograms for survival in RCC.

The *MSKCC nomogram* was developed for use in predicting disease recurrence after nephrectomy in localized RCC patients, and it consisted of TNM stage, tumor size, histology, and symptoms at presentation (Kattan *et al.* 2001). The MSKCC nomogram was reassessed in 2005. It focused on

conventional RCC and also included Fuhrman grade, histological necrosis, and microvascular invasion (Sorbellini *et al.* 2005).

At the Mayo Clinic, the *SSIGN-nomogram* was developed after Frank *et al.* (2002) found the following independent prognostic factors: TNM staging system, tumor size, nuclear grade and coagulative tumor necrosis. In the SSIGN nomogram, scores are presented as follows: +1 for pT2, +2 for pT3, +2 for pN1 or pN2, +4 for pM1, +2 for tumor size ≥ 5 cm, +1 for grade 3, +3 for grade 4, and +2 for tumor necrosis. Five-year cancer-specific survival rates were 99.4% for scores 0 to 1, and 7.4% for score 10. SSIGN has been validated and found to have a high degree of prognostic accuracy (Ficarra *et al.* 2007).

According to Lungberg *et al.* (1999), no follow-up in patients with diploid pT1-2 tumors or with aneuploid pT1 tumors of < 5 cm is needed.

Table 10. Prognostic algorithms and nomograms for survival in RCC.

Reference	Year	No. of Patients	Tumor Subtype	Prognostic Factors	Prognostic Information
Ljungberg *et al.* (Umeå)	1999	187	All	T-stage, DNA ploidy in T1-T2 tumors	Recurrence, Survival
Tsui *et al.* (2000a). (UCLA)	2000	643	All	TNM stage, nuclear grade, performance status	Survival
Kattan *et al.* (MSKCC)	2001	601	All	TNM stage, tumor size, histology, symptoms	Recurrence
Zisman *et al.* (2002b) (UCLA)	2002	292	All	Nuclear grade, performance status	Survival
Frank *et al.* (SSIGN, at Mayo Clinic)	2002	1801	Conventional, also metastatic	TNM stage, tumor size, nuclear grade, histologic necrosis	Recurrence
Patard *et al.* (Rennes University Hospital)	2004	4202	All	TNM stage, nuclear grade, performance status	Survival
Sorbellini *et al.* (MSKCC)	2005	701	Conventional	TNM stage, tumor size, nuclear grade, histologic necrosis, microvascular invasion, symptoms	Recurrence

MSKCC = Memorial Sloan Kettering Cancer Center
UCLA = University of California

2.2.4.2 PROGNOSTIC MODELS IN METASTASIZED DISEASE

Therapies for mRCC cause a wide variety of adverse effects, which reduce the quality of life. Determining the prognostic factors for survival in mRCC patients is valuable in directing therapy for those patients who would benefit from it.

In mRCC, many independent prognosticators have been reported. The most often presented factors are performance status, time to metastases, number of metastatic sites and prior nephrectomy. Several models have been developed for predicting the likelihood of response to therapy and to predict survival. Table 11 and Table 12 show the prognostic algorithms and nomograms in mRCC for response to treatment and long-term survival.

Table 11. Prognostic algorithms and nomograms for survival in mRCC between 1980 and 1999.

Reference	Year	No. of Patients	Therapy Administered	Tumor Subtype	Prognostic Factors	Prognostic Information
Maldazys and deKernion (UCLA)	1986	181	Chemotherapy	All	Performance status, disease-free interval, nephrectomy status, metastatic site	Survival
Elson *et al.* (ECOG)	1988	610	Unspecified	All	Performance status, time from diagnosis to treatment, nephrectomy status, no. of metastatic sites	Survival
de Forges *et al.* (Institut Gustave Roussy)	1988	134	Unspecified	All	Disease-free interval, presence of liver metastasis, no. and size of lung metastases, ESR / weight loss	Survival
Neves *et al.* (Mayo Clinic)	1988	158	Unspecified	All	Grade of the primary lesion, weight loss, no. of metastatic sites, nephrectomy status	Survival
Palmer *et al.* (Cetus)	1992	327	IL-2	All	Performance status, time from diagnosis to treatment, no. of metastatic sites	Survival
Fosså *et al.* (Multi-institutional study)	1994	295	IFN-α, chemotherapy	All	Performance status, ESR, ≤10% weight loss	Survival
Mani *et al.* (YCC)	1995	84	IFN-α, IFN-β, IFN-γ, IL-2	All	Performance status, sarcomatoid histology, bone metastases	Survival
Lopez Hänninen *et al.* (Medizinische Hochschule Hannover)	1996	215	IFN-α + IL-2 ± 5-FU ± 13CRA	All	ESR, LDH, neutrophil count, hemoglobin, extrapulmonary metastases, bone metastases	Survival
Motzer *et al.* (MSKCC)	1999	670	Cytokines, chemotherapy	All	Performance status, nephrectomy status, LDH, hemoglobin, corrected calcium	Survival

13CRA = 13-cis-retinoid acid
ECOG = the Eastern Cooperative Oncology Group
ESR=erythrocyte sedimentation rate
LDH=lactate dehydrogenase
MSKCC = Memorial Sloan Kettering Cancer Center
UCLA = University of California,
YCC = Yale Comprehensive Cancer Center

Table 12. Prognostic algorithms and nomograms for survival in mRCC between 2000 and 2008

Reference	Year	No. of Patients	Therapy Administered	Tumor Subtype	Prognostic Factors	Prognostic Information
Motzer *et al.* (MSKCC) (2002b)	2002	463	IFN-α	All	Performance status, time from diagnosis to start of therapy, LDH, hemoglobin, corrected calcium	Survival
Zisman *et al.* (UCLA)	2002	262	IL-2 or IFN-α (197 pts), other (65 pts)	All	T-stage, nodal involvement, nuclear grade, no. of symptoms, immunotherapy	Survival
Négrier *et al.* (Group Francais d'Immunotherapie)	2002	782	IFN-α ± IL-2	All	Performance status, no. of metastatic sites, disease-free interval, signs of inflammation, hemoglobin	Survival, rapid progression
Atzpodien (Medizinische Hochschule Hannover)	2003	425	IFN-α + IL-2 ± 5-FU ± 13CRA	All	Neutrophil count , LDH, CRP, time from diagnosis to start of therapy, no. of metastatic sites, bone metastases	Survival
Motzer *et al.* (MSKCC)	2004	251	New agents	All, if cytokine refractory disease	Performance status, hemoglobin, corrected calcium	Survival for those who enter clinical trials of new agents
Choueiri *et al.* (Cleveland Clinic Foundation)	2007	358	IFN-α ± IL-2 ± chemotherapy	All	Performance status, hemoglobin, no. of metastatic sites, involved kidney of primary tumor	Long-term survival
Cho *et al* (Yonsei University)	2008	197	Immunotherapy	All	Performance status, N stage, no. of metastatic sites, sarcomatoid differentiation, liver metastasis	Survival
Motzer *et al.* (MSKCC) (2008a)	2008	375	Sunitinib	Conventi onal RCC	Performance status, time from diagnosis to start of therapy, nephrectomy status, no. of metastatic sites, presence of liver or lung metastases, LDH, corrected calcium, hemoglobin, alkaline phosphatase, thrombosytosis	Probability of 12-month progression-free survival

LDH=lactate dehydrogenase
MSKCC = Memorial Sloan Kettering Cancer Center
UCLA = University of California,

One of the first publications on prognostic factors in mRCC was published in 1986. At *UCLA,* Maldazys and deKernion (1986) found that performance status, disease-free interval and presence of pulmonary metastases should be included as independent factors for predicting outcome of mRCC patients. Elson *et al.* (1988) found that age and sex were not independent prognostic factors. A scoring system was developed to classify patients with metastatic disease into five categories based on ECOG performance status (ECOG-PS), time from diagnosis to metastasis, prior chemotherapy, weight loss, and number of metastatic sites.

UCLA developed the mathematical equations for estimating survival after radical nephrectomy for education and counseling purposes (Patard *et al.* 2004, Zisman *et al.* 2002b). In mRCC patients, T-stage, nodal involvement, nuclear grade, number of symptoms, and previous immunotherapy were observed to be independent prognostic factors for survival, while in non-mRCC patients, only nuclear grade and performance status were independent prognostic factors (Zisman *et al.* 2002b).

In *a multi-institutional study,* prognostic factors and survival were analyzed in mRCC patients treated with either chemotherapy or IFN-α (Fosså *et al.* 1994). In those RCC patients with favorable risk, the three-year survival rate was 48% for IFN-α, and 15% for chemotherapy, but in those mRCC patients with poor or intermediate risk, no significant difference in survival was observed.

In the study of the *Group Francais d'Immunotherapie,* the following prognostic factors were observed: ECOG performance status, number of metastatic sites, disease-free interval, biological signs of inflammation, and hemoglobin level (Négrier *et al. 2*002). Négrier *et al.* (2002) also found four independent factors for rapid disease progression. These were presence of hepatic metastases, less than one-year disease-free interval from primary RCC tumor, more than one metastatic site, and elevated neuthrophil counts. If three of these factors were found, the probability of rapid progression within three months was more than 80%, regardless of the treatment.

MSKCC models for mRCC are among the most widely used models. In the first analysis, 670 mRCC patients were treated with chemotherapy or immunotherapy (Motzer *et al.* 1999). Independent prognostic factors for survival were: Karnofsky performance status less than 80%, absence of prior nephrectomy, baseline levels of serum lactate dehydrogenase more than 1.5 times the upper normal limit, hemoglobin below the lower limit of normality, and corrected serum calcium above 10 mg/dL (Motzer *et al.* 1999). The survival in the poor risk group was less than six months with both chemotherapy and IFN-α therapy (Motzer *et al. 1*999), and the difference in survival between chemotherapy and IFN-α therapy was greater in those patients who belonged to the groups of intermediate or favorable risk (Motzer *et al.* 1999). Motzer *et al.* (1999) also found that survival was greater for those RCC patients who were treated in the 1990's compared to those treated in earlier years.

After the establishment of the role of cytoreductive nephrectomy in mRCC patients prior to IFN-α therapy (Flanigan *et al.* 2001, Mickisch *et al.* 2001), the MSKCC model was reassessed (Motzer *et al.* 2002b). The new prognostic factor was an interval from diagnosis to treatment of less than one year. In the study of 463 patients treated with IFN-α as first-line therapy, median survival time was 13 months, and time to progression 4.7 months. Patients were categorized into three different risk

groups according to the number of risk factors predictive of short survival: zero scores for the group with favorable risk, one or two scores for the group with intermediate risk, and three or more scores for the group with poor risk. The patients were classified into three groups with three different predictive outcomes. Median survival time was 30 months for the group with favorable risk, 14 months for the group with intermediate risk, and five months for the group with poor risk. This model was validated independently at the Cleveland Clinic Foundation (Mekhail *et al.* 2005); it has been used in phase III trial design and in evaluation of the new, targeted agents (Escudier *et al.* 2007b, Hudes *et al.* 2007, Motzer *et al.* 2007).

Motzer *et al.* (2004) from MSKCC created a new model for those mRCC patients who have received prior cytokine therapy and are candidates for clinical trials of novel targeted agents as second line therapy. They found that poor performance status, low level of hemoglobin, and high level of corrected calcium represented poor prognosis. This model can be used in the clinical trial design of phase III trials as stratification factors and in interpreting the outcome of phase II trials.

A new MSKCC model for predicting the probability of 12-month progression-free survival in patients who receive sunitinib was published in 2008 (Motzer *et al.* 2008a).

Choueiri *et al.* (2007) at *Cleveland Clinic Foundation* developed a simple scoring system with three different risk groups for determining the long-term survival. They found in their study that independent prognostic factors for short-term survival were ECOG performance status higher than zero, number of involved metastatic sites more than two, baseline levels of hemoglobin below the lower limit of normal, and primary RCC in the left kidney in previously untreated mRCC patients who are treated with IFN-α or IL-2 with or without chemotherapy. In the study, 63% of all the patients were short-term survivors. The other two groups represent long-term survivors.

2.3 BIOMARKERS RELATED TO MOLECULAR MECHANISMS IN RCC

Carcinogenesis is a multistep process in which mutations accumulate with no proper activation of repair mechanisms. According to current knowledge, mistakes in deoxyribonucleic acid (DNA) repair are an important reason for carcinogenesis. Additionally, if cancer occurs, some defect in the immune system has taken place.

The following factors lead to carcinogenesis: 1) self-sufficient production of growth signals activated by oncogenes, 2) insensitivity to negative growth-inhibitory signals which leads to inactivation of tumor suppressor genes, 3) ability to avoid apoptosis, 4) limitless proliferative capacity, 5) induction of angiogenesis, and 6) ability to invade new organs and metastasize

(Hanahan and Weinberg 2000). Oncogenes promote growth as an accelerator, and tumor suppressor genes inhibit growth as a brake in the cell cycle. The activation of oncogenes and the inactivation of tumor suppressor genes are important phenomena in cancer development.

2.3.1 pVHL, VON HIPPEL-LINDAU PROTEIN, MODULATOR OF HYPOXIC RESPONSE

pVHL, a tumor suppressor gene product, is expressed especially in the kidney's proximal renal tubule (Corless *et al.* 1997, Iliopoulos *et al.* 1995). Approximately 61-75% of sporadic conventional RCCs contain mutations in *VHL*, in the short arm of chromosome 3 (3p25-26) (van Houwelingen *et al.* 2005, Gnarra *et al.* 1994, Maxwell *et al.* 1999), of which 50% show loss of heterozygosity (LOH) (Kovacs *et al.* 1997, Gnarra *et al.* 1994) and 10-20% silencing of the wild-type allele by promoter hypermethylation (Herman *et al.* 1994). VHL is associated with carcinogenesis.

The function of pVHL is ubiquitylation of hypoxia-inducible factor (HIF); therefore, it modulates the hypoxic response; VHL protein can bind to hypoxia inducible factor-1 alpha (HIF-1α) and target this factor for destruction in the presence of oxygen. HIF in turn controls the expression of several proteins, including carbonic anhydrase 9 (CA9) and proteins involved in angiogenesis, i.e. vascular endothelial growth factor (VEGF) and EPO, via oxygen-dependent ubiquitination (van Houwelingen *et al.* 2005, George and Kaelin 2003). Normally, VHL down regulates vascular endothelial growth factor (VEGF) by different pathways. In VHL-defective cancer cells, increased concentrations of VEGF and EPO are observed.

2.3.2 CA9, HYPOXIA ASSOCIATED ENZYME

CA9, a member of the carbonic anhydrase family, is suggested to play a role in the regulation of cell proliferation in response to hypoxic conditions. Low CA9 expression associates with the absence of VHL mutation and aggressive tumor characteristics in conventional RCC (Pantuck *et al.* 2007). CA9 may indicate those patients who benefit from IL-2, as low CA9 expression associates with lower survival compared to high CA9 expression in mRCC patients who receive IL-2 (Atkins *et al.* 2005, Bui *et al.* 2003). It has also been suggested that CA9 may indicate those patients who benefit from CA9-targeted therapies. It is also being investigated whether CA9 may indicate those patients who are potential candidates for adjuvant therapy.

2.3.3 p53, BIOMARKER OF CELL CYCLE POINT

p53, a tumor suppressor gene product, is a promoter of cell growth arrest and apoptosis (Choisy-Rossi and Yonish-Rouach 1998). Activated p53 elicits several cellular responses, including apoptosis and cell cycle arrest (Reich and Levine 1984), and responds to DNA damage at the

restriction checkpoint of the G1 phase of the cell cycle (May and May 1999). In normal cells, p53 is usually undetectable (Finlay *et al.* 1988). Mutant p53 accumulates in cell nuclei and can be immunostained (Reich and Levine 1984), whereas wild-type p53, because of its short half-life, is usually undetectable by routine immunohistochemistry (Reich and Levine 1984). p53 may be upregulated in part by VHL, accounting for some of the tumor suppressive functions of VHL in RCC (Galban *et al.* 2003).

Published results on the association of p53 with survival have been controversial, some studies suggesting positive p53 associating with poor survival (Shvarts *et al.* 2005 , Zigeuner *et al.* 2004, Uchida *et al.* 2002, Haitel *et al.* 2000, Moch *et al.* 1997, Uhlman *et al.* 1994), while others have observed no association (Itoi *et al.* 2004, Olumi *et al.* 2001, Rioux-Leclercq *et al.* 2000, Hofmockel *et al.* 1996, Bot *et al.* 1994, Lipponen *et al.* 1994). In the study of Phuoc *et al.* (2007), p53 was significantly associated with survival in univariate analysis, but the association was not independent. In some studies, the association of p53 and survival has been investigated in a group of RCC patients with both locally confined and primary metastatic RCC; thus, patient selection varies in different studies (Olumi *et al.* 2001).

2.3.4 Ki-67, PROLIFERATION BIOMARKER

Ki-67, a proliferation biomarker, is expressed throughout the active phases of the cell cycle, and serves as a good marker for proliferative activity in cell nuclei (Gerdes *et al.* 1984). Ki-67 accumulates during the cell cycle from G1 to mitosis, and is at its lowest level after mitosis (du Manoir *et al.* 1991). The percentage of nuclei staining by immunohistochemistry reflects Ki-67 expression (Olumi *et al.* 2001). Ki-67 has been reported to independently predict survival following nephrectomy in many studies (Dudderidge *et al.* 2005, Bui *et al.* 2004, Itoi *et al.* 2004, Rioux-Leclercq *et al.* 2000, Aaltomaa *et al.* 1997). Ki-67 has been observed to increase in sarcomatoid change (Kanamaru *et al.* 1999), indicating different protein expression profiles in different entities according to the Heidelberg classification.

2.3.5 COX-2, BIOMARKER FOR INFLAMMATION AND NEOPLASIA

Cyclo-oxygenase-2 (COX-2), an isoform of the COX^3 enzyme, is an inducible form of an enzyme involved in the first steps of prostaglandins and thromboxane synthesis. COX-2 converts arachidonic acid first into prostaglandin G2, and afterwards by peroxidase activity into prostaglandin H_2, a precursor of the prostaglandins (Taketo 1998). COX-2 is suggested to play a physiological role in fetal nephrogenesis (Khan *et al.* 2001). COX-2 increases in inflammation and neoplasia (Miyata *et al.* 2003, Hara *et al.* 2002, Maitra *et al.* 2002, Nose *et al.* 2002, *et al.* Taketo 1998), and is undetectable in most normal tissues (Mungan *et al.* 2006, Yoshimura *et al.* 2004).

The conversion of procarcinogens to proximate carcinogens is catalyzed by the peroxidase activity of COX-2 (Elinq *et al.* 1990).

COX-2 is highly induced by stimulus of oncogenes, cytokines, growth factors, and tumor promoters (Smith *et al.* 2000, Herschman 1996, Subbaramaiah *et al.* 1996). Associations between COX-2 over-expression and antiapoptotic ability, tumor invasiveness, tumor growth, angiogenesis, and immunosuppression, as well as multidrug resistance in cancer have been reported (Cao and Prescott 2002, Masferrer *et al.* 2000, Subbramaiah *et al.* 1996, Tsujii and DuBois 1995).

Cytoplasmic/membranous COX-2 staining by immunohistochemistry reflects COX-2 protein expression (Cho *et al.* 2005). The study results on associations of COX-2 with tumor stage, grade, and survival have been contradictory. Yoshimura *et al.* (2004) demonstrated that COX-2 was expressed at its highest in G1, as well as in pT1 RCC tumors, compared to other RCC tumors in grade and T-stage, while in Hashimoto *et al*'s study (2004), more COX-2 was found at the higher tumor grade, as well as stage. A significant association has been observed between COX-2 and Ki-67 expression (Miyata *et al.* 2003).

2.3.6 Her-2, BIOMARKER OF PROTO-ONCOGENE PRODUCT

Her-2, a proto-oncogene product, is a member of the ErbB family of receptor tyrosine kinases. Her-2 functions in secretory epithelial tissues, and regulates intracellular signaling cascades (Arteaga *et al.* 2001, Olayioye *et al.* 2000). Her-2 is over-expressed in approximately 20-30% of human adenocarcinomas (Latif *et al.* 2002, Lipponen *et al.* 1994, Slamon *et al.* 1989), and the over-expression is associated with metastatic phenotype and poorer prognosis, e.g. in breast and ovarian cancer (Slamon *et al.* 1989).

Gene amplification of *Her-2* can be investigated by cytogenetic analyses, such as fluorescent *in situ* hybridization (FISH), chromogenic *in situ* hybridization (CISH), and polymerase chain reaction (PCR). In breast cancer, FISH and CISH positivity are accurate predictors of response to trastuzumab (anti-Her2 therapy) (Isola *et al.* 2004, Lebeau *et al.* 2001). Receptor-mediated targeted tumor therapy with Herceptin® (RhuMAb HER-2), a recombinant humanized monoclonal anti-Her-2 antibody, has improved the survival of breast carcinoma patients both in adjuvant therapy and in therapy for metastatic disease (Smith *et al.* 2007, Montemurro *et al.* 2003).

Membranous staining of HER-2 in immunohistochemistry reflects HER-2 protein expression (Zhang *et al.* 1997). Her-2 receptor-specific tumor toxin, in an animal model, effectively reduced pulmonary tumors of advanced RCC (Maurer-Gebhard *et al.* 1998). Parallel associations of Her-2 expression between tumor stage and grade in RCC patients have been observed in many studies

(Zhang *et al.* 1997, Stumm *et al.* 1996), although in the study of Seliger *et al.* (2000) no such association was found. In the study of Hofmockel *et al.* (1997), higher tumor grades were seen when Her-2 expression was low, and higher T-stage associated with high Her-2. In the study of Phuoc *et al.* (2007), Her-2 protein expression did not correlate with Ki-67 protein expression.

In most *HER-2* gene amplification studies, *Her-2* gene amplification was observed neither by FISH analysis (Latif *et al.* 2002), messenger ribonucleic acid (mRNA) analysis (Stumm *et al.* 1996), nor PCR analysis (Selli *et al.* 1997, Zhang *et al.* 1997). Selli *et al.* (1997) found *HER-2* gene amplification in collecting duct RCC cases (45%). Therefore, *HER-2* gene amplification may be more pronounced in collecting duct RCC, than in other more common RCC types (Matei *et al.* 2005, Zhang *et al.* 1997). The association of *HER*-2 gene amplification and HER-2 protein expression with the prognosis of RCC patients has been estimated in few studies and the results have been contradictory (Phuoc *et al.* 2007, Lipponen *et al.* 1994). Further studies are needed to determine whether HER-2 protein expression or *HER-2* gene amplification may be used as prognostic factors in RCC patients.

2.4 THERAPIES FOR RCC

2.4.1 SURGERY

2.4.1.1 NEPHRECTOMY FOR LOCALIZED DISEASE

Nephrectomy is the only curative treatment for locally confined RCC (Robson *et al.* 2002). For RCC patients with isolated metastases, nephrectomy in conjunction with metastasectomy can be considered aiming at curative treatment, especially if the disease-free interval has been long (Kavolius *et al.* 1998).

Giuseppe Zambeccarrius performed a successful nephrectomy in a dog in 1678. Gustav Simon of Heidelburg performed the first successful intentional nephrectomy in a woman in 1869. Postoperative sepsis and massive bleedings after nephrectomy were common in those days. Partial nephrectomy was first performed for renal tumor excision in 1887 by Czerny.

In 1969, Robson and colleagues reported a technique for radical nephrectomy, which increased survival rates in RCC (Robson *et al.* 2002). It still remains the gold standard for the treatment of RCC. Radical nephrectomy includes removal of the kidney with intact Gerotas fascia and the ipsilateral adrenal gland with early vascular ligation.

Many patients may be over treated by removal of the adrenal gland, but this allows for correct staging, and long-term follow-up has indicated that its removal is not harmful (Hellström *et al.*

1997). The incidence of adrenal involvement in histologically confirmed RCC is 3-4%, and the risk of adrenal metastases has been associated with left-sided, upper pole, advanced T-stage, poor differentiation, and multifocality (Li *et al.* 1996, Sagalowsky *et al.* 1994).

The therapeutic value of lymph node dissection remains unproven (Mejean *et al.* 2003, Mickisch *et al.* 1999). There is evidence that retroperitoneal lymph node dissection (RPLND) may prolong survival in selected patients (Canfield *et al.* 2006). An increase in five-year survival from 65% to 80% was reported after lymphadenectomy with stage II RCC, and from 47% to 60% with stage III RCC (Golimbu *et al.* 1986). In a retrospective analysis, radical RPLND was beneficial in less than 4% of 1 035 patients without distant metastases (Schafhauser *et al.* 1999).

The rate of lymph node involvement ranges from 13% to 30% (Blom *et al.* 1999, Giuliani *et al.* 1990). Extensive lymph node dissection may improve staging (Blom *et al.* 1999). Locoregional lymph node involvement has been associated with unfavorable prognosis with a five-year survival of 5-30% (Bassil *et al.* 1985, Nurmi 1984). Independent lymph node involvement without distant metastases has been found to be rare, found in fewer than in 1% of the patients (Johnsen and Hellsten 1997).

2.4.1.2 NEPHRON-SPARING SURGERY

Radical nephrectomy is not suitable for patients with bilateral tumors or a small unilateral tumor in a solitary functioning kidney. Partial nephrectomy (PN), also called nephron-sparing surgery (NSS) (Novick 1995, Patard 1983), has been developed for such patients. PN is also indicated when a disease might threaten the future functioning of the contralateral kidney. Such a disease may be chronic pyelonephritis, renal artery stenosis, ureteral reflux, nephrosclerosis, or diabetes (Licht and Novick 1993). During the past decade, the trend has been to promote nephron-sparing surgery at the expense of radical nephrectomy (Gill *et al.* 2002). Recently, PN has also been performed in patients with a normally functioning contralateral kidney (Novick 1995).

After PN, in patients with a clearly localized RCC tumor of 4 cm or less in diameter, particularly for a low TNM stage, such as $T_1N_0M_0$, the survival rates have been observed to be comparable to those for radical nephrectomy (Lee *et al.* 2000, Belldegrun *et al.* 1999). No differences in survival rates have been observed between centrally or peripherally located tumors following PN (Hafez *et al.* 1998). The criterion for PN is that the tumor has not invaded vascular structures located in the center of the kidney. Of RCC cases, 10-25% are multifocal, and thus not suitable for PN (Whang *et al.* 1995).

After PN, the observed five-year cancer-specific survival is 88-100% (Fergany *et al.* 2000, Hafez *et al.* 1999), and the 10-year cancer-specific survival 73% (Fergany *et al.* 2000). A long-term (10-years) preservation of renal function has been achieved in 93% of patients (Fergany *et al.* 2000, Lau *et al.* 2000). Postoperative local tumor recurrence after PN has occurred in 3-6% of patients (Derweesh and Novick 2003, Hafez *et al.* 1999), but for the subgroup of T_{3b} RCC, a local recurrence rate of 10.6% has been observed (Hafez *et al.* 1997). It is suggested that local recurrence after PN is a manifestation of undetected microscopic multifocal RCC in the remaining kidney (Derweesh and Novick 2003). The risk of local recurrence after radical nephrectomy is not known, but it is very low (Derweesh and Novick 2003).

The maximum size of RCC suitable for PN has not been defined. Cancer-free survival is better in patients with tumors smaller than 4 cm than in patients with tumors between 4 and 7 cm (Hafez *et al.* 1999). Recently, also tumors of this size have been resected by partial nephrectomy with results that are comparable with radical nephrectomy (Leibovich *et al.* 2004). Data suggest that PN provides a long-term renal functional advantage over radical nephrectomy in those with an abnormal opposite kidney (Derweesh and Novick 2003, Lau *et al.* 2000). Quality of life is improved following PN (Clark *et al.* 2001), regardless of a complication rate of 9%, with the most bothersome being prolonged urinary fistula and delayed hemorrhage, which rarely require re-operation (Stephenson *et al.* 2004). PN may also be indicated in patients with a normal contralateral kidney, providing that the RCC tumor is single, small, and localized (Derweesh and Novick 2003). Patient selection has been a significant factor for outcome in this group (Derweesh and Novick 2003).

2.4.1.3 CYTOREDECTIVE NEPHRECTOMY

Cytoreductive nephrectomy has been performed since the 1960's to reduce symptoms from the primary tumor. It can be used to control flank pain, hematuria, anemia, hypercalcemia, and severe paraneoplastic syndrome. Embolization with postinfarction nephrectomy has also been used (Kurth *et al.* 1987). Angioinfarction instead of cytoredyctive nephrectomy may control local symptoms. Kim and Louie (1992) reported that in those patients with partial response to IL-2 treatment, the subsequent surgical resection of residual tumor may be beneficial.

Nephrectomy alone prolongs survival only in a minority of mRCC patients; it is more feasible in those patients with favorable prognostic factors such as low ESR and high hemoglobin (Onishi *et al.* 1989). Prior cytoreductive nephrectomy is justified in mRCC when later immunotherapy is planned (Flanigan *et al.* 2001, Mickisch *et al.* 2001). Radical nephrectomy followed by IFN-α monotherapy improves survival by 50% in patients with good performance status (Flanigan *et al.* 2001, Mickisch *et al.* 2001). However, in these studies, the combined morbidity rate was 18% due

to nephrectomy; 3% had a fatal complication. The patient selection for surgery is crucial due to high mortality rates (up to 17%) (Bennett *et al.* 1995). mRCC patients who have renal vein/inferior vena caval thrombus benefit from radical nephrectomy and thrombectomy, which improves disease-free survival (Parekh *et al.* 2005). Cytoreductive nephrectomy has also been associated with spontaneous regression in mRCC patients (Marcus *et al.* 1993, Gleave *et al.* 1998, Oliver *et al.* 1989).

2.4.1.4 METASTASECTOMY

Metastasectomy may prolong survival in patients with favorable prognostic factors (Samellas 1963). Surgery is potentially curative. Five-year survival rates from 20% to 70% have been achieved, when both primary tumor and solitary metastasis (mostly from lungs) have been resected (Kavolius *et al.* 1998, McNichols *et al.* 1981, Skinner *et al.* 1971). Resections of solitary second and third recurrences have been observed to increase five-year survival rates to 46% and 44%, respectively (Kavolius *et al.* 1998). Brain metastases are well-circumscribed with a surrounding pseudocapsule and they are often removable with low morbidity and mortality. The palliative benefit from the resection of brain metastases seems to be greater than from radiotherapy alone, and it also improves the performance status (Takashi *et al.* 1995). Long disease-free survival after adrenalectomy (with later steroid replacement) because of solitary adrenal gland metastasis has been achieved (Ertl and Darras 1999).

2.4.2 RADIOTHERAPY

Preoperative radiotherapy in local RCC has not been proved beneficial in prospective randomized studies. The applied radiation doses to the involved kidney and regional lymph nodes have been 30 Gy to 40 Gy with daily fractions of 2 Gy (Juusela *et al.* 1977). In this study, patients who received preoperative radiation therapy doses of 33 Gy with 15 fractions had a poorer five-year survival rate compared to those with only nephrectomy. However, preoperative radiotherapy has been observed to transform an inoperable tumor to an operable one in some RCC patients (van der Werf-Messing *et al.* 1973).

Many prospective randomized studies have found no benefit from postoperative adjuvant radiotherapy to the kidney bed and regional ipsilateral and contralateral lymph nodes (Kjaer *et al.* 1987, Finney 1973). In the study of Kjaer *et al.* (1987), the dose was 50 Gy in 20 fractions. Significant toxicity was observed in the stomach, duodenum, and liver.

Currently, according to retrospective studies, radiotherapy may be indicated in inoperable advanced local tumor stages and residual tumors, increasing the local control rate in RCC with lymph node

metastases, perinephric fat or adrenal invasion, or surgically positive margins (Kao *et al.* 1994, Rabinovitch *et al.* 1994, Stein *et al.* 1992). Although radiotherapy may decrease the local recurrence rate, no improvement in overall survival has been observed. Modern CT-assisted treatment planning decreases the toxicity of radiotherapy (Stein *et al.* 1992).

Palliative radiotherapy is used for ameliorating the symptoms due to metastases in, e.g. the brain, lung, or bone (Halperin and Harisiadis 1983). A typical palliative dose is 30 Gy in 10 fractions.

2.4.3 CHEMOTHERAPY

RCC has been proved to be unresponsive to chemotherapy with no improvement in survival. Vinblastine has been observed to have some activity in RCC with a response rate of less than 10%, except in one study where the response to vinblastine was 16% (Fosså *et al.* 1992a, Kuebler *et al.* 1984). Gemcitabine with capecitabine or 5-fluorouracil has a similar response rate of around 10% (Tannir *et al.* 2008, George *et al.* 2002), except in the study of Rini *et al.* (2000) where the rate was 17%. Gemcitabine with doxorubicin has antitumor activity in collecting duct, sarcomatoid, or with rapidly progressing RCC (Nanus *et al.* 2004, Milowsky *et al.* 2002). The addition of 5-fluorouracil to immunotherapy does not seem to increase the response rate (Négrier *et al.* 2000), but does increase the amount of adverse effects (Olencki *et al.* 2001). Capecitabine with thalidomide, docetaxel, or immunotherapy have produced response rates of less than 10% (Harshman *et al.* 2008, Marur *et al.* 2008, Wenzel *et al.* 2003). Treatment with pemetrexed also results in response rates of less than 10% (Thödtmann *et al.* 2003).

2.4.4 INTERFERON-α THERAPY

Immunotherapy was shown to be active in RCC by Tykkä *et al.* in 1978. Partially purified human leukocyte IFN-α (Cantell-type IFN) (Cantell and Hirvonen 1977) and lymphoplastoid IFN-α (Wellferon, Burroughs Wellcome Company) were first found to have activity in mRCC (Kirkwood *et al.* 1985, Neidhart *et al.* 1984, deKernion *et al.* 1983, Quesada *et al.* 1983). Subsequently, the cloning of the gene for IFN-α and recombinant genetic engineering allowed the production of rIFN-α (Pestka 1983). Two preparations of IFN-α are commercially available: rhIFN-α2a (Roferon®, Hoffman LaRoche) and rhIFN-α2b (Intron®, Schering-Plough Laboratories). It is generally accepted that there is no difference in the efficacy of these two preparations, although no direct comparison has been made. In addition, rhIFN-α2c (Boehringer Ingelheim) has been licensed in some countries. The US Food and Drug Administration (FDA) approved rIFN-α for clinical use in hairy cell leukemia in 1986 (Pestka 1997), and afterwards for many other malignancies. IFN-α is active in multiple tumors, e.g. in cutaneous T-cell lymphomas, superficial bladder cancer, malignant neuroendocrine tumors, Kaposi sarcoma, and malignant melanoma. For

RCC, IFN-α therapy is preferentially used in Europe, while IL-2, the other cytokine therapy, is predominantly used in North America.

2.4.4.1 RESPONSE TO IFN-α

The response rate to IFN-α is approximately 12%, ranging most often from 10% to 20% (Table 13). The majority (approximately 70%) of objective responses are partial responses (PR). The median duration of response is six to eight months, and rarely exceeds two years (Fosså 1988). In the Cochrane meta-analysis, based on four studies, on average, a 2.8 month improvement in median survival was achieved with IFN-α therapy compared to controls, although, the confidence limits could not be estimated (Coppin *et al.* 2007). Using overall survival as the appropriate measure of benefit, IFN-α has been associated with an increase in median survival of 3.8 months, a reduction in one-year mortality of 44%, and a reduction in two-year mortality of 26%, compared to non-immunotherapy controls (Coppin *et al.* 2007, MRCRCC 1999, Pyrhönen *et al.* 1999). Three percent of patients have shown prolongation of survival due to IFN-α (Minasian *et al.* 1993). IFN-α is effective in conventional type mRCC but its efficacy in other mRCC types is uncertain (Motzer *et al.* 2002a).

Table 13 shows the results of IFN-α treatment analysed in terms of survival in randomized studies. IFN-α is effective and increases survival in RCC patients with good prognosis. Pyrhönen *et al.* (1999) suggested in their study (IFN-α versus IFN-α plus vinblastine) that the survival benefit of IFN-α may be greater in those patients with adverse prognostic factors, such as poor performance status, age over 60 years, and male gender (Pyrhönen *et al.* 1999). In the study by Négrier *et al.* (2007), IFN-α was not beneficial in patients with intermediate prognostic factors (Négrier *et al.* 2007). IFN-α produces long-lasting remission in some patients (Motzer *et al.* 2000a, Minasian *et al.* 1993).

The prognostic value of secondary leucopenia as response to IFN-α therapy has been found contradictory in different studies (Buzaid *et al.* 1987, Muss *et al.* 1987, Umeda and Niijima 1986, Kirkwood *et al.* 1985, Quesada *et al.* 1985b).

Pegylated IFN-α (PEG-IFN-α) has a longer half-life compared to standard IFN-α, which enables weekly doses. PEG-IFN-α is effective on a weekly schedule at a dose of 4.5 μg/kg subcutaneously (Feldman *et al.* 2008, Motzer *et al.* 2001a) with median overall survival of 31 months and comparable safety with standard IFN-α (Feldman *et al.* 2008, Motzer *et al.* 2001a).

Table 13. Benefit from IFN-α analysed in randomized studies.

Author	Year	No. of pts	Therapy	Dose (million units MU), schema and route	CR + PR (%)	Median overall survival (months)	Survival benefit
Steineck *et al.*	1990	30	IFN-α2a	10-50 MU/m^2 im tiw until progression	6	7	No
		30	MPA	1000mg im tiw for 5 wks, thereafter 1000mg im q1wk until progression	3	7	
Kriegmair *et al.*	1995	41	IFN-α2b + vinblastine	IFN-α 8 MU sc tiw, vinblastine 0.1 mg/kg iv q3wk until progression or no change after a period of 3 months	20.5	16	No
		35	MPA	500 mg im q1wk until progression or no change after a period of 3 months	0	10	
Pyrhönen *et al.*	1999	79	IFN-α2a + vinblastine	IFN-α max 18 MU tiw sc, until 12 months or 3 months after CR, but in PR and SD beyond 12 months + vinblastine 0.1 mg/kg iv q3wk	16.5	16.9	Yes
		81	vinblastine	vinblastine 0.1 mg/kg iv q3wk until 12 months or 3 months after CR, but in PR and SD beyond 12 months	2.5	9.5	
MRCRCC *et al.*	1999	167	IFN-α-2b	10 MU sc tiw until 3 months, 23 patients continued after 12 weeks	16*	8.5	Yes
		168	MPA	300 mg po daily, 3 months	2*	6	
Négrier *et al.*	2007	122	IFN-α	9 MU sc tiw, max 6 months	4.4*	15.2	No
		125	IL-2	9 MU sc, max 6 months	4.1*	15.3	
		122	IFN-α + IL-2	IFN-α 6 MU tiw + IL-2 9 MU sc, max 6 months	10.9*	16.8	
		123	MPA	200mg po daily, 6 months	2.5*	14.9	

* Response at 3 months.
qXwk = every X weeks
tiw = three times a week
im = intramuscularly
sc = subcutaneously
iv = intravenously
po = orally
MPA = medroxyprogesterone acetate

Performance status is the most consistently reported prognostic factor in mRCC. Remission is more likely in those patients with only lung metastases. However, this may be in part a result of bias relating to the different radiological methods for evaluating lesions. Brief lesions are more likely to be seen by X-ray in the lung than by scintigraphy or CT in bone, liver or other inner organ.

2.4.4.2 ANTI-IFN ANTIBODIES

Antibodies have frequently been observed in patients receiving recombinant IFN-α2a, but less frequently in patients receiving IFN-α2b. On the contrary, antibodies have not been observed in patients receiving lymphoplastoid IFN. This preparation of interferon (IFN) is genetically identical to its natural form. The most frequently detected antibodies are IFN-binding and neutralizing

antibodies (Prümmer 1993, Fosså *et al.* 1992b, Figlin *et al.* 1988, Buzaid *et al.* 1987, Quesada *et al.*1985a). Prednisone may be capable of blunting the formation of anti-IFN antibodies (Ernstoff *et al.* 1990). However, prednisone has not been associated with decreased survival (Fosså *et al.* 1992b). The data suggest that anti-IFN antibodies rarely cause resistance to IFN-α.

2.4.4.3 *DOSAGE AND SCHEDULE*

The optimal dose and treatment schedule of IFN-α therapy has not yet been defined (Coppin *et al.* 2007). The dose of IFN-α has varied in different studies from 1 million units (MU) to 36 MU. Low doses of less than 5 MU have been observed to be ineffective (Quesada *et al.* 1985b, Kirkwood *et al.* 1985, Muss *et al.* 1987). A dose of Cantell-type IFN of 1 MU was compared to a dose of 10 MU, with IFN-α being administered intramuscularly daily. None of the patients responded to 1 MU, but 19% responded to 10 MU (Kirkwood *et al.* 1985).

Toxicity of IFN-α is dose-dependent. High doses of IFN-α result in excessive toxicity requiring dose reduction and maybe discontinuation (Krown 1987, Trump *et al.* 1987). Fosså *et al.* (1988 and 1986) observed severe adverse effects with doses of IFN-α ranging from 18 to 36 MU three times a week (tiw). For example, 7% of patients developed mental confusion and 4% visual disturbances due to retinal exudation. In more than half of the patients, IFN-α had to be reduced, delayed or discontinued due to toxic effects (Fosså *et al.* 1988, Fosså *et al.* 1986). In the study by Muss *et al.* (1987), the amount of grade 3 and 4 toxicity was greater in the group of patients who received an IFN-α dose from 30 to 50 MU/m^2 intravenously for five consecutive days every three weeks, compared to those patients with 2MU/m^2 subcutaneously tiw. The response rates were 10% for the former group, and 7% for the latter group. IFN-α administered subcutaneously or intramuscularly provides more prolonged systemic exposure than when administered intravenously. In a study by Pyrhönen *et al.* (1999), the dose of IFN-α was escalated from the starting dose of 3 MU tiw to 18 MU tiw. Thereafter, for patients unable to tolerate 18 MU, the dose was reduced to 9 MU tiw.

2.4.4.4 QUALITY OF LIFE (QoL)

IFN-α therapy is safe and no IFN-α-related deaths have been reported (Coppin *et al.* 2007), but IFN-α causes many adverse effects affecting the quality of life. Almost all patients experience symptoms of a flu-like syndrome, such as fever, fatigue, taste change, loss of appetite, and myalgia. As the dose of IFN-α increases, depression, anorexia, malaise, leucopenia, and elevation of liver function tests become common. Fatigue and depression result in reduction of physical activity in mRCC patients receiving IFN-α. In the study of Steineck *et al.* (1990), very high elevation of liver function tests associated with intolerable tiredness. Severe neuropsychiatric effects during IFN-α

therapy, such as confused state of mind, are rare (Renault *et al.* 1987). Depression has successfully been treated with antidepressant agents. The association between toxicity and remission has not been defined.

In the study of Négrier *et al.* (2007), formal quality of life was assessed using the EORTC QLQ-C30 instrument. At three months, 16% of patients with IFN-α therapy had impaired quality of life, while the same was true for 11% of patients with medroxyprogesterone acetate (MPA) therapy. Reducing the dose ameliorates the symptoms. Dose reductions or discontinuations of IFN-α therapy are common events in various studies.

2.4.4.5 NEPHRECTOMY PRIOR TO IFN-α

Initial cytoreductive nephrectomy prior to planned IFN-α treatment improves survival and delays time to progression, despite an unimproved response rate in mRCC (Flanigan *et al.* 2001, Mickisch *et al.* 2001). Nephrectomy followed by IFN-α monotherapy improved one-year survival by 47%; the improvement in median survival was nearly five months (Flanigan *et al.* 2001, Mickisch *et al.* 2001). The eligibility of patients was restricted; these studies included only patients with good performance status (ECOG 0-1). The dose of IFN-α was 5 MU and most often IFN-α was started within four week after nephrectomy. The response rate was only 6% in both studies combined. The mechanism of improved survival is currently unknown. The benefit of using nephrectomy in mRCC patients prior to other forms of systemic therapy remains unproven.

2.4.4.6 IFN-α VERSUS OTHER THERAPIES

When comparing IFN-α to chemotherapy, hormonal therapy, or biological response modifiers (Motzer *et al.* 2001b), such as thalidomide (Motzer *et al.* 2002c), the effectiveness of IFN-α has been found to be superior or similar. However, of the modern targeted therapies, sunitinib, a VEGF receptor tyrosine kinase inhibitor, has been shown to produce a superior objective response rate and progression-free survival in the sunitinib group as compared to IFN-α (Motzer *et al.* 2007).

2.4.4.7 IFN-α PLUS OTHER THERAPIES

There have been efforts to enhance the efficacy of IFN-α by additional agents, but so far there is no evidence of enhanced survival except with bevacizumab (Rini *et a. l*2004) for increasing efficacy. In randomized studies, combining low dose IL-2 (LD-IL-2) with IFN-α increases the response rate compared to either therapy alone; the combined therapy response rate being 18.6% versus approximately 7% (Négrier *et al.* 2007, Boccardo *et al.* 1998, Négrier *et al.* 1998, Vuoristo *et al.* 1994, Vogelzang *et al.* 1993). However, similar response rates have been achieved by IFN-α alone

in other studies. Additionally, due to higher toxicity, the combination of LD-IL-2 and IFN-α led to a decreased quality of life in 33% of patients, compared to 16% for either therapy alone (Négrier *et al.* 2007).

The additional agents have included hormones such as toremifene and MPA (Oh *et al.* 2002, Porzsolt *et al.* 1988), chemotherapies such as vinblastine (Fosså *et al.* 1992c, Kellokumpu-Lehtinen and Nordman 1990) and 5-fluorouracil (Lopez Hänninen *et al.* 1996), other biological therapies, such as IFN-β (Mani *et al.*1995), IFN-γ (Mani *et al.*1995), and 13CRA (Aass *et al.* 2005, Motzer *et al.* 2000b). Additionally, aspirin (Creagan *et al.* 1991), prednisone (Fosså *et al.*1992b), and cimetidine with coumarin (Sagaster *et al.* 1995) for alleviating adverse effects of therapy, have been used.

2.4.4.8 IFN-α AS SECOND LINE THERAPY

Second-line immunotherapy with IFN-α or IL-2 (IFN-α after failure of IL-2, or IL-2 after failure of IFN-α) is not effective (Escudier *et al.* 1999).

2.4.4.9 IFN-α AS ADJUVANT THERAPY

IFN-α as adjuvant therapy following nephrectomy has been investigated in patients at high risk of relapse (Messing *et al.* 2003, Pizzocaro *et al.* 2001, Prümmer 1993). An early pilot study was promising as a significant increase in NK cell activity was detected after initiation of IFN-α (Takahashi *et al.* 1994). However, current evidence does not support the use of IFN-α as adjuvant therapy in RCC (Messing *et al.* 2003, Pizzocaro *et al.* 2001), although Pizzocaro *et al.* (2001) found a protective effect in the subgroup of pN2/pN3 patients.

2.4.4.10 BIOLOGICAL EFFECTS OF IFN-α

There are two types of human IFNs: IFN-α, the leucocyte IFN, and IFN-β, the fibroblast IFN. These are called type I IFNs, while IFN-γ, the immune IFN, is called type II IFN (Zav'Yalov and Zav'Yalova 1997). IFN-α was discovered by Isaacs and Lindemann in 1957 while searching for a substance that blocked viral infection of cells.

IFN-α is a glycosylated polypeptide of 16-25 kDa. Fourteen genes, considered tumor-suppressor genes, on chromosome 9p21 encode 12 distinct protein forms of IFN-α (Pestka 1997). IFN-α is produced in response to enveloped viruses, virus-infected cells, bacteria, tumor cells, and double-stranded RNA (Ruszczak and Schwartz 1997). IFNs were named for their ability to mediate viral

interference, where one virus interferes with the replication of a second (Pfeffer and Donner 1990). Lymphocytes and macrophages produce IFN-α.

IFN-α is pleiotropic cytokine with immunomodulatory, antiangiogenic, proapoptotic effects, and antiviral and antiproliferative effects. IFN-α has direct antiproliferative effects on renal-tumor cells (Nanus *et al.* 1990, Kuebler *et al.* 1987). IFN-α has different cytostatic and immunomodulatory effects on mammalian cells (Pestka 1983). The exact antitumor mechanisms against RCC have not been defined.

The inhibitory effect of IFNs is not specific to the phase of the cell cycle (Kasuya *et al.* 2001). It is known that IFN-α enhances class I major histocompatibility complex (MHC) expression of cells. Moreover, IFN-α has the capacity to affect cellular differentiation.

IFN-α has multiple immunomodulatory effects, such as activating monocytes and macrophages, and induction of IL-2 receptors (Holan *et al.* 1991). IL-2 is produced by T and NK cells and plays a pivotal role in the regulation of lymphocyte activation and proliferation (Trinchieri *et al.* 1996). IFN-α acts as a positive and negative regulator of NK cells, and modifies the susceptibility of target cells to lysis (Ernstoff *et al.* 1983). IFN-α increases the amount of T-lymphocytes. IL-2 is presumed to activate NK cells, T-lymphocytes and lymphocyte-activated killer cells and tumor-infiltrating lymphocyte cells (Trinchieri *et al.* 1984). Phagocyte receptors are crucial for the cytotoxic function of neutrophils and macrophages (Wallace *et al.* 1994).

2.4.5 INTERLEUKIN-2

The development of HD-IL-2 therapy was based on animal model data (Rosenberg *et al.* 1985). HD-IL-2 appears to benefit some mRCC patients by producing durable objective responses (CRs or PRs) (Fyfe 1995). When comparing HD-IL-2 therapy to combined LD-IL2 and IFN-α, or to LD-IL-2 or IFN-α therapy alone, the response rate increases by adding another cytokine to IFN-α, but is highest with HD-IL-2 therapy. The reported response rates have typically been more than 20% with HD-IL-2, from 10% to 20% with LD-IL-2 plus IFN-α, or less than 10% with LD-IL-2 alone (McDermott *et al.* 2005, Négrier *et al*. 1998), but lower responses with HD-IL-2 have also been reported (Wagner *et al.* 1999). Remissions have not translated into a similar hierarchy for overall survival. HD-IL-2 may improve survival in the subgroup of patients with the poorest prognosis, such as those with primary tumor still in place or with either liver or bone metastases (McDermott *et al.* 2005). Of patients treated with HD-IL-2, 10-20% are estimated to live for 5 to 10 years (Fisher *et al.* 2000). However, patients for HD-IL-2 therapy should have (very) good performance status and organ function, such as cardiac function, since the treatment is toxic. Therefore, HD-IL-2

is the therapy of choice for only few patients only. Additionally, for other types of RCC than the conventional type, HD-IL-2 should not be considered, because the efficacy of cytokine therapy in other types of RCC is uncertain (Rosenberg *et al.* 1993). For mRCC, IL-2 therapy is predominantly used in the USA, while IFN-α in Europe (Decatris *et al.* 2002). HD-IL-2 therapy is not a common therapy because of its toxicity with multi-organ system effects, such as hypotension, tachyarrhythmias, capillary leak syndrome, and renal or hepatic failure (McDermott *et al.* 2005, Yang *et al.* 2003b). In the United States, HD-IL-2 therapy is usually administered by bolus, while in Europe it is administrated continuously. LD-IL-2 has been used subcutaneously (Négrier *et al.* 2007, Atzpodien *et al.* 2002) and intravenously (Négrier *et al.* 2007).

The additional agents connected with LD-IL-2 therapy have been lymphocyte-activated killer cells (Rosenberg *et al.* 1993), tumor-infiltrating lymphocytes (Figlin *et al.* 1999), IFN-β (Witte *et al.* 1995), histamine (Donskov *et al.* 2005) and melatonin (Lissoni *et al.* 2000), but there is no evidence has that these affect survival.

2.4.6 TARGETED THERAPIES

The molecular pathways with multiple targets that are of special interest in RCC are angiogenesis and intracellular signal transduction pathways.

Sunitinib, inhibitor of VEGF receptor tyrosine kinase, has been investigated in conventional RCC in a randomized study (Motzer *et al.* 2007). The response rate to sunitinib was superior compared to IFN-α, 47% and 12%, respectively; the difference was statistically significant. Median progression-free survival with sunitinib was superior to IFN-α; 11.0 months and 5.0 months, respectively; the difference was also statistically significant. Median progression-free survival was superior for those patients with good or intermediate prognosis (Motzer *et al.* 2007). Sunitinib may also be effective in papillary and chromophobe RCC (Choueiri *et al.* 2008). Administration of sunitinib in mRCC patients without nephrectomy may be feasible, and lead to a reduction in tumor size that can facilitate subsequent surgical resection (Bex *et al*. 2009, Thomas *et al.* 2009). The most often seen adverse effects with sunitinib are diarrhea, fatigue, nausea, anemia, and leucopenia. QoL was significantly improved by sunitinib when the functional assessment of the cancer therapy general scale (FACT-G) and the functional assessment of the cancer therapy-kidney symptom index (FKSI) self-assessment tools were used (Motzer *et al.* 2007). Sunitinib has shown to be potentially cost-effective as a second-line treatment for cytokine-refractory mRCC compared with the best supportive care, including palliative biochemotherapy (Purmonen *et al.* 2008).

Sorafenib, inhibitor of VEGF receptor tyrosine kinase, which can also suppress immune responses by inhibiting dendritic cell function (Hipp *et al.* 2007), was superior to placebo at a dose of 400 mg twice daily as a second-line therapy in patients with prior cytokine therapy (Escudier *et al.* 2007a). Progression-free survival for the patients given sorafenib was 5.5 months, while for placebo, it was 2.8 months. The risk of brain metastases was reduced by sorafenib; these were observed in 3% of patients with sorafenib, and in 12% of patients with placebo (Stein 2006). The adverse effects caused by sorafenib included cardiac ischemia, hypertension, hand-foot syndrome, and diarrhea. Sorafenib was found by QoL measures to ameliorate symptoms. Bone pain was found to be more common in those patients who were given placebo (Bukowski *et al.* 2007). The study was discontinued early when progression-free survival was clearly better in the sorafenib group. Sorafenib may also be effective in papillary and chromophobe RCC (Guevremont *et al.* 2009).

Temsirolimus, also called rapamycin, is metabolized to sirolimus, and is an inhibitor of an intracellular kinase called mTOR (mammalian target of rapamycin), disrupting cell cycle progression and angiogenesis. Temsirolimus is a macrolide antibiotic and immunosuppressive drug, derived from the *Streptomyces* species. A phase III study compared temsirolimus at a dose of 25 mg i.v. weekly to IFN-α at a dose of 3 MU tiw (Hudes *et al.* 2007) in patients with intermediate or poor prognosis. Progression-free survival for patients with temsirolimus was 3.8 months, while for IFN-α it was 1.9 months. Overall survival for patients on temsirolimus was 10.9 months, while for IFN-α it was 7.3 months; the difference was statistically significant. Even in patients with non-conventional RCC, overall survival was statistically significantly improved with temsirolimus compared to IFN-α (Dutcher *et al.* 2009). Grade 3 and 4 toxicity was more often seen in the patients on IFN-α compared to those on temsirolimus. The typical adverse effects caused by temsirolimus are anemia, thrombocytopenia, hypertriglyceridemia, hypercolesterolemia, hyperglycemia, rash, acne, and increased creatine (Bellmunt *et al*. 2008). There is an increased risk of stomatitis and weight loss if temsirolimus and IFN-α are administered together (Bellmunt *et al*. 2008).

Everolimus, an orally administered inhibitor of mTOR, has an encouraging antitumor activity against mRCC. Progression-free survival of more than or equal to six months for approximately 70% of patients, and a median overall survival of 22.1 months have been observed (Amato *et al.* 2009). In the patients with prior progression on sunitinib or sorafenib, median progression-free survival of four months was found in the group on everolimus compared to 1.9 months in the placebo group (Motzer *et al.* 2008b). Stomatitis, rash and fatigue are the typical side effects caused by everolimus (Motzer *et al.* 2008b). Pneumonitis has been observed in 8% of patients, and the severity of pneumonitis may be as high as grade 3 (Motzer *et al.* 2008b).

The addition of *bevacizumab*, an antiangiogenesis monoclonal antibody, to IFN-α in conventional RCC has been examined in a placebo-controlled study (Escudier *et al.* 2007b). IFN-α plus bevacizumab 10 mg/kg every two weeks gave a major remission rate of 31%, compared to 13% with IFN-α plus placebo. Median progression-free survival was 10.2 months for IFN-α plus bevacizumab and 5.4 for IFN-α plus placebo; the difference was statistically significant. The overall survival analysis showed a trend in favor of IFN-α plus bevacizumab. Therefore, the monitoring committee recommended that those patients with IFN-α plus placebo who had not progressed should change to IFN-α plus bevacizumab. Proteinuria and hypertension are the most often found adverse effects of this therapy, attributable mainly to bevacizumab.

2.4.7 OTHER THERAPIES

Nonmyeloablative allogeneic hematopoetic stem-cell transplantation can induce regression in mRCC patients who have not responded to prior IFN-α or IL-2 (Takahashi *et al.* 2008, Childs *et al.* 2000). Response rates with hormonal therapy, such as progestin, tamoxifen or toremifen have been approximately 10% (Gershanovich *et al.* 1996, Igel'nik *et al.* 1991, Jacqmin *et al.* 1988).

Vaccines and gene therapies are potential future therapies, particularly when the disease burden is low. On the basis of their potent antigen-presenting potential, dendritic-cell-based therapies have been investigated (Guse *et al.* 2009, Hawkins *et al.* 2009, Gitlitz *et al.* 2003). Also T-lymphocyte-based gene therapies have been developed to generate cytotoxic T lymphocytes (Mulders *et al.* 1998). WX-G250 is a chimeric monoclonal antibody that binds to carbonic anhydrase 9, which is expressed in approximately 95% of conventional RCCs (Bleumer *et al.* 2006). The assumed working mechanism of WX-G250 is by antibody-dependent cell-mediated cytotoxicity (ADCC) (Bleumer *et al.* 2006).

3 AIMS OF THE PRESENT STUDY

I To investigate the efficacy and tolerance of prolonged and intermittent administration of IFN-α_{2a} in mRCC in a phase II clinical study.

II To investigate the changes in the blood neurophils and monocyte receptor profile during IFN-α_{2a} administration in mRCC.

III To explore the prognostic significance of molecular markers of p53, Ki-67 and COX-2 in RCC according to the occurrence of metastases and survival.

4 MATERIALS AND METHODS

The patient characteristics in the different studies are presented in Table 14. No mRCC patient had biochemotherapy before IFN-α treatment, and after progression to IFN-α, no one was treated with bevacizumab or tyrosine kinase inhibitors. Histopathological samples of the studies were re-evaluated: the tumors were categorized according to the Heidelberg classification (Kovacs *et al.* 1997), and re-graded according to the WHO classification (Mostofi *et al.* 1998) by an experienced pathologist (Karl-Ove Söderström). For T-staging categorization, the 2002 updated UICC pTNM classification system of renal carcinomas was used (Sobin and Wittekind 2002). Table 15 shows the follow-up of the patients in the studies.

Table 14. Characteristics in different studies.

Characteristics	**No. (%)**	**No. (%)**	**No. (%)**	**No. (%)**	**No. (%)**
Publication	**I**	**II**	**III**	**IV**	**V**
Total no. of RCC pts	75 (100)	117 (100)	18 (100)	117 (100)	102 (100)
Sex					
Female	29 (39)	47 (40)	6	54 (46)	44 (43)
Male	46 (61)	70 (60)	12	63 (54)	58 (57)
Age					
years, median [range]	63 [43-77]	63 [38-78]	64 [52-77]	61 [37-82]	61 [37-79]
Time to metastases in M0-pts					
months, median [range]	1 [0-156]	0 [0-157]	22 [0-82]		
WHO performance status					
< 2	57 (76)	80 (68)			89 (87)
= 2	18 (24)	37 (32)			13 (13)
T-stage					
T1		20 (20) [1]		42 (37)	36 (36)
T2		19 (19) [1]		29 (26)	26 (26)
T3		50 (50) [1]		35 (31)	31 (31)
T4		12 (12) [1]		7 (6)	7 (7)
Tumor grade					
G1		38 (37) [2]		34 (29)	27 (26)
G2		39 (38) [2]		57 (49)	51 (50)
G3		25 (25) [2]		26 (22)	24 (24)
No. of metastases					
M0	0 (0)	0 (0)		51 (44)	45 (44)
M1	75 (100)	117 (100)		66 (56)	57 (56)
Site of metastases					
lung	52 (69)		3, other 5		
skin or soft tissue	28 (37)				
bone	26 (35)				
lymph node	19 (25)				
liver	11 (15)				
pleural	10 (13)				
Heidelberg classification type					
conventional				101 (86)	86 (84)
papillary				4 (3)	4 (4)
chromophobe				6 (5)	6 (6)
unclassified				6 (6)	6 (6)
Prior therapy					
Nephrectomy	70 (93)	107 (91)	17	117 (100)	102 (100)
Radiotherapy to kidney	1 (1)	2 (2)			
Response to IFN-α					
complete response	5 (6.6) [3]	9 (7.7) [4]			
partial response	8 (10.7) [3]	11 (9.4) [4]			
stable disease	32 (42.7) [3]	49 (41.9) [4]			
progressive disease	27 (36.0) [3]	48 (41.0) [4]			

[1]Sixteen patients were not evaluable for T-stage.
[2]Fifteen patients were not evaluable for tumor grade.
[3]Three patients were non-evaluable as IFN-α was discontinued before 4 weeks
[4]An intention-to-treat analysis.

Table 15. Follow-up of RCC patients after nephrectomy and mRCC patients after the beginning of IFN-α. Staging RCC was based on the American Joint Committee on Cancer staging (Miller *et al.* 1981).

Staging investigation:	Follow-up interval in RCC	Follow-up interval in mRCC
Follow-up evaluation:		
symptoms of the disease	every 6 months	every 1-4 weeks, thereafter bimonthly
signs of the disease		
performance status		
neurological status		
body weight		
response to IFN-α	N/A	
Laboratory evaluation:		
blood counts	every 6 months	every 1-4 weeks, thereafter bimonthly
serum concentrations of calcium		
liver enzymes		
creatinine		
Radiological evaluation:		
chest X-ray and abdominal US / chest and abdominal CT	every 6 months if signs of disease manifestation	alternative evaluation method, every 3-4 months
bone scintigraphy	if bone pain	if bone pain

4.1 EFFICACY AND TOLERABILITY OF PROLONGED ADMINISTRATION OF IFN-α_{2a} IN mRCC IN PHASE II CLINICAL STUDIES. (I, II)

4.1.1 PATIENT ELIGIBILITY

Between December 1994 and December 2002, 117 patients with mRCC gave informed consent to participate in the recombinant IFN-α2a (Roferon®) study. The inclusion criteria were: age younger than 80, performance status of 0-2 (WHO), serum creatinine concentration of less than 200 umol/l, and serum concentrations of liver enzymes less than twice the upper reference limit. Patients with brain metastases, other malignancies, and severe concomitant disease, or with a life expectancy of less than three months were excluded.

4.1.2 TREATMENT SCHEDULE

Patients were treated with subcutaneous IFN-α three times a week. The dose was increased weekly from 4.5 MU to the maximal tolerable dose of 9, 12, 13.5 or 18 MU. The maximum dose was to be reached during the first four weeks. The maintenance dose was chosen on the basis of the patient's tolerance of adverse effects. The treatment cycle consisted of three weeks' treatment with IFN-α followed by a one-week pause. IFN-α was planned to be continued until progression of over 25% in tumor measurements (PD), substantial adverse effects, or for up to two years. Adverse effects were recorded according to ECOG toxicity criteria (Oken *et al.* 1982).

IFN-α was self-administered on an outpatient basis. In addition to the injection of IFN-α, patients were recommended to use naproxen prophylactics to ameliorate flu-like symptoms. The treatment protocol was designed by Professor E. Salminen. The patients were treated by IFN-α until December 2002, and followed at the Department of Oncology and Radiotherapy of Turku University Hospital, until December 2008.

4.1.3 STAGING AND RESPONSE ASSESSMENT

The evaluation of the response followed the criteria of the World Health Organization (WHO) (Table 16). For the patient to qualify as a responder, two successive measurements at least four weeks apart confirming response (CR or PR) were required. A PR in bone metastases was defined as clinically stable lesions with a decrease in uptake in skeletal scintigram and normalizing of alkaline phosphatase. The response duration was measured from the first observed response.

Table 16. Evaluation of the response following the criteria of the World Health Organization (Miller *et al.* 1981).

Type of response	Abbreviation	Description of the response
complete response	CR	Disappearance of all known disease, determined by two observations not less than four weeks apart
partial response	PR	A 50% or more decrease in total tumor load of the lesions that have been measured to determine the effect of therapy by two observations not less than four weeks apart. Bi-dimensional: single lesion, greater than or equal to 50% decrease in tumor area (multiplication of longest diameter by the greatest perpendicular diameter)
stable disease	SD	A 50% decrease in total tumor size cannot be established nor has a 25% increase in the size of one or more measurable lesions been demonstrated.
progressive disease	PD	A 25% or more increase in the size of one or more measurable lesions or the appearance of new lesions.

4.1.4 STATISTICS

The association of prognostic factors with survival time or time to progression was analyzed using survival analysis applying Kaplan-Meier estimation, log-rank test and Cox's regression analysis. The associations between separate prognostic factors were analyzed with cross-tabulation and Pearson's chi-square test. p-values less than 0.05 were interpreted as statistically significant. Computing was performed with SAS System version 9.2 (2002, SAS Institute Inc., Cary, NC, USA).

4.2 CHANGES IN BLOOD NEUTROPHIL AND MONOCYTE RECEPTOR PROFILE DURING IFN-α_{2A} ADMINISTRATION IN mRCC (III)

Eighteen mRCC patients, aged 52 to 77 years, receiving IFN-α were included in the study. Their phagocyte receptor expressions (FcγRI, FcγRII and FcγRIII) and complement receptors (CR1 and

CR3) were studied in neutrophils and monocytes. No previous radio-, chemo-, or hormonotherapy had been given. The initial dose of IFN-α was 4.5 MU s.c. tiw, followed by escalation up to 9.0-13.5 MU tiw. This dose was continued intermittently with a three-week-on one-week-off schedule. As controls, samples from 39 healthy subjects were studied.

Samples from three patients were collected after the maintenance level of IFN-α was reached, during the treatment weeks prior to and after the rest week, and during the rest week, in order to study receptor expression during the treatment cycle, and evaluate the significance of the sampling time.

4.2.1 REAGENTS

Hanks balanced salt solution without Ca^{2+} and Mg^{2+} ions (CMF-HBSS, pH 7.4) was prepared and supplemented with 0.1% gelatine. FITC-conjugated anti FcγRI (CD64), anti-FcγRIII (CD16), anti-CR1 (CD35) and mouse IgGl isotype control antibody and PE conjugated anti-FcγRII (CD23), anti-CR3 (CDIIb), mouse IgG1 isotype antibody and mouse IgG2a isotype antibody were purchased from Immunotech (Marsseille, France).

4.2.2 COLLECTION AND PREPARATION OF SAMPLES

EDTA-anticoagulated (1.5mg EDTA/ml, blood) blood samples were collected from the 18 study subjects prior to and during the first two months of treatment. After the maintenance level was reached, samples were collected from three patients during the treatment cycle. Blood erythrocytes were lysed with 0.83% ammonium chloride (1.5 ml blood, 8.5 ml ammonium chloride) at +20 °C for 15 minutes. After centrifugation (400 x g for 10 minutes at +4 °C), the leukocytes were re-suspended in 500 µl ice-cold CMF-HBSS. The leukocyte count was determined with a Coulter counter model S blood cell analyzer (Coulter Electronics Inc., Hialeah, FL, USA).

4.2.3 MEASUREMENTS OF RECEPTOR EXPRESSION

Leukocytes ($3x10^5$) were incubated with monoclonal antibodies (mAb) in 12x75 mm polystyrene vials for 30 minutes at +4°C. The incubation volume was 90 µl. The control sample was incubated with isotype-matched mAbs directed to an irrelevant antigen. After incubation, the cells were washed with cold CMF-HBSS. Leukocytes were re-suspended in 500 µl cold CMF-HBSS and analysed in a Coulter EPICS (Coulter, Miami, Florida, USA) flow cytometer. The fluorescence of 5000 cells was measured using logarithmic amplification. A relative measure of receptor

expression was obtained by determining the mean log fluorescence intensity. The percentage of positive cells was generally 98-100 unless otherwise indicated.

4.2.4 IN VITRO EXPERIMENTS

Heparin anticoagulated blood samples, containing 0, 2.25, 11, or 55 ng/ml interferon-α were incubated at 37°C for 60 minutes. Whole blood lacing interferon-α served as control. After incubation, the red blood cells were lysed with 0.83% ammonium chloride, and the receptor expression was measured.

4.2.5 STATISTICS

Analysis of variance for repeated measurements was used for comparing differences between groups at different time points (SAS 6.12 version, proc mixed). Pair-wise comparison was tested by the Tukey HSD test with confidence limits between means. Confidence intervals for group means were calculated using the Scheffe method. The two-sample t-test was used for comparison of controls to treated patients. Values obtained in different treatment phases were analysed with one-way ANOVA. The level of significance was $p<0.05$.

4.3 PROGNOSTIC SIGNIFICANCE OF MOLECULAR MARKERS OF p53, Ki-67 AND COX-2 IN RCC ACCORDING TO OCCURRENCE OF METASTASES (IV, V)

4.3.1 PATIENTS, STAGING, AND HISTOLOGY

The study included samples from 117 patients with local or metastatic RCC treated in Turku University Hospital. Consecutive samples were collected from patients with local RCC treated between 1986 and 1996, with metastatic RCC between 1995 and 2001. As the patients with local disease had been free of metastases for at least 7.5 years, their samples were from an earlier period than the samples from patients with metastatic disease. The patients of the study were divided into three categories according to the occurrence of metastases. The patients with M0 staging (pTNM classification system) at primary diagnosis were divided into two subcategories: the first group, no metastases (nm), i.e. those patients whose RCC had no metastases within the follow-up of 7.5 years, and the second group, late metastases (lm), i.e. those patients whose RCC developed later metastases (= metachronous metastases) after the primary diagnosis. The third group, primary metastases (pm), was formed by those RCC patients who were M1 patients at primary diagnosis (pTNM classification system), (= synchronous metastases).

4.3.2 IMMUNOHISTOCHEMICAL STAINING AND SCORING OF p53, Ki-67 AND COX-2

From archival paraffin-embedded blocks, containing well-preserved cancer tissue, 5-μm-thick sections were cut, deparaffinized with xylene, and rehydrated through a graded series of alcohol. For antigen retrieval, the samples were boiled for 10 minutes in a microwave oven in 10mM sodium citrate buffer (pH 6.0). An automated processor (TechMate 500, DAKO) was used for immunohistochemical staining. Steps were performed in the immunostainer using the avidin-biotin-peroxidase staining methods.

Table 17. Evaluation of immunoreactivity of p53, Ki-67, and COX-2.

Monoclonal antibody	Antigen to detect	Antibody dilution	Counting of immunoreactivity	Classification of staining in the sample
DO-7 (Dako, Denmark)	p53	1:300	Percentage of carcinoma cells exhibiting p53 nuclei staining	As continuous data from undetectable levels (0%) to homogeneous (100%).
MIB-1 (Dako, Denmark)	Ki-67	1:100	Percentage of carcinoma cells exhibiting Ki-67 nuclei staining	As continuous data from undetectable levels (0%) to homogeneous (100%).
COX-2 (Dako, Denmark)	COX-2	1:100	a) Percentage of carcinoma cells exhibiting COX-2 nuclei staining b) Degree of intensity (absent/weak, pale, strong)	As three classes: 0 (no), absent/weak intensity in less than 10% of cancer cells; 1 (low), pale intensity in 10% or over of cancer cells; and 2 (high), strong intensity in majority of cancer cells.

The samples were incubated with commercial monoclonal antibody at optimal dilution, for 27 minutes as shown in Table 17, after which they were visualized by avidin-biotin-peroxidase staining. The immunoreactivity of p53, Ki-67, and COX-2 was counted for each tumor slide. Also, peritumoral inflammation as immunologic effect was analyzed: the amount of peritumoral lymphocytes was counted as 0 = no.; 1 = mild; 2 = moderate or 3 = severe (Tuna *et al.* 2004).

The 10% cut-off value was selected to achieve statistically reliable results, as well as in accordance with a previous study on the subject (Olumi *et al.* 2001). Staining without the primary antibody served as negative control. No significant background staining was detectable. The reliability of staining was measured by standard positive controls used as weekly standard controls in the routine pathological laboratory.

Immunohistochemistry of p53, Ki-67, and COX-2 was scored as a consensus of two investigators (Minna Kankuri-Tammilehto, Karl-Ove Söderström). The pathologist (Karl-Ove Söderström) also analyzed the reaction of peritumoral inflammation.

4.3.3 STATISTICAL ANALYSIS

Univariate associations between the variables were evaluated using contingency tables and χ^2 or Fisher's exact test. When metastatic group, tumor size, and grade were the dependent variables, the univariate and multivariate associations of dependent variables and the prognostic factors, p53 and Ki-67, were analyzed using logistic regression analysis (Hosmer *et al.* 2000). As the ordinal-type dependent variables consisted of more than two categories, the cumulative logistic models (proportional odds model) were used instead of the traditional binary logistic regression analysis. The results of logistic regression were quantified by calculating odds ratios (OR) and cumulative odds ratios (COR) with 95% confidence intervals (95% CI). Kaplan-Meier survival curves for p53 and Ki-67 were calculated for patients with primary metastases (pm) or later metastatic disease (lm), and the curves were compared using the Log-Rank test. Additionally, for the patients with later metastatic disease (lm), prognostic value of the clinicopathological variables and biomarkers, p53 and Ki-67, for metastases-free and overall survival were analyzed using the Cox proportional hazards model. In all tests, p-values less than 0.05 were considered statistically significant. Statistical calculations were performed using SAS System for Windows, release 8.02/2001 (SAS Institute, Cary, NC).

The associations between patient characteristics and COX-2 expression were evaluated using cross-tabulation, χ^2 or Fisher's exact test, and one-way analysis of variance (age). The Cox proportional hazards model was used to analyze the association of the clinicopathological variables, the biomarkers, p53 and Ki-67, and COX-2 expression, with metastases-free survival and overall survival. Variables significantly associated with survival in univariate Cox models were included in the multivariate Cox model. The results were quantified by calculating hazard ratios with 95% confidence intervals. Survival curves were calculated using the Kaplan-Meier method. The differences between the curves were tested with the log-rank test.

The association of COX-2, p53 and Ki-67 with the metastatic group was analyzed using univariate and multivariate multinomial logistic regression. The results were quantified by calculating odds ratios with 95% confidence intervals. In all tests, p-values less than 0.05 were considered statistically significant. Statistical calculations were performed using SAS System for Windows, release 8.02/2001 (SAS Institute, Cary, NC).

5 RESULTS

5.1 EFFICACY AND TOLERANCE OF PROLONGED ADMINISTRATION OF IFN-α_{2a} IN mRCC IN PHASE II CLINICAL STUDIES. (I, II)

At the time of analysis the median progression-free survival was eight months, and the median overall survival 19.1 months. The five-year survival rate was 16%. There were statistically significant differences in survival by response to treatment (log-rank test, $p<0.001$, Figure 2). In four (20%) of 20 responders the IFN-α dose was under 9 MU 3 tiw. The dose for the remaining 16 (80%) responders was between 9 and 18 MU 3 tiw. The mean duration of treatment with interferon-α was 11 months (range 2 weeks to 32 months).

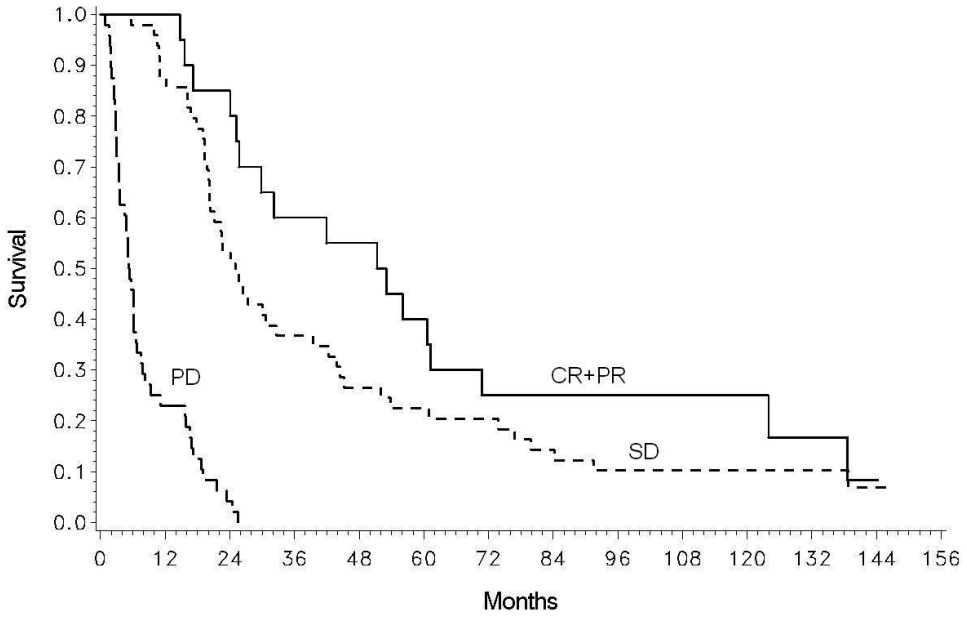

Figure 2. Kaplan–Meier survival curves for responding patients, patients with stable and progressive disease (log-rank P< 0.001). CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

The patients were classified according to the Cleveland Clinic Foundation scoring system into three risk groups (Choueiri *et al.* 2007). The median overall survival and five-year survival proportions, as well as the number of objective responses (CR + PR) in each risk group are shown in Table 18. Figure 3 shows the Kaplan-Meier survival curves for the three risk groups.

Table 18. Survival for risk groups according to Cleveland Clinic Foundation scoring system.

Risk group	Number of poor prognostic factors	No. of patients	No. of objective responses (CR+PR)	Proportion of five-year survivors (%)[1]	Median overall survival (mos) [range]
Low	<2	60 (52%)	13 (22%)	23	24.3 [19.1 to 32.7]
Intermediate	=2	33 (29%)	6 (18%)	15	14.7 [5.6 to 22.3]
High	>2	22 (19%)	1 (5%)	0	6.2 [3.0 to 9.4]

[1]p-value <0.001

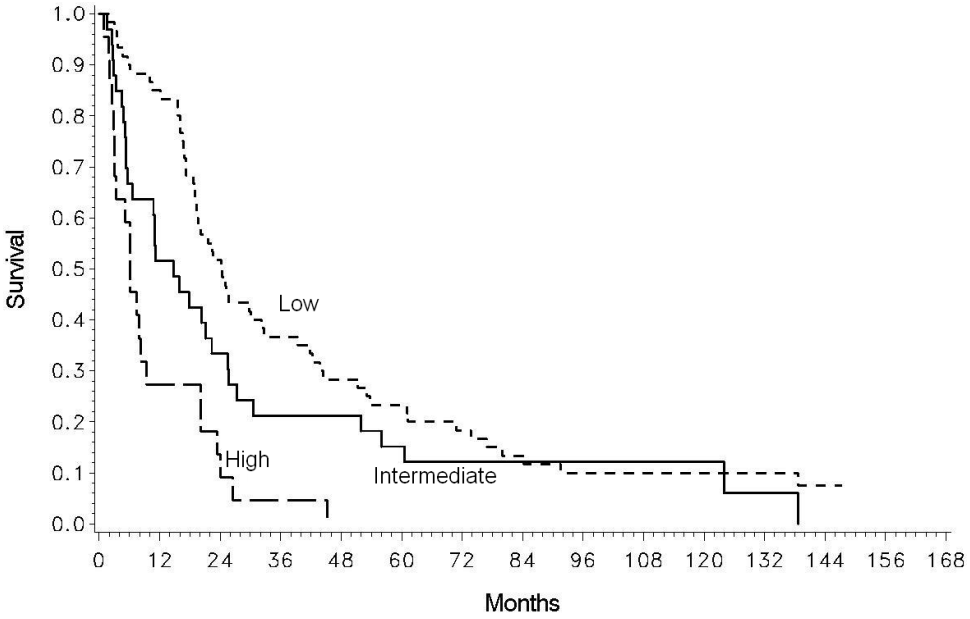

Figure 3. Kaplan–Meier survival curves for risk groups according to the Cleveland Clinic Foundation scoring system (log-rank p<0.001).

5.2 CHANGES IN BLOOD NEUTROPHIL AND MONOCYTE RECEPTOR PROFILE DURING IFN-α_{2A} ADMINISTRATION IN MRCC (III)

The phagocyte receptor expression in kidney cancer patients (n=18) compared with normal controls (n=39) is presented in Table 19 (neutrophils) and Table 20 (monocytes). The mean fluorescence intensity of neutrophils labeled with anti-CR3 mAbs was significantly higher in renal cancer patients than in controls (p=0.002). Furthermore, the proportion of FcγRI positive neutrophils was significantly raised in patients with renal cancer when compared to controls (p=0.0003). No alterations were observed in CR1, FcγRII or FcγRIII levels between healthy controls and cancer patients.

In the monocytes of the cancer patients, mean fluorosence intensities of all phagocyte receptors, except FcγRIII, were raised when compared to controls (Table 20). Furthermore, the proportion of FcγRIII positive monocytes was significantly increased (Table 20). No significant changes were observed in the proportion of monocytes expressing other phagocyte receptors.

Table 19. Mean fluorescence intensity of Fcγ- and complement receptor expression in neutrophils.

	Controls		Patients		
Neutrophils	**Mean**	**Std**	**Mean**	**std**	**p-value**
CR1	8.04	3.35	7.04	2.49	0.2656
CR3	8.34	4.67	14.87	7.34	0.0020
FcγRI %	28.87	20.46	52.37	23.06	0.0003
FcγRI	1.60	0.37	1.61	0.30	0.9468
FcγRII	6.80	3.08	7.98	3.29	0.1966
FcγRIII	117.26	32.70	113.53	35.30	0.6983

Healthy control subjects, n=39
Patients with mRCC prior to IFN-α therapy, n=18

Table 20. Mean fluorescence intensity of Fcγ- and complement receptor expression in monocytes.

	Controls		Patients		
Monocytes	**Mean**	**std**	**Mean**	**std**	**p-value**
CR1	8.48	2.86	10.34	3.49	0.0379
CR3	8.39	4.49	21.12	11.30	0.0002
FcγRI	8.24	1.81	10.06	3.56	0.0534
FcγRII	7.17	3.23	11.22	4.27	0.0002
FcγRIII	4.21	2.52	4.10	1.74	0.8635
FcγRIII %	42.06	16.90	54.43	18.80	0.0166

Healthy control subjects, n=39
Patients with mRCC prior to IFN-α therapy, n=18

To investigate the effects of IFN-α on receptor expression, samples during treatment and recovery phases were collected for analysis. In neutrophils, the expression of CR1 receptor decreased significantly during the treatment week (p=0.0114, baseline vs. treatment), whereas the others remained fairly constant. In monocytes, the receptor level of FcγRI increased significantly during treatment (p=0.0027, baseline vs. treatment), whereas in CR1, FcγRII and FcγRIII expression, a transient and statistically non-significant change was observed. The results are presented in Table 21 for neutrophils and in Table 22 for monocytes.

Table 21. Mean fluorescence intensity of Fcγ- and complement receptor expression in neutrophils.

	Before treatment		Treatment		Recovery		
Neutrophils	**Mean**	**std**	**Mean**	**std**	**Mean**	**std**	**p-value**
CR1	7.04	2.49	4.79	3.47	5.75	2.16	0.0263*
CR3	14.87	7.34	15.12	6.77	11.09	3.58	0.2154
FcγRI %	52.37	23.06	52.55	28.7	52.15	28.7	0.8315
FcγRI	1.61	0.30	1.66	0.27	1.60	0.41	0.8324
FcγRII	7.98	3.29	7.23	3.05	6.55	2.69	0.1750
FcγRIII	113.53	35.30	114.7	27.20	102.8	37.6	0.6524

* = CR1, baseline vs. treatment p=0.0114
Patients with mRCC prior to IFN-α therapy and recovery, n=18

Table 22. Mean fluorescence intensity of Fcγ- and complement receptor expression in monocytes.

	Before treatment		Treatment		Recovery		
Monocytes	**Mean**	**std**	**Mean**	**std**	**Mean**	**std**	**p-value**
CR1	10.34	3.49	8.29	4.76	9.76	4.27	0.2031
CR3	21.12	11.30	27.11	15.00	15.13	8.38	0.0959
FcγRI	10.06	3.56	13.98	5.07	10.85	3.19	0.0102*
FcγRII	11.22	4.27	10.71	2.96	8.99	2.74	0.2332
FcγRIII	4.10	1.74	4.16	1.57	4.31	1.29	0.9044
FcγRIII %	54.43	18.80	54.40	19.60	50.51	13.7	0.3430

* = FcγRI, baseline vs. treatment p=0.0027
Patients with mRCC prior to IFN-α therapy and recovery, n=18

5.3 PROGNOSTIC SIGNIFICANCE OF MOLECULAR MARKERS OF p53, Ki-67 AND COX-2 IN RCC ACCORDING TO OCCURRENCE OF METASTASES (IV, V)

Both p53 and Ki-67 were markers for overall survival in the pm/lm patients (Figure 4 and Figure 5, respectively). The median overall survival was 24 months in p53-positive, and 59 months in p53-negative patients (Log-rank test, p=0.030), and 24 months in Ki-67-positive, and 63 months in Ki-67 negative patients (Log-rank test, p=0.031).

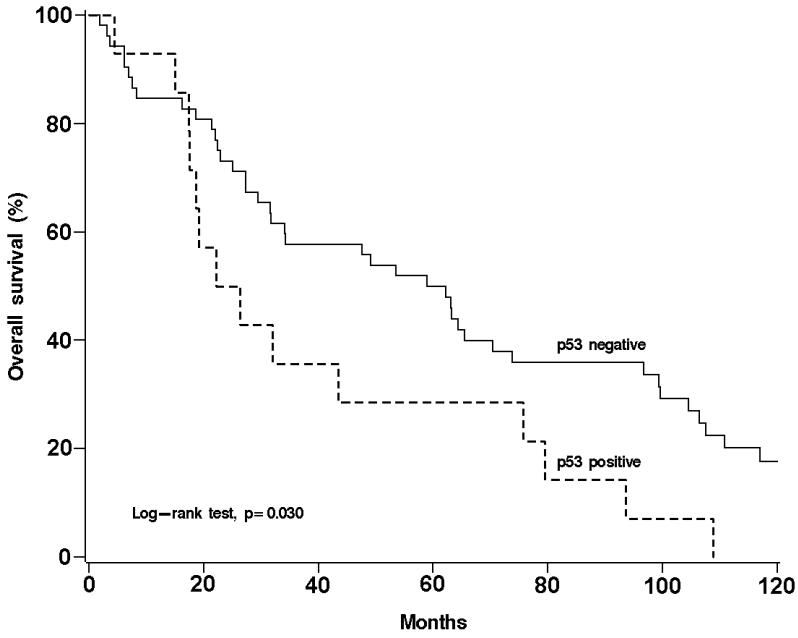

Figure 4. Kaplan-Meier survival curve for p53 in mRCC (n=66).

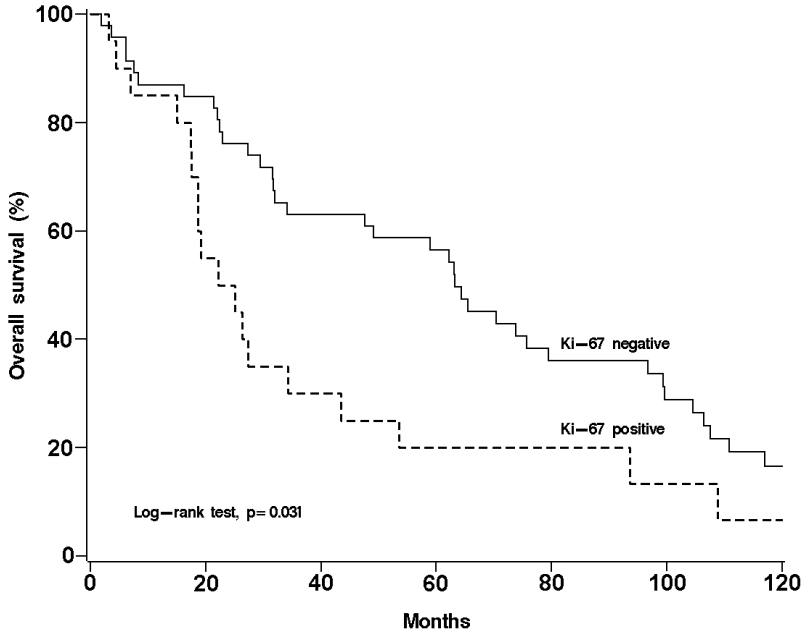

Figure 5. Kaplan-Meier survival curve for Ki-67 in mRCC (n=66).

In mRCC, COX-2 negativity/Ki-67 positivity was associated with shorter overall survival from nephrectomy when compared to COX-2 positivity/Ki-67 negativity (median overall survival time 19 vs. 97 months) (HR=3.5, 95% CI 1.5-8.1, p=0.004) (Figure 6, Log-rank test). Additionally, double positivity (HR=0.4, 95% CI 0.1-1.1, p=0.083) or double negativity (HR=0.5, 95% CI 0.3-1.0, p=0.061) of COX-2/Ki-67 was associated with longer overall survival when compared to COX-2 negativity/Ki-67 positivity (Figure 6, Log-rank test).

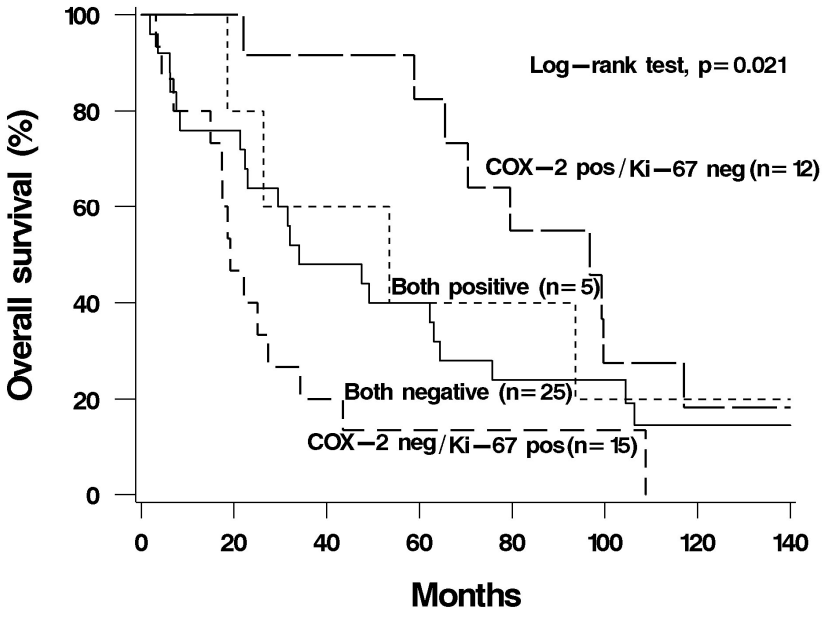

Figure 6. The prognostic value of covariation of COX-2/Ki-67 for overall survival from nephrectomy in RCC patients with metastases (either primary presentation or later) (n=57, Kaplan-Meier method): the median overall survival time was 97 months with COX-2 positivity/Ki-67 negativity, and 19 months with COX-2 negativity/Ki-67 positivity (p=0.004).

The prognostic value of variables for overall survival in the pm/lm group of patients is presented in Table 23 (Cox regression analysis). COX-2 negativity was associated with shorter overall survival, when compared to COX-2 positivity (median overall survival time 28 vs. 94 months)(p=0.027). The higher the T-stage (T3,T4), the shorter was the overall survival (p=0.012); patients with high T-stage (T3,T4) had double the risk of death compared to patients with low T-stage (T1,T2). Patients with grade 3 tumors had a 2.5 times higher risk of death when compared to patients with grade 1 tumors (p=0.039). p53 and Ki-67 negativity showed a trend toward longer overall survival (p=0.063 and p=0.068, respectively). In multivariate analysis, only T-stage was an independent variable for overall survival.

The median metastases-free survival was shorter with COX-2 negative tumors when compared to those with COX-2 positive tumors (15 vs. 46 months)(HR=2.5, 95% CI 1.1 - 5.3, p=0.020, Log-rank test) (Figure 7).

Table 23. Prognostic value of variables for overall survival in RCC patients with metastatic disease (n=57).

Variable	Univariate analysis p-value	Hazard ratio	95% confidence interval	Multivariate analysis[4] p-value	Hazard ratio	95% confidence interval
T-stage (3+4 vs. 1+2)[1]	0.012	2.3	1.2 to 4.4	0.048	2.0	1.0 to 3.9
Tumor grade[2]	0.109[2]					
2 vs. 1	0.277	1.5	0.7 to 3.3	0.582	1.3	0.6 to 2.9
3 vs. 1	0.039	2.5	1.0 to 6.0	0.264	1.7	0.7 to 4.1
COX-2 (negative vs. positive)	0.027	2.1	1.1 to 3.9	0.105	1.8	0.9 to 3.6
p53 (positive vs. negative)	0.063	1.8	1.0 to 3.4			
Ki-67 (positive vs. negative)	0.068	1.7	1.0 to 3.1			
Sex (female vs. male)	0.119	1.6	0.9 to 2.8			
Age at nephrectomy (< 65 yrs. vs. others)	0.417	1.3	0.7 to 2.3			
Heidelberg[3] (conventional vs. other types)	0.296	1.4	0.7 to 2.8			

Analyzed using Cox regression analysis. ns=not significant. Analyzed from nephrectomy.
[1] One patient was not evaluable for T-stage.
[2]Overall p-value for difference between tumor grades.
[3]According to Heidelberg classification
[4]The variables that were positive in univariate analysis were analyzed in multivariate analysis.

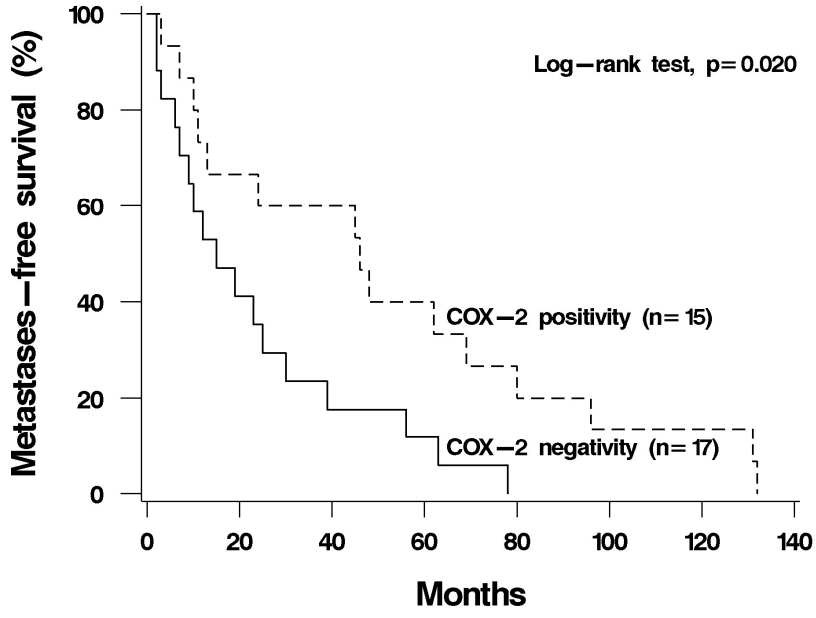

Figure 7. The prognostic value of COX-2 for metastases-free survival from nephrectomy in RCC patients who later developed metastatic disease (n=32, Kaplan-Meier method): the median metastases-free survival time was 46 months with COX-2 positivity, and 15 months with COX-2 negativity.

The prognostic value of variables for metastases-free survival in the lm group of patients is presented in Table 24 (Cox regression analysis). COX-2 was the only variable to have prognostic value for metastases-free survival.

Table 24. Prognostic value of variables for metastases-free survival in RCC (lm patients, n=32).

Variable	Univariate analysis p-value	Hazard ratio	95% confidence interval
T-stage (3+4 vs. 1+2)[1]	0.089	1.9	0.9 to 4.1
Tumor grade[2]	0.218		
2 vs. 1	0.886	0.9	0.4 to 2.1
3 vs. 1	0.101	2.6	0.8 to 8.3
COX-2 (negative vs. positive)	0.024	2.5	1.1 to 5.3
p53 (positive vs. negative)	0.684	1.2	0.5 to 3.2
Ki-67 (positive vs. negative)	0.196	1.8	0.7 to 4.2
Sex (male vs. female)	0.823	1.1	0.5 to 2.2
Age at nephrectomy (65 or older vs. < 65 yrs)	0.668	1.2	0.6 to 2.5
Heidelberg[3] (Other types vs. conventional)	0.989	1.0	0.3 to 2.9

Analyzed using Cox regression analysis. Analyzed from nephrectomy.
[1]One patient was not evaluable for T-stage.
[2]Overall p-value for difference between tumor grades.
[3]According to Heidelberg classification

6 DISCUSSION

6.1 IFN-α IN mRCC

6.1.1 RESPONSE TO AND LONG-TERM SURVIVAL WITH PROLONGED USE OF IFN-α THERAPY

Randomized trials have indicated that IFN-α increases the survival of RCC patients (MRCRCC 1999, Pyrhönen *et al.* 1999). Therefore, before the era of novel targeted therapies, IFN-α was considered as the standard comparator for first-line therapy of mRCC. The current questions concerning the use of biological response modifiers, such as IFN-α, are the timing (Motzer *et al.* 2007, Stadler *et al.* 2007) and the optimal dosage of IFN-α, alone or in combination with targeted therapies (Bracarda *et al.* 2007).

One of the aims of this study was to investigate whether prolonged IFN-α therapy with intermittent administration is feasible and effective. In most previous studies, the IFN-α treatment duration has been 6 to 24 weeks (Négrier *et al.* 2007, Atzpodien *et al.* 2002, MRCRCC 1999), and most responses have been observed within 8 - 24 weeks (Négrier *et al.* 2007, MRCRCC 1999). Since host immune mechanisms are apparently important in regulating tumor growth and in patients responding to IFN-α (Motzer *et al.* 1996, Marcus *et al.* 1993, Rosenberg *et al.* 1993, Oliver *et al.* 1989), a prolonged schedule of IFN-α administration was used in the present study.

The patients in the current study achieved median overall survival of 19.1 months, which was longer compared to the weighted average median survival of 11.4 months in a meta-analysis of 644 mRCC patients treated with IFN-α (Coppin *et al.* 2007). In the study of MRCRCC (1999), when comparing IFN-α for 12 weeks versus medroxyprogesterone acetate, the median survival time was 8.5 months in the IFN-α group and 6 months in the other group. In the study of Négrier *et al.* (2007), the median overall survival was 15.2 months for the IFN-α group, and 16.8 months for the IFN-α plus IL-2 group, with treatment duration of 24 weeks. Pyrhönen *et al.* (1999) observed median overall survival of 16.9 months for IFN-α plus vinblastine following a median treatment duration of 6.0 months. The results of the current study show that the overall survival was even longer despite the treatment cycle pause, compared to other reports on continuous IFN-α therapy with or without additional agents.

Previously reported five-year long-term survival of patients with IFN-α based therapy has been approximately between 9% and 16% (Atzpodien *et al.* 2002, Marincola *et al.* 1995, Fosså *et al.* 1992c). In all these studies, additional agents have been used, e.g. IL-2 (Marincola *et al.* 1995), 5-

fluorouracil (Atzpodien *et al.* 2002), vinblastine (Fosså *et al.* 1992c), and 13-cis-retinoid acid (Atzpodien *et al.* 2002). Only few reports of long-term outcome with IFN-α therapy alone in mRCC have been published. In the study of Minasian *et al*. (1993), a five-year survival rate of only 3% was reported with IFN-α monotherapy. In that study, the planned treatment duration was three months in one trial and until progression of disease in the other trials; the median treatment duration was not reported. The current long-term outcome study (an intention-to-treat analysis) shows that 16% of mRCC patients with prolonged IFN-α therapy achieved five-year survival.

Stratifying the patients into different prognostic groups, e.g. according to the Cleveland Clinic Foundation scoring system (Choueiri *et al*. 2007), enables better comparison of the results in different studies. The results of the current study demonstrate that approximately one out of four patients in the low risk group achieved five-year survival, and even in the intermediate risk group, approximately one in seven patients survives for at least five years. With prolonged IFN-α, the median overall survival in the low risk group was 24.3 months. Even in the intermediate risk group it was better, 14.7 months, than the weighted average median survival of 11.4 months in the previously mentioned meta-analysis in mRCC patients (Coppin *et al.* 2007). The benefit of IFN-α in mRCC patients with intermediate risk has been discussed recently. In the study of Négrier *et al* (2007), the survival benefit was not observed in patients with intermediate prognostic factors, but Pyrhönen *et al.* (1999) observed in their study (IFN-α plus vinblastine versus vinblastine) that the survival benefit of IFN-α may be greater in those patients with adverse prognostic factors, such as poor performance status, age over 60 years, and male gender. In the present study, the considerably large number of patients with intermediate risk and, additionally, the rather good median overall survival, may indicate that the intermediate risk group patients would also benefit from prolonged IFN-α therapy.

The objective response rate of 17% in the current study is comparable to rates in other studies with IFN-α alone (Négrier *et al.* 2007, MRCRCC 1999), or IFN-α with IL-2 (Négrier *et al.* 2007), and IFN-α with vinblastine (Pyrhönen *et al.* 1999). In the present study, patients with stabilized disease (42%) evidently benefitted from the prolonged treatment. The survival of patients with stabilized disease was close to the survival of responding patients. Those patients who achieve objective responses (CR + PR) reach ten times better median overall survival compared to those with progressive disease. The complete responders had metastases mainly in the lungs (7 out of 9 complete responders). In addition, one of the complete responders had bone metastases, which disappeared as observed by bone scintigraphy, and a decrease in alkaline phosphatase to normal levels. One responder had liver metastases, which disappeared as observed by CT. It has been suggested that responses to IFN-α rarely last more than two years (Wirth 1993). The data from this

study support the fact that durable responses to IFN-α are possible, and that a response duration for CR patients of a median of four years, and even more than 11 years, is possible to achieve. In the current study, four patients (3%) achieved partial response after 12 months of treatment, one of them at 17 months after the onset of therapy. One of these patients later achieved a complete response at 22 months after the onset of therapy. In one patient, the metastases seemed at first to progress slightly at 12 months and then decreased in size. This observation of late responses is a new clinical finding. This is important since response to IFN-α is a significant prognosticator for overall survival in mRCC patients. The observation of late responses in the present study indicates that prolonged IFN-α may be beneficial, as the overall survival and five-year survival data indicate.

Median progression-free survival was eight months with the present IFN-α treatment. After the progression during IFN-α therapy, no patients were treated with bevacizumab or tyrosine kinase inhibitors. Two of the patients received HD-IL-2 therapy after the progression without success. In addition, two patients were treated with capecitabine without response. Therefore, the achieved five-year survival rate is specifically due to IFN-α therapy.

The incidence of primary or late brain metastases due to RCC has been reported to be as high as 11% in a large autopsy series (Saitoh 1981). In the present study, no brain metastases were present at the beginning of the treatment since it was an exclusion criterion. The incidence of brain metastases during IFN-α or after discontinuation of IFN-α was one in seven patients: one third developing brain metastases during the IFN-α therapy, and two thirds after discontinuation of the IFN-α therapy. The RCC patients with pulmonary metastases were the most likely subjects to develop brain metastases. This is in accordance with previous reports, where the lungs are the most common metastatic location associated with brain metastases (Mori *et al.* 1998). We suggest that this is due to the fact that patients with lung metastases have the longest survival and, consequently are the most susceptible to brain metastases. The median overall survival after detection of brain metastases was only 2.7 months, which is comparable to findings in other studies (Wronski *et al.* 1997, Decker *et al.* 1984). The median detection time of brain metastases was 21 months after the start of IFN-α therapy. Men were more likely to suffer from brain metastases than women. Brain metastases are a late manifestation of the disease and are usually associated with progression also in extracranial sites. More effective treatments than are currently available are needed for those mRCC patients who develop brain metastases; both to treat actual brain metastases, as well as the extracranial disease.

The frequency of spontaneous regression in mRCC has been a controversial issue (Gleave *et al.* 1998, Marcus *et al.* 1993). Spontaneous regression has been suggested for approximately 1% of

RCC patients, although a higher percentage of up to 6.6% has been reported for selected patient groups (Gleave *et al.* 1998). In the present study, two patients (2%) had spontaneous regression prior to IFN-α therapy, one of whom, with lung metastases, achieved complete response to IFN-α, the other, with pleural and para-aortic lymph node metastases, had a stable disease. Spontaneous regression has most often occurred in RCC patients with pulmonary metastases (Gleave *et al.* 1998), as was also the case in this study.

6.1.2 CLINICOPATHOLOGICAL PROGNOSTIC FACTORS IN MRCC PATIENTS

Several prognostic models including different clinicopathological prognostic factors for survival have been created for mRCC. The heterogeneity of RCC within the same T-stage and grade has resulted in a need for prognostic models for prognostication and treatment modality selection. The Cleveland Clinic Foundation scoring system (Choueiri *et al*. 2007) was used in this study as the prognostic factors used in the model are easily applied and were available from the patient records. The widely used MSKCC 2002 prognostic model (Motzer *et al.* 2002b) could not have been used as LDH and corrected calcium values were not routinely recorded for the patients, as the study included patients from 1994 onwards. Nor could the Négrier *et al.* 2002 model have been used as the CRP value was not routinely recorded for the patients.

According to previous reports, prognostic models are good tools for prognostication and patient selection for risk groups. In clinical work, prognostic models could be more widely used to guide the laboratory schema. According to this study, the Cleveland Clinic Foundation scoring system differentiates well three different prognostic groups. However, different prognostic models are needed for different treatment modalities, for example, the MSKCC 2008 prognostic model (Motzer *et al.* 2008a) has been created for those RCC patients with sunitinib therapy.

In metastatic RCC, the following independent clinical prognostic factors for poor survival have been reported, e.g. poor performance status, high number of metastatic sites, and Hb level lower than normal baseline (Négrier *et al* 2002, Motzer *et al.* 1999). Similarly, in the present study, all these, as well as other metastatic site than the lung, and the presence of bone or liver metastases, were significant predictors of poor survival in Cox regression analysis. In multivariate analysis, response to IFN-α was associated with prolonged progression-free survival. De Forges *et al.* (1988) included the presence of liver metastasis in their prognostic model, but in the newer prognostic models, metastatic site has rarely been included (Motzer *et al.* 2004, Motzer *et al.* 2008a). Performance status is the most consistently used prognostic factor in the models. According to the current study, performance status and response to treatment are the most important clinical predictors in mRCC. Of course, response to treatment cannot be included in prognostic models at

the initiation of IFN-α, but it can be used when following and evaluating the patient's response to predict their prognosis. The presence of lung metastases was a significant prognostic factor for five-year survival in multivariate analysis. It has been previously reported that remission is more likely in those patients with only lung metastases, approximately 30%. This may be in part due to the methods of measuring pulmonary lesions. Small lesions are more likely to be seen by X-ray in the lung than by scintigraphy or CT in bone, liver or other viscera. Thus, it is possible that lung metastases are observed earlier than other metastases. In future, prognostic models should be developed by adding novel prognostic factors such as biomarkers.

6.1.3 TOXICITY OF IFN-α COMPARED TO OTHER THERAPIES

The intermittent administration of IFN-α was chosen since continuous therapy as reported, and also in our experience, has been associated with significant toxicity. The treatment with IFN-α was planned to be continued for 24 months or as long as progression or severe adverse effects were encountered. In some responding patients with good performance status, IFN-α therapy was continued for even longer.

Toxicity of IFN-α is dose-dependent and high doses of IFN-α result in excessive toxicity requiring dose reduction or discontinuation (Krown 1987, Trump *et al.* 1987). In this study, as in the studies of Minasian *et al*. (1993) and Steineck *et al*. (1990), the highest tolerable dose was defined for each patient by escalating the dose in the beginning of treatment. Previous studies have indicated that doses of 5 to 18 MU of IFN-α three times a week seem to be effective and tolerated (Krown 1987, Muss *et al.* 1987, Kirkwood *et al.* 1985). This is supported by the present result; only two patients had a dose of lower than 4.5 MU, one in a responding patient.

Altogether 8% of patients discontinued the treatment because of fatigue, elevation of liver enzymes, or cardiac arrhythmias. The degree of discontinuation is low compared to other studies with IFN-α (Fosså 1988, Fosså *et al.* 1986). In the study of Muss *et al.* (1987), the frequency of grade 3 and 4 toxicity was greater in the group of patients who received an IFN-α dose from 30 to 50 MU/m^2 intravenously for five consecutive days every three weeks compared to those patients with 2 MU/m^2 subcutaneously tiw. In our study, grade 3 toxicity was rare. In the long-term analysis of 117 mRCC patients, no life-threatening side-effects were observed. However, for patients with cardiac problems, such as cardiac arrhythmias or insufficiency, IFN-α therapy must be carefully considered as IFN-α may cause sudden cardiac death (Olencki *et al.* 2001). IFN-α is also neurotoxic, causing depression, and in rare cases, confusion, but in this study mostly of grade 1 toxicity. Fatigue, fever, poor appetite, nausea, abnormal liver enzymes, and muscular or joint pain were found in more than 20% of patients in the current study. These data show that prolonged and

intermittently administered IFN-α is well tolerated; the one-week pause every four weeks allows most patients to continue prolonged treatment with the highest tolerable dose.

The toxicity profile of IFN-α is different to that of sunitinib, which often causes diarrhea, fatigue, and nausea, and may cause anemia, leucopenia, hypertension, migraine, palmar-plantar erythrodysesthesia, hypothyreosis, and decreased blood glucose level (Gore *et al. 2*009). In more rare cases, sunitinib causes cardiac failure, venous thromboembolic events, and pulmonary events, such as dyspnea. Both IFN-α and sunitinib may in very rare cases cause encephalopathy syndrome (Cumurciuc *et al.* 2008, Mitsuyama *et al.* 1992). Neither the subcutaneously administered IFN-α nor the orally administered sunitinib require ward or policlinical resources as do the intravenous therapies. The different toxicity profiles of IFN-α and sunitinib enable the choosing of medication, paying attention to the patients' concomitant diseases and medication.

6.1.4 TIMING OF IFN-α IN THE ERA OF NOVEL TARGETED THERAPIES

In the era of novel targeted therapies, the choices for mRCC therapy have increased. Sunitinib, sorafenib, temsirolimus, everolimus, and bevacizumab have been approved for clinical use in the EU between 2006 and 2009. Sunitinib improves disease-free survival with acceptable toxicity. The addition of bevacizumab to IFN-α in conventional RCC increases the median progression-free survival. With IFN-α plus bevacizumab, fatigue, stomatitis and hematological toxicity have been less common compared to sunitinib therapy. Temsirolimus has recently been observed to improve overall survival of mRCC patients, even those with non-conventional RCC (Dutcher *et al.* 2009). As a second-line therapy in cytokine refractory mRCC, bevacizumab increases time to progression for patients in the intermediate risk group with good performance status (Yang *et al.* 2003a), while sorafenib increases progression-free survival. After the progression on other targeted therapies, everolimus prolongs progression-free survival (Motzer *et al.*2008b). ESMO, NCCN, and EAU have created guidelines for the treatment of RCC, INF-α has been defined as an optional therapy (Escudier *et al.* 2009, Motzer *et al.* 2009, Ljundberg *et al.* 2007).

The current status of biological response modifiers to treat mRCC is not established. Sunitinib or bevacizumab plus IFN-α are currently considered the drugs of choice for good or intermediate risk groups (Escudier *et al.* 2007b, Motzer *et al.* 2007). For the poor risk group, temsirolimus or sunitinib is recommended (Motzer *et al.* 2007, Hudes *et al.* 2007). The progression-free survival of the patients with IFN-α in the present study was better compared to the progression-free survival with IFN-α or IFN-α with placebo in the studies of sunitinib and bevacizumab with IFN-α (Motzer *et al.* 2007, Escudier *et al.* 2007b). IFN-α based treatment duration in those studies was less than or equal to 5.1 months, whereas, in the present study, the treatment duration was longer (mean 11

months), a duration evidently made possible evidently by the one-week pause every four weeks. The long treatment duration may be the reason for better progression-free survival in the present study. The median overall survival and long-term survival analyses of sunitinib and bevacizumab with IFN-α are still ongoing.

Many ongoing trials are trying to answer the question of timing of IFN-α. According to the data of the present study, prolonged IFN-α therapy may also be considered as an additional choice for first-line therapy, especially in those patients whose concomitant disease or medication does not allow the use of sunitinib or bevacizumab. Prolonged IFN-α therapy may not be beneficial in patients in the poor risk group but it is beneficial in those in the intermediate risk group. Previously, conventional RCC has been proven to have the best response to and most favorable outcome from IFN-α compared to the other RCC subgroups (Motzer *et al.* 2002a). Differences in survival between IFN-α and the novel therapies cannot be reliably compared, as long-term survival data on novel therapies are not yet available. However, comparing prolonged IFN-α therapy to sunitinib, or bevacizumab with IFN-α in a randomized trial would give more exact information about whether there are differences in survival.

Response rates in mRCC vary greatly in different studies. In different sunitinib trials, response rates have varied from 9% to 44% (Gore *et al.* 2007, Motzer *et al.* 2007) With temsirolimus therapy, objective responses were infrequent as was the case with IFN-α therapy (Dutcher *et al.* 2009). Prior observations have shown that the difference in remission rates with different cytokine therapies has not been found to be a reliable surrogate for survival in mRCC (Coppin *et al.* 2007). The two following examples describe this phenomenon. Initial nephrectomy prior to planned IFN-α for mRCC patients improves survival and delays time to progression despite an unimproved response rate (Flanigan *et al.* 2001, Mickisch *et al.* 2001). Also, combining LD-IL-2 with IFN-α increases the response rate compared to one of the therapies alone, but the improvement in response rate does not translate into better survival (Négrier *et al.* 2007, Négrier *et al.* 1998). For this reason, survival data are more precise than response rates for comparison of results in different studies.

High-dose-IL-2 (HD-IL-2) may increase the complete response rate and improve survival in conventional RCC patients with the poorest prognosis (Coppin *et al.* 2007, Spanknebel *et al.* 2005); those with primary tumor still in place or with either liver or bone metastases (McDermott *et al.* 2005). HD-IL-2 has not been compared to LD-IL-2 or IFN-α in a randomized trial, but it has been observed that HD-IL-2 may cause durable responses in 7-8% of patients (McDermott *et al.* 2005, Yang *et al.* 2003b). HD-IL-2 therapy may increase cardiac toxicity, when administered after

VEGFR-TKI therapy (vascular endothelial growth factor receptor tyrosine kinases). HD-IL-2 is the therapy of choice for only a few patients because of its high toxicity; patients have to have (very) good performance status and organ function, such as cardiac function (Spanknebel *et al.* 2005). The immunohistochemical analysis of CA9 expression can find those patients who will benefit from IL-2 therapy (Atkins *et al.* 2005, Bui *et al.* 2003): higher CA9 expression predicts longer survival compared to low CA9 expression.

Currently, patients should be treated in trials, if possible, to obtain more knowledge about the timing of different therapies. Also, more survival data with different therapies are needed from non-conventional types of RCC. PEG-IFN-α is more convenient to administer and has potential for increased efficacy and less toxicity compared to IFN-α (Sunela *et al.* 2009, Feldman *et al.* 2008); this should also be further assessed in clinical trials. Assessing the patients into different prognostic groups is nowadays possible with many models; e.g. for IFN-α with MSKCC (Motzer *et al.* 2002b), the Cleveland Clinic Foundation (Choueiri *et al.* 2007), or Group Francais d'Immunotherapie (Négrier *et al.* 2002), and for sunitinib with MSKCC (Motzer *et al.* 2008a). In future, vaccine delivery systems for generation of immune responses against RCC with IFN-α (Hawkins *et al.* 2009, Viaud *et al.* 2009) or alone (Gitlitz *et al.* 2003), and gene therapy (Guse *et al.* 2009) as targeted routine therapies may be used clinically for mRCC patients. Additionally, vaccine therapy is currently the only promising systemic therapy in the adjuvant setting for RCC patients.

6.2 BIOLOGICAL EFFECTS OF IFN-α

IFN-α is a pleiotropic cytokine and it has immunomodulatory, antiangiogenic, proapoptotic, antiviral and antiproliferative effects. IFN-α has different cytostatic and immunomodulatory effects (Pestka 1983). IFN-α is known to activate monocytes and NK cells. The exact antitumor mechanisms against RCC have not been defined. For targeted therapy, knowledge of specific types of the biochemical derangements created by IFN-α is needed. In this study, the impact of IFN-α on phagocyte receptors for IgG and complement in monocytes and neutrophils was investigated. The impact of IFN-α on phagocyte receptors has not been previously reported.

According to the present study, in mRCC patients in neutrophils the expression of CR3 receptors and the proportion of FcγRI positive neutrophils was significantly raised. As CR3 is previously known to serve as an adhesion molecule, which binds neutrophils to other cells, e.g. cancer cells, the observed activation may reflect a response of neutrophils to cancer cells. FcγRI has previously been shown to be important in cell-mediated cytoxocity. In monocytes, the expression of all phagocyte receptors, except FcγRIII, were raised when compared to controls. In neutrophils, IFN-α

treatment lowered the elevated receptor expressions. During early treatment (<2 months) a significant decrease in the expression of CR1 receptors in neutrophils was observed. In monocytes, a significant activation of the expression of FcγRI receptors was observed. The data of the current study show that changes in receptor expression reflect the inflammatory activation of phagocytes in mRCC. IFN-α, both in vivo and in vitro, modulates the expression of phagocytic receptors.

The observed receptor expression in mRCC patients in the present study differs from previous observations in patients with infectious disease, as reported by Leino *et al.* (1997). The observed receptor expression during IFN-α therapy differs from previous observations after the induction of IFN-γ reported by Buckle and Hogg (1989). Previous clinical studies with mRCC have indicated a lack of efficacy of IFN-γ, whereas IFN-α improves overall survival in mRCC with a response rate of 14% to 30% (MRCRCC *et al.* 1999, Pyrhönen *et al.* 1999, Gleave *et al.* 1998). In this study, the differences in the receptor expression reflect the impact of IFN-α. The differences are not similar to what has been observed due to infectious disease or IFN-γ.

This sensitive, although arbitrary, method of investigation is useful in characterizing and differentiating specific clinical conditions. Although it is not sensitive enough for cancer diagnosis, it may add information on specific treatment effects and immunomodulation. This is in agreement with our clinical observation of early radiological progression in some patients responding later when treatment with IFN-α was continued. By using whole blood leukocytes, expression provoked by purification procedures can be avoided (Leino *et al.* 1997).

6.3 CLASSIFICATIONS ACCORDING TO MORPHOLOGY AND GENETIC FINDINGS IN RCC

The Heidelberg classification, which was published in 1997, subclassifying RCCs, was the first to combine morphology and genetic findings, and is considered a pioneer work in the field. Therefore, it was the natural choice in the studies of p53, Ki-67 and COX-2 at the time. Later, in 2004, WHO published the reassessed classification which is also based on both genetic and pathological abnormalities. The Heidelberg classification is still widely used worldwide. Both the Heidelberg and the WHO 2004 classifications contain conventional, papillary, chromophobe and collecting duct RCCs, which are the most often observed subtypes in RCC. In the Heidelberg classification, other RCC types are classified as unclassified type of RCC. In the WHO 2004 classification, many rare subtypes are added. These rare subtypes expand our knowledge of the biology of RCCs. In statistical regression analysis, the Heidelberg classification is a useable and clear classification, because the very rare subtypes, excluding collecting duct RCC, are classified as unclassified RCC. It is known that conventional, papillary, chromophobe and collecting duct RCCs differ from each

others in the five-year DSS in localized RCC, and response to treatment modalities in mRCC. However, in studies, neither the Heidelberg nor the WHO 2004 classification are found as independent prognostic factors for survival in localized or metastatic RCC. In future, classifications according to both morphology and genetic findings will be developed as new knowledge of the RCC biology is acquired.

6.4 T-STAGE AND GRADE AS PROGNOSTIC FACTORS IN RCC

Tumor stage and grade have previously been identified as the most important prognostic factors in RCC. The current study indicates that T-stage is a prognostic factor for metastases-free and overall survival in RCC patients. In patients who later developed metastatic disease, high T-stage caused twice the risk of metastatic disease and three times the risk of death compared with low T-stage. This indicates that as the tumor size increases, the more aggressive its growth becomes and the more probable is tumor cell dissemination, as can be expected. These results parallel a previous observation (Kirkali *et al.* 2001), and confirm recent analyses on the predictive power of T-stage in the 1997 and 2002 pTNM classification (Sobin and Wittekind 2002, Tsui *et al.* 2000a, Javidan *et al.* 1999). A uniform staging classification, the TNM staging system, has increased the co-operation between oncologists and pathologists concerning the outcome of RCC patients (Javidan *et al.* 1999).

Previously, it has been suggested that T-stage is not an important prognostic factor in the survival of patients who have neither lymph node nor distant metastases (Giuliani *et al.* 1990). However, the therapeutic value of lymph node dissection remains unproven (Mickish 1999). For this reason, extensive lymph node dissection was not carried out, and no systematic data on metastatic lymph nodes in nephrectomized patients were available in the present study. Current results suggest that T-stage alone is a valuable prognostic factor for survival, even when the status of lymph nodes is unknown.

In the present study, T-stage was found to be an important factor in predicting the survival of patients who underwent nephrectomy. Therefore, T-stage can be used in estimating the correct duration and frequency of surveillance of RCC patients after nephrectomy. Additionally, high T-stage has been used as an inclusion criterion for adjuvant treatments in trials (Atzopodien *et al.* 2005, Repmann *et al.* 2003).

Moreover, T-stage seems to be an independent prognostic factor in mRCC patients. In this study, the association between T-stage and overall survival was also found in those with primary metastases at the time of nephrectomy. T-stage is not typically used in prognostic models in

mRCC, a UCLA model (Zisman *et al.* 2002b) being an exception. T-stage seems to be a good tool in prognostic evaluation in mRCC patients and could be included in prognostic models.

Nuclear grade is typically used in prognostic models in locally confined RCC. In the present study, in the group of patients with later metastases, tumor grade was not associated with overall survival. Several other studies have also failed to demonstrate any difference in the survival of patients with different grades (Uchida *et al.* 2002, Rioux-Leclercq *et al.* 2000, Usubutum *et al.* 1998). This is partly because, as yet, no consensus has been reached on a universal tumor grading system (Kanamaru *et al.* 2001). However, the present results did point out differences in metastases-free survival between the highest and the lowest grades, although when all three grades were included the differences were no longer statistically significant. Similar results were also found in other studies on RCC. Overall, histopathological grade seem to be imprecise for prognostic evaluation in RCC patients (Uchida *et al.* 2002, Rioux-Leclercq *et al.* 2000, Lanigan *et al.* 1994).

6.5 BIOMARKERS AS PROGNOSTIC FACTORS IN RCC

Molecular tumor markers are expected to revolutionize the staging of RCC in the future (Srigley *et al.* 1997), as nowadays stratifying the patients into risk groups is largely done on the basis of clinopathological factors, e.g. clinical stage of the disease. Advances in the understanding of the pathogenesis, behavior, and molecular biology of RCC may help to better predict tumor prognosis, and thus improve survival of RCC carcinoma patients when a more tailored therapy can be given to each individual patient. Biomarkers, such as p53, Ki-67 and COX-2, are candidates for defining prognostic subgroups (Delahunt *et al.* 2002), and for guiding targeted therapies (Choisy-Rossi and Yonish-Rouach 1998, May and May 1999), as shown in the current study, where p53, Ki-67 and COX-2 had prognostic value in predicting survival.

In the current study, the p53 and Ki-67 analyses were performed not only in the group of all RCC types, but also in the conventional type RCC subgroup, to achieve a more homogenous group. However, the results were similar in both analyses, maybe due to the high proportion of conventional type RCC of all RCC types (more than 80%). Therefore, in other studies, the analyses were performed in all types of RCC. The size and distribution of the patient material in the study were typical of other RCC studies.

6.5.1 BIOMARKERS IN RELATION TO T-STAGE, GRADE OR OCCURRENCE OF METASTASES

The association between p53 and Ki-67 protein expressions in the present study is in accordance with findings in other studies (Olumi *et al.* 2001, Rioux-Leclercq *et al.* 2000), indicating that p53

accumulation and increased cell proliferative activity are parallel phenomena in RCC. The present finding of no association between COX-2 and p53 is in accordance with a previous observation (Cho *et al.* 2005), but the finding of no association between COX-2 and Ki-67 differs from a previous observation (Miyata *et al.* 2003).

p53 seems to associate weakly with tumor grade, as the association was seen only in univariate analysis. Nor was an association between p53 and grade observed in a previous microarray study (Zigeuner *et al.* 2004). In both studies, the nuclear grade was determined according to the WHO guidelines. The present results and others (Dudderidge *et al.* 2005, Rioux-Leclercq *et al.* 2000) show an association between Ki-67 and high T-stage and metastases development, indicating that Ki-67 is a marker for aggressive disease in RCC with an increased risk of early metastases development. In the present study, no association between COX-2 and tumor grade or T-stage was found. Published associations between COX-2 and T-stage or tumor grade in RCC have been contradictory. Yoshimura *et al.* (2004) demonstrated that COX-2 expression was highest in G1, as well as in pT1 RCC tumors, compared to other grades and stages, while in Hashimoto *et al.*'s study (2004), the results were the opposite, with increased COX-2 expression in the higher tumor grade and stage.

The current study indicates that the proportion of COX-2 positive tumors is highest in RCC with the ability to develop later metastases, when compared to both RCC without metastatic potential and RCC with primary metastases. To our knowledge, this finding is new. Previously, Miyata *et al.* (2003) observed that positive COX-2 expression associated with primary metastases in univariate analysis (when M0-patients were compared to M1-patients). Cho *et al.* (2005) found no association between positive COX-2 expression and metastases (when M0-patients were compared to M1-patients, or appearance of metastatic disease was compared to non-metastatic disease). In those studies, the method of analysis differs from that of the present study, where patients were divided into three categories according to the appearance of metastases. According to the present study, metastases-free survival is longer in patients with COX-2 positive tumors. The median metastases-free survival was 46 months in RCC with COX-2 positivity compared to 15 months in RCC with COX-2 negativity. The present results indicate that COX-2 positivity associates with the delay of metastatic formation in RCC patients who do not have disseminated disease at presentation. The results of the current study indicate that COX-2 negativity associates with an aggressive phenotype in mRCC disease.

6.5.2 BIOMARKER ASSOCIATION WITH SURVIVAL

MRCC is an extremely heterogeneous disease, with patients having an overall survival from a few months to several years, and to date, no biomarker is capable of predicting the survival of mRCC patients. Earlier published results on the associations of p53 and Ki-67 protein expression with survival have been controversial. Some studies have suggested that positive p53 protein expression associates with poor survival (Shvarts *et al.* 2005, Uchida *et al.* 2002), while others have observed no association between p53 and survival (Haitel *et al.* 2000, Rioux-Leclercq *et al.* 2000). In a tissue array study on metastasized patients, overexpression of p53 was associated with impaired DSS in renal carcinoma (Kim *et al.* 2004b). Dudderidge *et al.* (2005) found Ki-67 to be an independent prognostic factor for disease-free survival in RCC, but opposite results have also been published (Donskov *et al.* 2004, Yildiz *et al.* 2004). The present study supports the finding that there is no association between p53 or Ki-67 alone and survival in RCC patients. The difference between the previous and the present study was in the classification of metastases: Kim and coworkers classified both distant and local lymph node metastases as metastatic disease, whereas in the present study, only tumors with distant metastases were classified as metastatic.

The present study indicates that p53 and Ki-67 are not able to predict which patients will develop metastatic disease after nephrectomy, but interestingly, they predict poor survival in mRCC patients. Therefore, p53 and Ki-67 can help in determining metastatic patients with a poor prognosis and, e.g. those who might benefit from aggressive treatment, such as high-dose interleukin-2 (Spanknebel *et al.* 2005) or temsirolimus (Hudes *et al.* 2007).

Few studies have reported the results of an association between COX-2 expression and survival in RCC patients. Previously, Miyata *et al.* (2003) found that the five-year survival of patients with COX-2 positive tumors from nephrectomy was 66%, and of COX-2 negative patients 91% (Miyata *et al.* 2003). In Miyata's study, the patients were 86% M0 and 14% M1 at nephrectomy. Previously, to our knowledge, no results of COX-2 and overall survival in mRCC patients have been published. The current study indicates that COX-2 positivity predicts improved overall survival in patients with mRCC treated with IFN-α. This is in line with the previous study of Rini *et al.* (2006), in which COX-2 positivity associated with longer time to progression in the patients treated with celecoxib plus interferon-α. According to the present study, there is no association between COX-2 staining and response to IFN-α alone, while Rini *et al*. (2006), in a small-scale, study reported that all the RCC patients with objective responses to celecoxib plus interferon-α expressed COX-2 staining. Additionally, the present study indicates that COX-2 does not associate with the Heidelberg classification, which is in line with a previous result (Yoshimura *et al.* 2004).

6.5.3 INCIDENCE OF p53, Ki-67, AND COX-2 EXPRESSIONS

The incidence of p53- and Ki-67-positive expression in RCC tumors in the present study was low but similar to that in other RCC studies (Kirkali *et al.* 2001, Haitel *et al.* 2000, Rioux-Leclercq *et al.* 2000). It is known that in addition to melanoma, RCC belongs to tumors with a low incidence of *p53* mutations compared to, e.g. prostate and bladder cancer (Haitel *et al.* 2000, Kirkali *et al.* 2001, Rioux-Leclercq *et al.* 2000). The low *p53* mutation in different cancers (Olivier *et al.* 2002) and the low immunohistochemical staining of RCC tissue blocks for the p53 protein in this and other studies (Haitel *et al.* 2000, Rioux-Leclercq *et al.* 2000) suggest that mutations in *p53* result in an accumulation of the p53 protein. In the study of Oda *et al.* (1995), p53 expression was found only in those components with *p53* mutations, mainly in the sarcomatoid components. The 10% cut-off value of p53 and Ki-67 was selected to achieve statistically reliable results, and in accordance with previous studies on the subject (Olumi *et al.* 2001). Previously published reports indicate that the proportion of COX-2 positive cells varies in human RCCs (Cho *et al.* 2005, Miyata *et al.* 2003). In the present study, weak intensity of COX-2 staining was considered as COX-2 negative, which resulted in a lower number of positive COX-2 cells than in some other RCC studies (Tuna *et al.* 2004, Cho *et al.* 2005). For comparison, in the study of Miyata *et al.* (2003), the criterion for positive COX-2 expression was 5%, whereas in the present study it was considered to be 10%. Also different antibodies have been used in other studies (Rini *et al.* 2006, Cho *et al.* 2005, Hashimoto *et al.* 2004). This fact and the criteria for immunohistochemical classification may contribute to the difference in the results. Validation of immunohistochemical methods is needed before the methods could be widely adopted for in clinical use.

6.5.4 COMBINING MARKERS

In the present study, p53, Ki-67 and COX-2 were associated with metastatic appearance and survival. In multivariate analysis, COX-2 and Ki-67 were independent variables, indicating that they are both stronger biomarkers than p53 for the development of metastases in RCC. However, combining markers may specify prognostic subgroups better than observing a single marker. As shown in a study by Haitel *et al.* (2000), p53 was not an independent predictor for survival, but p53 and mdm2, a negative regulator of p53, showed a strong association with poor survival. In the present study, in RCC patients, double positivity for p53 and Ki-67 expression seems to indicate a higher probability of metastases than either marker alone. Additionally, combining COX-2 and Ki-67 increases their ability to predict survival in mRCC. In this study, median overall survival time of RCC with COX-2 negativity/Ki-67 positivity was 19 months, which was almost five times shorter than of RCC with COX-2 positivity/Ki-67 negativity. Median overall survival time of RCC with COX-2 negativity alone was 28 months, which was three times shorter than that of RCC with COX-2 positivity.

Prognostic markers can be used in patient counseling, to select treatment modalities, and to determine eligibility for clinical trials. Different prognostic models have been created to specify the prognosis of RCC patients (Motzer *et al.* 2004, Motzer *et al.* 1999); they typically include conventional prognostic markers. However, combining biomarkers and conventional clinical markers seems to predict DSS more accurately than grade or TNM stage alone, both in locally confined and metastatic RCC (Kim *et al.* 2004b).

6.5.5 TRENDS IN THE USE OF BIOMARKERS

Prospective clinical trials on the clinical use of p53, Ki-67, and COX-2 protein expression in predicting overall survival could answer the question of whether the expression of these biomarkers can be reliably used in mRCC. The present data show that these biomarkers cannot predict response to IFN-α, whether these biomarkers can predict response to novel targeted therapies should be investigated in trials.

The new era of genetic cancer studies shows great promise in terms of patient evaluation for new targeted therapies or immunotherapy. By means of the tissue microarray technique, thousands of tumors can be investigated simultaneously to determine the protein expression profile. However, creating a consensus in the tissue microarray construction protocol is challenging, as RCC is a relatively large-size tumor of a highly heterogenous nature (Signoretti *et al.* 2008). At current, whole tissue sections are considered the gold standard, but the more cores per tumor are sampled the fewer errors are introduced by limited sampling. Using gene chips to profile kidney tumors defines the genes that determine patient survival and response to therapy, thus enabling precise prognosis determination and individual treatment planning (Tan *et al.* 2008). Additionally, tissue microarrays enable the analysis of protein expression profiles in specimens to determine their potential clinical significance and role in RCC biology.

7 SUMMARY AND CONCLUSIONS

The present study led to the following conclusions.

I

Performance status and response to IFN-α are independent prognostic factors for overall survival in mRCC. Those patients who achieve objective responses (CR + PR) to prolonged IFN-α achieve twice as long median overall survival compared to those with stabilized disease, and ten times longer compared to those with progressive disease. Late objective responses can be seen even at 17 months after the initiation of therapy. For CR patients, a median response duration of four years is achievable. Approximately one in six patients is able to reach five-year survival, and patients with lung metastases are the most likely to achieve long-term survival. Also, a significant subgroup of patients in the intermediate risk group, seems to benefit from prolonged IFN-α therapy. T-stage is an independent prognostic factor for overall survival in those patients with primary metastases at the time of nephrectomy. Additionally, more than one metastatic site, bone or liver metastases, and Hb level lower than normal at baseline, are significant prognostic factors in mRCC. One in seven patients seems to develop brain metastases after the start of IFN-α as a late manifestation of the disease.

The highest tolerable doses of up to 18 MU of IFN-α three times a week seem to be effective. Responses are typically seen between the doses of from 9 to 18 MU. The rate of discontinuation of prolonged IFN-α therapy because of fatigue, elevation of liver enzymes or cardiac arrhythmias is low, approximately 8%, and the treatment does not seem to have a life-threatening effect. Prolonged IFN-α is feasible; the one-week pause every four weeks allows most patients to continue prolonged treatment at the highest tolerated dose.

In the era of novel targeted therapies, one choice in the treatment of mRCC patients is still IFN-α with a highest tolerated dose of 3 to 18 MU three times a week in a treatment cycle of three weeks, followed by a one-week pause. Prolonged treatment of more than 12 months in stable and responding patients is beneficial. Prolonged IFN-α therapy is beneficial in patients with good and intermediate risk.

II

Changes in receptor expression reflect the inflammatory activation of phagocytes in mRCC compared to a healthy control group. In mRCC patients in neutrophils the expression of CR3 receptors and the proportion of FcγRI positive neutrophils increases. As CR3 is previously known to serve as an adhesion molecule, which binds neutrophils to other cells, e.g. cancer cells, the observed activation may reflect a response of neutrophils to cancer cells, and FcγRI is previously known to be important in cell-mediated cytoxocity. The observed receptor expression in mRCC before and during IFN-α treatment differs from previous observations of patients with infectious disease, or after the induction of IFN-γ. IFN-α, both in vivo and in vitro, modulates the expression of phagocytic receptors. Although investigation of the impact of IFN-α on phagocyte receptors is not sensitive enough for cancer diagnosis, it may add information about specific treatment effects and immunomodulation.

III

p53 associates weakly with tumor grade. Ki-67 associates with T-stage and metastatic development, indicating that Ki-67 is a marker for aggressive disease in RCC with an increased risk of early metastases development. The proportion of COX-2 positive tumors is highest in RCC with the ability to develop later metastases, when compared to both RCC without metastatic potential, and RCC with primary metastases. Metastases-free survival is longer in patients with COX-2 positive tumors compared to COX-2 negative tumors. These data show that COX-2 negativity associates with an aggressive phenotype in mRCC disease. COX-2 and Ki-67 alone are stronger biomarkers than p53 for the development of metastases in RCC.

p53 or Ki-67 alone are not valuable prognostic markers in locally confined RCC, but they can predict poor survival in mRCC. Therefore, p53 and Ki-67 can help in determining metastatic patients with a poor prognosis and, e.g. those who would benefit from high-dose IL-2 or temsirolimus. COX-2 positivity predicts improved overall survival in patients with mRCC treated with IFN-α. p53, Ki-67, and COX-2 cannot predict response to IFN-α. Investigating the ability of p53, Ki-67, and COX-2 protein expression to predict overall survival in a prospective clinical trial would answer the question of whether these biomarkers can be reliably used in mRCC.

Combining the results of COX-2 and Ki-67 expression, may predict overall survival in mRCC. In predicting the development of metastases in nephrectomized RCC patients, COX-2 alone or a covariation of p53 and Ki-67 seem to have prognostic value. Combining p53 or COX-2 with Ki-67 may result in more specific prognosis staging in RCC than observing a single marker. In future,

using the tissue microarray technique, the protein expression profile with several biomarkers can be determined quickly.

These findings increase our understanding of the molecular biology of locally confined RCC patients and metastatic RCC patients with IFN-α therapy. These markers might be useful as a part of the factors in prognostic models, and warrant further studies. The findings of such a study might be translated into prognostic tools that could be used in clinical work.

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