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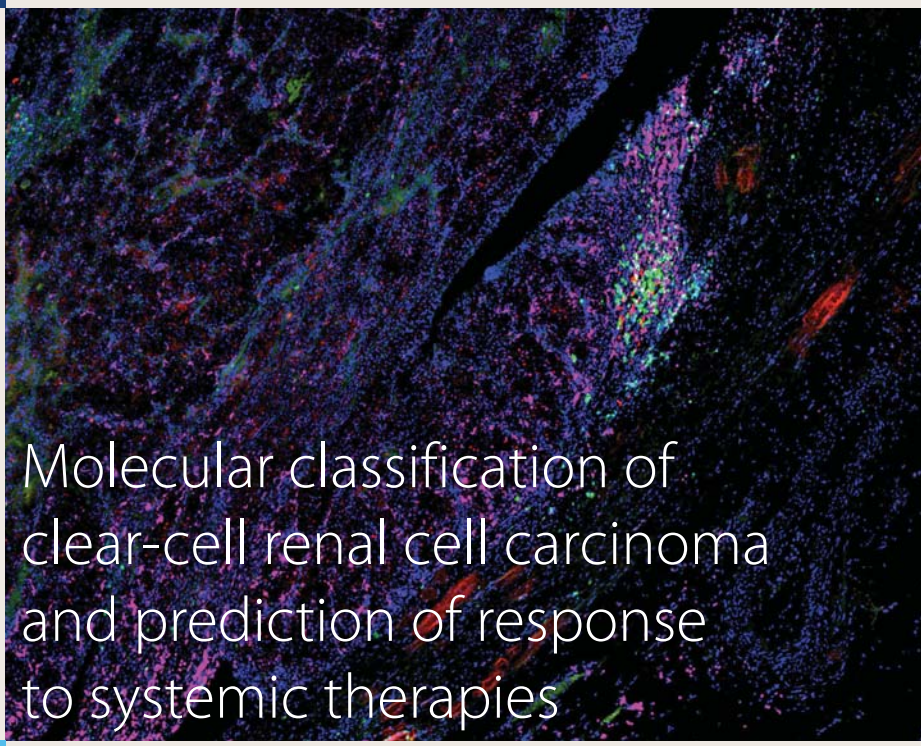
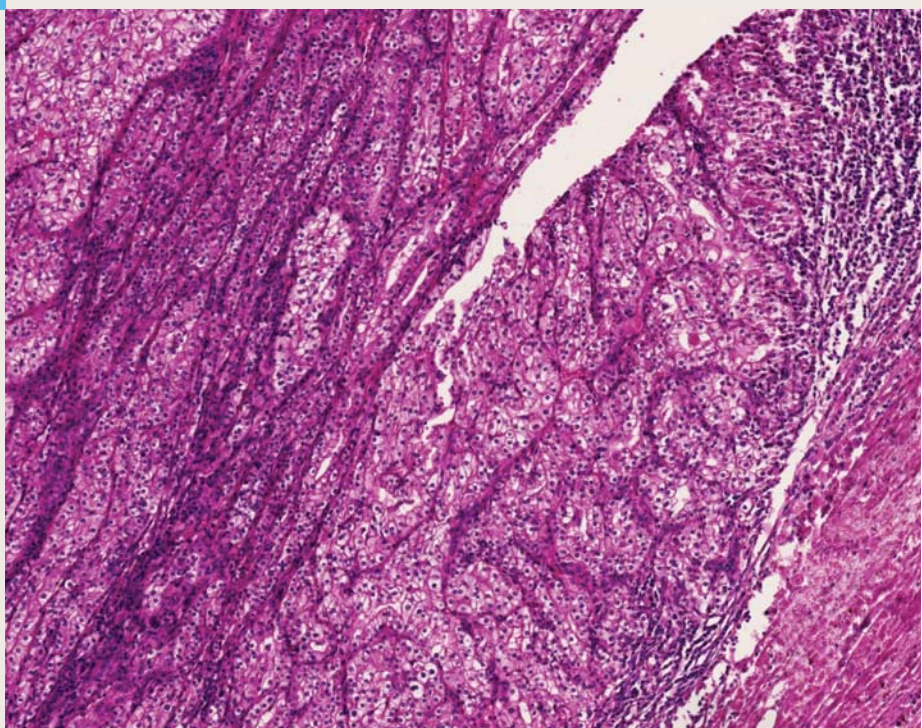


Molecular classification of clear-cell renal cell carcinoma and prediction of response to systemic therapies

Annelies Verbiest



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June 2020

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Annelies Verbiest

Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in the Biomedical Sciences

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June 2020

Table of Contents

Gratitude 7

Chapter 1 Introduction 11

Epidemiology and distinguishing features of renal cell carcinoma 14
- Epidemiology 14
- Genetic alterations 14
- Immune microenvironment 16

The contemporary therapeutic landscape of metastatic renal cell carcinoma 17
- Role of surgery 18
- First-line systemic treatment: ICI combinations 18
- Further line systemic treatment: TKI monotherapy 20
- Non-clear-cell RCC 20

The urgent need for predictive biomarkers 23
- Emerging biomarkers 23
- Open questions 25

ccRCC can be divided into four molecular subtypes 26
- The ccrcc1 to -4 molecular subtypes 26
- Other transcriptome-based ccRCC molecular subtypes 29

Chapter 2 Objectives 31

Chapter 3 Molecular subtypes of clear-cell renal cell carcinoma are associated with outcome during pazopanib therapy in the metastatic setting 35

Chapter 4 Non-surgical ablative treatment of distant extracranial metastases for renal cell carcinoma: a systematic review 45

Chapter 5 Long-term outcomes in clear-cell renal cell carcinoma patients treated with complete metastasectomy 59

Abstract 61

Introduction 62

Methods 63
- Statistical analysis 63

Results 64
- Outcomes after first metastasectomy 65
- Outcomes after repeat metastasectomy 65
- Systemic therapy after metastasectomy 66
- Comparison of outcomes of patients with and without metastasectomy 66

Discussion 68

References 70

Chapter 6 Molecular subtypes of clear-cell renal cell carcinoma are prognostic for outcome after complete metastasectomy 73

Chapter 7 Pro-angiogenic gene expression is associated with better outcome on sunitinib in metastatic clear-cell renal cell carcinoma 85

Chapter 8 Clear-cell renal cell carcinoma: molecular characterization of IMDC risk groups and sarcomatoid tumors 99

Chapter 9 MicroRNA expression profiles in molecular subtypes of clear-cell renal cell carcinoma are associated with clinical outcome and repression of specific mRNA targets 115

Abstract 117

Introduction 118

Results 119

Exploratory unsupervised clustering reveals two miRNA clusters that overlap with molecular subtypes 119

Molecular subtype-specific miRNA expression is involved in pathways that regulate tumor biology 120

MiRNA expression is correlated with both overall survival and molecular subtype 127

MiRNA expression is not predictive for response to sunitinib or pazopanib 128

Discussion 129

Conclusion 131

Methods 132

Small RNAseq preprocessing 132

Unsupervised clustering 133

Differential expression 133

Functional characterization 133

Correlation with clinical outcome 134

References 134

Supplemental tables and figures 138

Chapter 10 Identification of molecular subtypes in clear-cell renal cell carcinoma on paraffin-embedded tissue and molecular characterization of metastatic lesions 143

Summary 145

Disclaimer 146

Introduction 146

Preliminary results and discussion 147

The ccrcc4 subtype is highly inflamed 150

Ccrcc1 to -4 subtypes correlate well with the predictive IMmotion150 signatures 150

Ccrcc1 to -4 molecular subtypes outperform Angio score for outcome on anti-angiogenic therapy 152
The molecular subtypes and myeloid cell infiltration are prognostic for overall survival 154
Ccrcc1 tumors appear driven by chr14q loss 154
Ccrcc3 tumors overexpress mitochondrial pathways 154
Metastases often share the same subtype within patients 156
Angiogenic signature is highly conserved within patients 156
Sarcomatoid dedifferentiation is conserved within patients and less frequent in metastases 156
Conclusion and ongoing work 158
Methods 159
Method details 159
Quantification and statistical analysis 160
Analysis of clinical outcomes 162
References 163
Supplemental tables and figures 165

Chapter 11 Polymorphisms in the Von Hippel–Lindau gene are associated with overall survival in metastatic clear-cell renal-cell carcinoma patients treated with VEGFR tyrosine kinase inhibitors 169

Chapter 12 Concluding discussion 179

Molecular characterization of ccRCC 181
Molecular subtype-specific gene expression 181
microRNA expression patterns 182
Sarcomatoid RCC 182
Metastatic lesions 182
Biomarkers in the context of surgical ccRCC treatment 183
Current clinical questions and future work 183
Cytoreductive nephrectomy 184
Adjuvant therapy for localized disease 185
Biomarkers in the context of systemic ccRCC treatment 181
Current clinical questions and future work 186
References (Introduction and concluding discussion sections) 188

Chapter 13 Scientific abstract 195

Chapter 14 Scientific acknowledgements 199

Chapter 15 Personal contribution 201

Chapter 16 Conflict of interest statement 205

Chapter 17 Bibliography 207
Peer-reviewed publications 207
Papers posted on preprint server 209
Congress abstracts 210

Gratitude

"Och kijk! Nu is niets anders nog belangrijk!"

Ik citeer Benoits eerste reactie op het beeldje van de 12-weken echo, dat flikkert op het scherm van mijn gsm. Een omhelzing is op dat moment nog toegelaten: dat is zijn tweede reactie. Het is 28 februari 2020, minder dan vier maanden voor mijn thesisverdediging. Het zal dan ook niemand verbazen dat ik van alle mensen Benoit als eerste ontzettend wil bedanken. Het was een voorrecht om deze vier jaar je eerste thesisstudent te zijn. De vanzelfsprekendheid waarmee je je op hetzelfde niveau plaatst tijdens discussies, en het vertrouwen waarmee je mij het onderzoek mee laat vormgeven, zijn ontnuchterend en tegelijk de best mogelijke motivatie om te denken alvorens te doen. Bij jou vervloeien mens, wetenschap, familie en zingeving naadloos tot één geheel. Die bijzondere drive, tesamen met de nauwgezetheid en de focus waarmee je over de jaren aan je projecten blijft werken, zijn een inspiratie.

Het gezegde luidt dat je kinderen sterke wortels moet geven voordat ze vleugels krijgen. Mijn familie heeft gezorgd voor een rijke voedingsbodem waarin de liefde voor mens en wetenschap konden wortelen. Ook na het uitvliegen ben ik hen veel verschuldigd: voor praktische hulp en voor hun unieke combinatie van onvoorwaardelijke bewondering en nuchterheid ("Dus ASO, dat is dan dokter in niks?"). Kathleen, jou moet ik als zus expliciet bedanken om na vele jaren toch te aanvaarden dat je een goede arts kan zijn als je geen verkoudheid kan genezen. Tom, jij bent mijn grootste supporter al vanaf het moment waarop ik je acht jaar geleden verklaarde dat ik oncoloog zou worden: "Dat is fantastisch! Normale mensen moeten op het werk hun beste glimlach opzetten en reageren dan thuis hun frustraties af. Maar jij zal zo ernstig moeten zijn op je werk, dat je alle vrolijkheid voor thuis kan sparen!" En hoewel het er op oncologie een stuk vrolijker aan toe gaat dan men zou vermoeden, is het onmogelijk om niet spontaan gelukkig te worden wanneer ik bij jou en Elise thuis mag komen. Dank voor een warme thuis!

Gesproken over een thuishaven: dank aan de hele ploeg van oncologie om mij deel te doen voelen van een team waar ik af en toe kon aanmeren. Het is een plezier om opgeleid te worden in een omgeving die vertrouwen ademt. De manier waarop de stafleden de haast onmogelijke verwachtingen inlossen die aan hen worden gesteld als clinici, wetenschappers en opleiders, is een voorbeeld voor mij. Aan verpleging, logistiek, secretariaat en alle andere paramedici kan ik maar één ding zeggen: jullie zijn mijn voornaamste argument om jonge collega's te overtuigen van medische oncologie! Wie kiest voor zorg in de oncologie doet dat met volle overtuiging, en het is dan ook elke dag een plezier om in zo'n team te werken! Maar het plezier beperkt zich niet collega's alleen. We hebben allemaal ontdekt dat de zorg voor mensen met kanker niet de Griekse tragedie is die men zich daarbij voorstelt. Veel van onze patiënten ontdekken in zichzelf een kracht en een sereniteit die elke dag opnieuw bewondering oproept. Velen geven zelf meer zorg dan ze ontvangen – aan hun familie, en ook aan hun zorgverleners. Wij mogen niet enkel de uitdaging aangaan om kanker te onderzoeken en te behandelen, maar ook om met deze mensen op pad te gaan. Dankzij hen is dit veelal geen opgave, maar een voorrecht.

Bij de persoonlijke bedankingen is mijn voornaamste bezorgdheid die om mensen te vergeten: er hebben er zovelen deze vier jaar een onmisbare steen bijgedragen. Bedankt aan het team van LEO om een vaste uitvalsbasis te creëren, en in het bijzonder aan Aga voor de oprechte interesse en immer snelle en nuttige feedback op elk manuscript. Dank aan Jessica's team op INSERM, met onder andere Gabrielle en Stefano, om me de weg te wijzen in onze weefselcollectie en naar jullie befaamde bakker, aan wiens baguettes en frambozentaart ik nog steeds krokante herinneringen koester. Bedankt aan Marcella Baldewijns voor de enthousiaste pathologiereview, aan Thomas op VIB voor de RNA sequencing, aan Isabelle, Sara en Frederik op CME voor de samenwerking en heel in het bijzonder dank aan het team van pathologie dat ons toegang heeft verschaft tot de schier oneindige weefselbank van Gasthuisberg: Tom, Magda, Geert, Kathleen, Eef, Wilfried, Sabrina en co. Kevin en Ineke, bedankt voor de gezelligheid op bureau. Kom op tegen Kanker en FWO wil ik bedanken voor hun geloof in dit onderzoek. En tot slot aan Eduard: merci om ons project met zoveel enthousiasme voort te zetten! Kwaliteitsvol onderzoek is maar

mogelijk als het wordt warm gehouden, en ik ben dan ook ontzettend blij dat ons project nu bij jou in goede handen is.

Als bovenstaande paragrafen iets leren, dan is het wel dat solovliegen niet bestaat. Dat maken we nu zelf mee. De coronapandemie heeft op dit moment niet zijn voorspelde apocalyptische koers gevolgd, omdat we daar allemaal samen voor zorgen. Niemand gelooft dat ze het grote verschil maakt door een barbecue te vervangen door een videocall – maar het resultaat is overduidelijk. In onze snel veranderende wereld is het bijna onmogelijk geworden om het grote plaatje te zien, laat staan de impact van je eigen werk daarop. Maar we zien wel hoe onze samenwerking en nieuwsgierigheid ons razendsnel voortstuwen. Niemand weet hoe ons werk en ons leven er over twintig jaar zullen uitzien.

En dat maakt het net zo spannend.

Chapter 1

Introduction

Chapter 1: Contents

Epidemiology and distinguishing features of renal cell carcinoma 14
- Epidemiology 14
- Genetic alterations 14
- Immune microenvironment 16

The contemporary therapeutic landscape of metastatic renal cell carcinoma 17
- Role of surgery 18
- First-line systemic treatment: ICI combinations 18
- Further line systemic treatment: TKI monotherapy 20
- Non-clear-cell RCC 20

The urgent need for predictive biomarkers 23
- Emerging biomarkers 23
- Open questions 25

ccRCC can be divided into four molecular subtypes 26
- The ccrcc1 to -4 molecular subtypes 26
- Other transcriptome-based ccRCC molecular subtypes 29

The thesis manuscript is a scientific publication and as such should consist of objective facts and validated findings. In this first paragraph however, I am taking the liberty of adding an opinion: oncology is one of the most exciting fields to be working in right now. On top of that, renal cell carcinoma is one of the most thrilling tumors to be working on. A few decades ago, it morphed from a dreaded chemo- and radioresistant disease into a first beacon of hope for cytokine-based immunotherapy. It then quickly spearheaded the anti-angiogenic revolution, becoming a prime target for the new wave of precision molecules that were poured into the clinic. After the start of this thesis, in the heyday of angiogenesis inhibitors, renal cell carcinoma went on to prove itself an excellent target for the novel immune checkpoint inhibitors and recently even more so for combinations of these molecules. Along with the entire field, this biomarker-focused thesis has reinvented itself a couple of times. The field of renal cell carcinoma is a wonderful micro-example of our rapidly evolving world: our combined efforts are changing it so quickly, that no single person can fully grasp where we are heading. It is humbling to be part of, and exciting every time we catch a glimpse of what is still to come.

– Er gaat meer boven mijn pet dan er onder –

Toon Hermans

Epidemiology and distinguishing features of renal cell carcinoma

– Key message –

Clear-cell RCC (ccRCC) make up >80% of kidney carcinomas. They are hallmarked by ubiquitous loss of the Von Hippel Lindau (VHL) gene, which leads to accumulation of Hypoxia Inducible Factor (HIF) proteins despite normoxic conditions. This in turn results in increased angiogenesis, metabolic alterations and apoptosis resistance. Besides ubiquitous VHL loss, ccRCC display notorious intra- and intertumor heterogeneity on genetic, histological and clinical levels. For reasons that remain incompletely understood, they are also immunogenic tumors with high levels T-cell infiltration. Their signature hypervascularity and immunogenicity have made them preferred targets for treatment with angiogenesis inhibitors and immunotherapy.

Epidemiology

RCC are tumors originating from the renal epithelium, that account for >90% of kidney carcinomas. They rank in the top ten of most frequent cancers and are responsible every year for 295.000 new diagnoses and 134.000 deaths worldwide. (1,2) In Belgium, about 1700 people per year are diagnosed with RCC. (3) The median age at diagnosis is 64 years, with a male to female ratio of 2:1. In recent decades, both the incidence and survival of RCC have steadily increased until recently reaching a plateau. This evolution can be largely attributed to increased imaging, which has led to more and earlier incidental detection of RCCs. A little under one third of patients are metastatic at diagnosis, with another half of those with initially localized disease developing metachronous metastases later on. (4) RCCs can be divided into several histological subtypes that display different clinical behavior. Clear-cell RCC (ccRCC) make up the largest majority, accounting for over 80% of RCC. Among the other subtypes, which are typically grouped together as non-clear-cell RCCs (non-ccRCC), papillary and chromophobe RCC are the most frequent histologies, whereas other types of non-ccRCC have incidences of less than five percent (Figure 1.1, Table 1.1). (5)

Genetic alterations

ccRCC are hallmarked by ubiquitous loss of the *VHL* gene, through mutation, deletion, arm level loss of chromosome 3p or promotor methylation. (4) Under

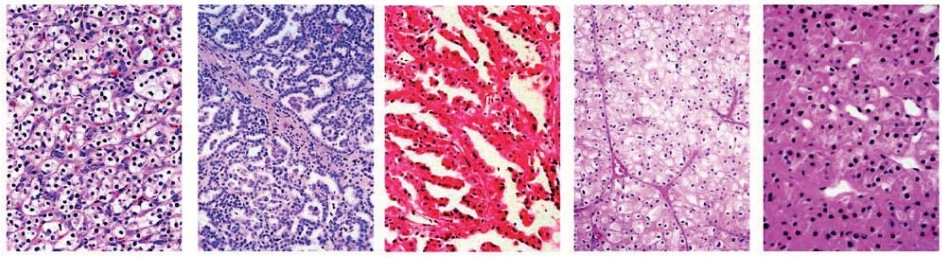

Figure 1.1. *Major histological subtypes of RCC.*
Adapted from Bottaro et al, Clinical Cancer Research 2005. (6) Copyright held by AACR

Table 1.1. *World Health Organization (WHO) classification of tumors of the kidney.*

Renal cell tumours	
Clear cell renal cell carcinoma	8310/3
Multilocular cystic renal neoplasm of low malignant potential	8316/1*
Papillary renal cell carcinoma	8260/3
Hereditary leiomyomatosis and renal cell carcinoma–associated renal cell carcinoma	8311/3*
Chromophobe renal cell carcinoma	8317/3
Collecting duct carcinoma	8319/3
Renal medullary carcinoma	8510/3*
MiT family translocation renal cell carcinomas	8311/3*
Succinate dehydrogenase–deficient renal carcinoma	8311/3
Mucinous tubular and spindle cell carcinoma	8480/3*
Tubulocystic renal cell carcinoma	8316/3*
Acquired cystic disease–associated renal cell carcinoma	8316/3
Clear cell papillary renal cell carcinoma	8323/1
Renal cell carcinoma, unclassified	8312/3
Papillary adenoma	8260/0
Oncocytoma	8290/0
Metanephric tumours	
Metanephric adenoma	8325/0
Metanephric adenofibroma	9013/0
Metanephric stromal tumour	8935/1
Nephroblastic and cystic tumours occurring mainly in children	
Nephrogenic rests	
Nephroblastoma	8960/3
Cystic partially differentiated nephroblastoma	8959/1
Paediatric cystic nephroma	8959/0
Mesenchymal tumours	
Mesenchymal tumours occurring mainly in children	
Clear cell sarcoma	8964/3
Rhabdoid tumour	8963/3
Congenital mesoblastic nephroma	8960/1
Ossifying renal tumour of infancy	8967/0
Mesenchymal tumours occurring mainly in adults	
Leiomyosarcoma	8890/3
Angiosarcoma	9120/3
Rhabdomyosarcoma	8900/3
Osteosarcoma	9180/3
Synovial sarcoma	9040/3
Ewing sarcoma	9364/3
Angiomyolipoma	8860/0
Epithelioid angiomyolipoma	8860/1*
Leiomyoma	8890/0
Haemangioma	9120/0
Lymphangioma	9170/0
Haemangioblastoma	9161/1
Juxtaglomerular cell tumour	8361/0
Renomedullary interstitial cell tumour	8966/0
Schwannoma	9560/0
Solitary fibrous tumour	8815/1
Mixed epithelial and stromal tumour family	
Cystic nephroma	8959/0
Mixed epithelial and stromal tumour	8959/0
Neuroendocrine tumours	
Well-differentiated neuroendocrine tumour	8240/3
Large cell neuroendocrine carcinoma	8013/3
Small cell neuroendocrine carcinoma	8041/3
Phaeochromocytoma	8700/0
Miscellaneous tumours	
Renal haematopoietic neoplasms	
Germ cell tumours	
Metastatic tumours	

The morphology codes are from the International Classification of Diseases for Oncology (ICD-O) {917A}. Behaviour is coded /0 for benign tumours; /1 for unspecified, borderline, or uncertain behaviour; /2 for carcinoma in situ and grade III intraepithelial neoplasia; and /3 for malignant tumours. The classification is modified from the previous WHO classification {756A}, taking into account changes in our understanding of these lesions.
*New code approved by the IARC/WHO Committee for ICD-O.

Reproduced from the WHO International Agency for Research on Cancer
(WHO classification of tumors of the urinary system and male genital organs, 4th edition 2016). (5)

normoxic conditions, the VHL protein is responsible for ubiquitylation of HIF1α and HIF2α through the E3 ligase complex, thereby inducing their proteasome-mediated degradation. Loss of *VHL* mimics cellular hypoxia, resulting in the aberrant accumulation of HIF proteins that activate pathways leading to increased angiogenesis, metabolic alterations and apoptosis resistance. This cascade is responsible for the typical histological features of ccRCC: clear cells filled with lipid vesicles, surrounded by an extensive vascular network. The HIF-induced aberrant angiogenesis is mediated by Vascular Endothelial Growth Factor (VEGF). For this reason, the last 15 years have seen the establishment of several tyrosine kinase inhibitors of the VEGF-receptor (VEGFR-TKIs) as a solid backbone of ccRCC treatment: sunitinib, pazopanib, cabozantinib, axitinib, sorafenib, tivozanib and lenvatinib. (7)

ccRCC vary widely in their clinical behavior, ranging from very indolent to highly aggressive diseases. They also display a marked intra- and intertumor genetic heterogeneity. Genetic driver events besides *VHL* loss are frequent, but usually subclonal: they include mutations in *PBRM1* (30-40%), *SETD2* (10%), *BAP1* (5-10%), *KDM5C* (5%), *MTOR* (5%), *PTEN* (4%) and other genes. (8) Interestingly, *PBRM1, SETD2* and *BAP1* are all involved in chromatin and histone regulation and are situated on the short arm of chromosome 3p, in the vicinity of *VHL*. Arm level losses of chr3p are frequent in ccRCC and result in haploinsufficiency of all four tumor suppressor genes. Mutations in these chromatin-regulating genes on the other hand, are typically mutually exclusive and carry different prognostic implications. (9–11)

Immune microenvironment

It has long been known that ccRCC are immunogenic tumors. Complete regression of metastases, due to an abscopal effect after cytoreductive nephrectomy, can be observed rarely but consistently. In the cytokine era, high dose interleukin 2 and interferon alpha could induce durable responses in a small fraction of patients. (12) Moreover, ccRCC have the highest cytolytic scores among the 18 tumor types from The Cancer Genome Atlas (TCGA). (13) The reasons for this particular immunogenicity are however not completely understood. In contrast to other immunogenic tumors such as melanoma, smoking-related lung carcinoma or mismatch repair deficient colon carcinoma, ccRCC carry only a modest tumor mutational burden. (14) One theory poses that they are relatively rich in indel mutations, which are more likely to create recognizable neoantigens compared to point mutations. But in a randomized phase II trial there was no link between indel load and $T_{effector}$ (T_{eff}) cell signature.

(15,16) Some studies have suggested a role of aberrantly expressed retroviruses in eliciting an immune response. (13,17,18)

Although immune responses are clearly present in the majority of metastatic ccRCC, these responses seem often poorly functional. (19) Higher infiltration rates by cytotoxic CD8+ T-cells signal a poor prognosis in metastatic ccRCC, in contrast towith most other tumor types and in contrast to early ccRCC, were CD8+ T-cell infiltration is favorable. (20–23) ccRCC often seem to fail to organize effective priming and maturation of cytotoxic T-cells in tertiary lymphoid structures or antigen-presenting intratumoral niches. Indeed, one ccRCC often lack functional tertiary lymphoid structures or other antigen-presenting intratumoral niches, that can effectively prime and mature cytotoxic T-cells. When present, these niches are associated with good prognosis. One study showed that in a small subset of ccRCC, that contained tertiary lymphoid structures with antigen-presenting dendritic cells, CD8+ T-cell infiltration was indeed correlated with better prognosis instead of worse. (21) Another recent study showed that intratumoral lymphoid aggregates, in which antigen-presenting cells interacted with CD8+ T-cells, were frequent in localized ccRCC that did not relapse, but absent in tumors that relapsed early. (23)

The contemporary therapeutic landscape of metastatic renal cell carcinoma

– Key message –

Treatment strategies for metastatic RCC have been turned upside down quite a few times over the past decades. When it comes to systemic therapies, cytokine-base immune therapies have been largely replaced by angiogenesis inhibitors, which are now again challenged by immune checkpoint inhibitors (ICI). In 1st line, combination regimens using an ICI backbone with another ICI or angiogenesis inhibitor are now the standard of care for all patients. In later lines, angiogenesis inhibitors remain active and can be used sequentially both after ICI and after previous angiogenesis inhibitors. In patients who have become resistant, the mTOR inhibitor everolimus remains an option. Local treatment can be appropriate in patients with favorable features for whom immediate start of systemic therapy is not necessary. Options are cytoreductive nephrectomy, or even, in highly selected patients, radical ablative treatment of all disease localizations. A proposed treatment algorithm is illustrated in Figure 1.2.

Role of surgery

In the setting of localized RCC, for which (partial) nephrectomy is the gold standard, there is no place for (neo-)adjuvant systemic therapy. Several trials of adjuvant sunitinib, sorafenib and pazopanib have failed to demonstrate benefit. (24,25) Only the S-TRAC trial, testing adjuvant sunitinib in high risk ccRCC, showed some benefit in disease-free survival, but at the cost of considerable toxicity and without effect on overall survival (OS). (26) The neo-adjuvant use of VEGFR-TKIs in order to downstage locally advanced tumors is not recommended as standard practice, but can be considered in selected cases that are primarily inoperable. Trials testing (neo-)adjuvant ICI are ongoing.

In metastatic disease, the role of cytoreductive nephrectomy has recently been redefined. Where cytoreductive nephrectomy could prolong OS in the cytokine era, the CARMENA trial has now shown that patients who need to start sunitinib immediately at time of diagnosis, derive no OS benefit from cytoreductive nephrectomy: it is therefore no longer recommended in this setting. (27) In contrast, patients for whom start of systemic therapy can be deferred, or those with symptomatic tumors, were not included in this trial: for them, cytoreductive nephrectomy remains the standard of care. Of note, both the CARMENA and SURTIME trials have shown that deferred nephrectomy, after start of sunitinib, is feasible and safe. (27,28) Trials investigating the place of cytoreductive nephrectomy in the context of ICI are ongoing.

For selected patients with oligometastatic RCC, radical local treatment by metastasectomy or stereotactic body radiotherapy can be offered after multidisciplinary review. (29)metastasectomy still remains the only potentially curable intervention and plays an important role both in disease control, cancer-specific survival (CSS Afterwards, patients should be offered active surveillance without systemic therapy, as two trials testing pazopanib and sorafenib after complete metastasectomy did not show any benefit. (30,31) Trials testing ICI combined with local treatment are ongoing. Some emerging treatment strategies include local treatment of oligoprogressive metastases while continuing systemic therapy for responding lesions, but these approaches should still be considered experimental.

First-line systemic treatment: ICI combinations

In 2017, the ICI + ICI combination nivolumab + ipilimumab demonstrated a clear OS benefit over sunitinib in 1st line, in patients with Intermediate or Poor risk according to the International Metastatic ccRCC Database Consortium (IMDC)

criteria (hazard ratio, HR, 0.63). (32,33) Responses rates (RR) and progression-free survival (PFS) were also increased (42% *vs* 27% and 11.6mo *vs* 8.4mo, HR 0.82). Interestingly, sunitinib yielded higher RR (52%) and PFS (25mo) in IMDC Good risk tumors, whereas the effects of nivolumab + ipilimumab were similar across IMDC risk groups.

In 2018, the ICI + VEGFR-TKI combination pembrolizumab + axitinib has proven itself superior over sunitinib in all IMDC risk groups (RR 59% vs 36%, HR PFS 0.69, HR OS 0.53). (34) Another combination, avelumab + axitinib, showed improved PFS over sunitinib, but OS data were immature and lacked a signal towards OS benefit: recent guidelines therefore do not currently recommend it as 1st line option. (7,35,36) Other phase III trials testing ICI + VEGFR-TKI combinations are ongoing. Of note, the combination of ICI with a VEGFR-TKI is supported by a strong scientific rationale. VEGF exerts well-known immune suppressive effects, which can dampen the response to ICI. In preclinical models, antiangiogenic therapy can decrease immunosuppressive cells (myeloid derived suppressor cells, regulatory T-cells), decrease immunosuppressive cytokines (IL-10, TGF-b), activate expression of immune checkpoints by tumor cells, facilitate homing of lymphocytes and increase expansion of tumor infiltrating lymphocytes. (37,38) Therefore, the addition of a TKI does not only provide an additive anti-angiogenic effect, but acts synergistically to boost the immune invigorating effects of ICIs.

Despite these tremendous advances, several open questions remain in 1st line. Most importantly, it is currently not possible to judge whether nivolumab + ipilimumab or pembrolizumab + axitinib are preferred in IMDC Intermediate/ Poor risk patients. Response rates to pembrolizumab + axitinib seem higher based on the registration trials, but this might be expected as it also targets the VEGF-pathway, and follow-up is too short to assess the OS plateau. In PD-L1 positive tumors, complete response rates to nivolumab + ipilimumab reached an impressive 16% in the Checkmate214 trial, but the definition of PD-L1 positivity was stricter compared to the Keynote426 (pembro + axi) trial and PD-L1 positivity is not used for patient selection in the clinic. A first real-world retrospective comparison of 188 patients receiving either nivolumab + ipilimumab or an ICI + VEGFR-TKI combination did not signal improved benefit of either strategy, but consisted of very heterogeneous populations. (39) Long term survival data, real world experience with toxicity and emerging molecular biomarkers will guide treatment decisions in the future. The optimal duration of treatment in case of long-lasting remission is currently unknown.

Further line systemic treatment: TKI monotherapy

ccRCC is known first and foremost as an angiogenic disease, with data from the TKI era indicating continued benefit of VEGFR-TKIs in early and later treatment lines. Emerging evidence is now supporting the benefit of VEGFR-TKIs after previous ICI. Several small prospective trials and retrospective series have reported response rates of 18 to 47% and PFS of 6 to 9 months on TKI after previous ICI or ICI combinations. (40–46)

There is insufficient evidence to recommend any specific TKI from 2nd line on. In Belgium, the recently revised reimbursement criteria put forward cabozantinib as the preferred 2nd line treatment after ICI, in patients with good performance status (KPS ≥ 70%). Indeed, cabozantinib is a TKI with pleiotropic effects that extend beyond VEGFR-inhibition (such as MET inhibition), which has proven its efficacy in RCC. A small phase II trial in Intermediate/Poor risk patients in 1st line, showed improved PFS and a trend towards OS benefit compared with sunitinib. (47) After previous TKI treatment, OS was also improved with cabozantinib compared with everolimus: a finding that also held true in the subgroup of patients who had also received previous ICI. (42,48) A recent large real-world retrospective series has suggested continued efficacy of cabozantinib from 2nd to 4th line, with response rates of about 25% both after VEGFR-TKI and ICI. (49) In patients with poor performance status or other contra-indications, TKIs with a more attractive safety profile can be used. Importantly, every TKI should be administered at the highest tolerable dose, as this clearly improves outcomes. (50–52)

From 3rd line on, any VEGFR-TKI can be used. Abundant data support the continued efficacy of TKI after previous TKI, mostly if a patient has experienced long-lasting remission on earlier therapy. (48,53,54) In TKI-resistant patients, the mTOR inhibitor everolimus remains a valid option. Although responses are rare and PFS usually short, some patients can still achieve durable responses.

There is no evidence that supports the use of ICI after previous failure of ICI. In a very small retrospective series, 3 of 5 patients immediately progressed upon rechallenge with ICI. Importantly, the other two (1 partial response and 1 stable disease) had discontinued 1st line ICI combination after less than three months, for other reasons than disease progression. (43)

Non-clear-cell RCC

Non-clear-cell histologies make up <20% of RCC, with papillary and chromophobe accounting for 80% of these. As these subtypes are usually

excluded from clinical trials, data to guide treatment decisions are scarce. In general, the same strategy is recommended as in ccRCC, but it is encouraged to include patients in clinical trials if possible. (7) The sensitivity of non-ccRCC to ICI in 1st line was demonstrated prospectively in the Keynote427 trial (26% RR to pembrolizumab) and in a small retrospective study (28% RR to nivolumab + ipilimumab). (55,56) VEGFR-TKIs have shown efficacy in various non-clear-cell histologies, both in trial and real world settings, in first and later lines.

Of note, papillary RCC often harbor *MET* mutations or *MET* amplification, making them intuitive candidates for treatment with MET inhibitors. Several MET inhibitors have shown activity in papillary RCC, of which cabozantinib is the only one available in Belgium. (57) It is therefore first choice after 1st line ICI combinations.

Collecting duct carcinoma (Bellini duct carcinoma) are highly aggressive tumors that arise from renal collecting tubules and are notoriously TKI-resistant. Limited data suggest activity of ipilimumab + nivolumab as 1st line treatment for these tumors. (56) In case of progression, they should preferentially be treated with cisplatinum-based chemotherapy.

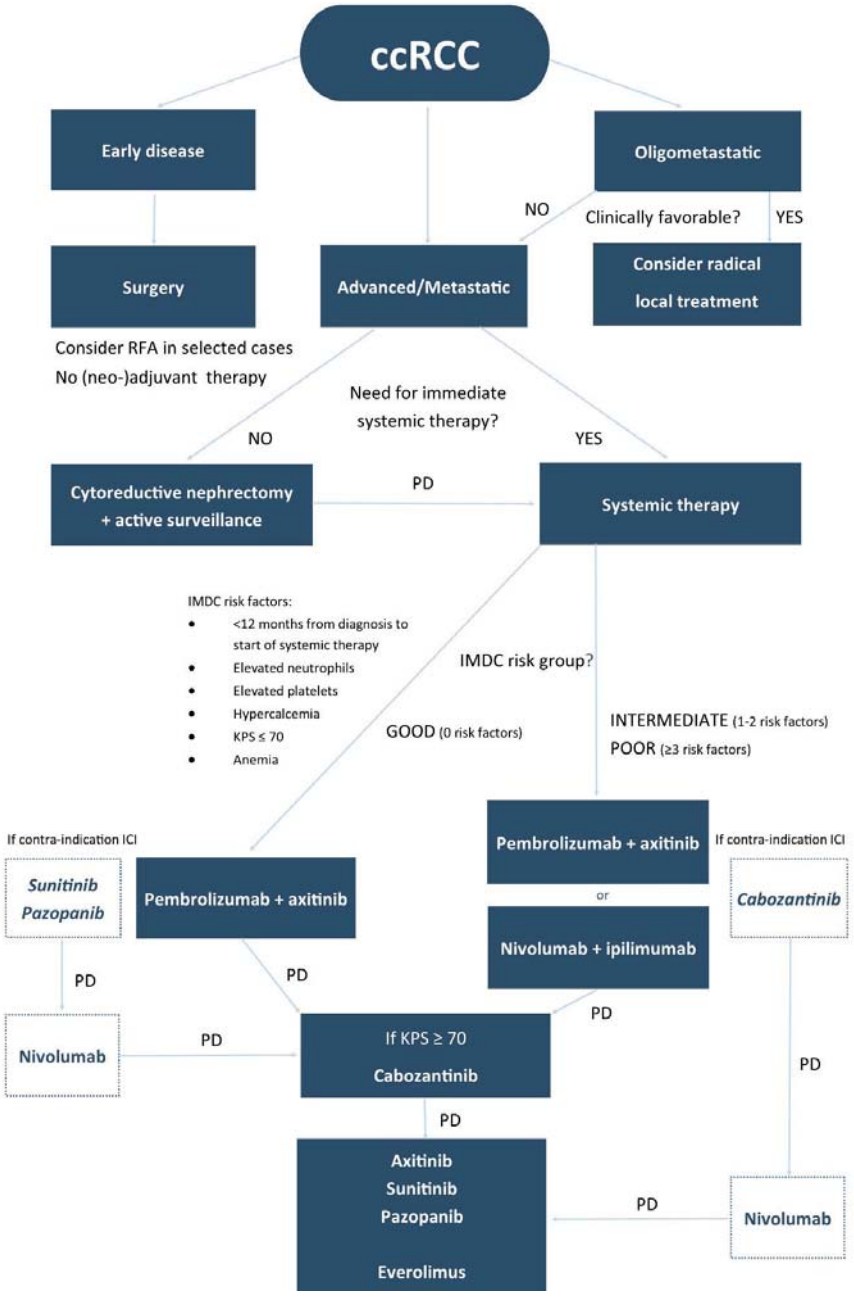

Figure 1.2. *Simplified treatment algorithm for ccRCC*
This figure also features in "An update on the management of metastatic clear-cell renal cell carcinoma: the BSMO expert panel recommendations". (58)

The urgent need for predictive biomarkers

– Key message –

At this moment, no reliable biomarkers are clinically available to guide treatment decisions. A favorable risk according to the clinical IMDC criteria and increased expression of angiogenic genes have been associated with response to VEGFR-TKIs. Sarcomatoid features, a $T_{effector}$-cell gene expression signature and PD-L1 positivity are associated with response to ICI.

Emerging biomarkers

Despite the current abundance of therapeutic molecules with different modes of action, only a subset of patients responds to any given treatment and we are not currently able to adequately identify them. For example, in unselected populations in 1st line, response rates to sunitinib reach about 30-35%, to ipilimumab + nivolumab 39% and to pembrolizumab + axitinib 59%. For this last combination however, it is unknown which and how many patients benefit only from the VEGFR-TKI or the ICI, and who needs the synergy of the combination to achieve a durable response.

In the TKI era, it became clear that patients who experience toxicity from VEGFR-TKIs such as arterial hypertension, or need dose reductions, have an increased chance of response. However, such on-target biomarkers merely reflect adequate drug exposure and are useful for dose optimization but not primary patient selection. Only since 2018, well after the start of this PhD, some predictive biomarkers have been proposed for VEGFR-TKIs and ICI.

Angiogenesis inhibitors

The IMDC risk score was developed as a prognostic model during the TKI era, to estimate the prognosis of patients treated with 1st line VEGFR-TKIs. (33) The score consists of six clinical risk factors: anemia, elevated platelets, elevated neutrophils, hypercalcemia, Karnofsky performance status ≤70, <1 year between diagnosis and systemic treatment. Patients with zero risk factors are considered Favorable risk (±15%, OS 43mo), those with 1-2 risk factors Intermediate risk (±60%, OS 23mo) and those with ≥3 risk factors Poor risk (±25%, OS 8mo). Beside a solely prognostic value, the Checkmate214 trial showed in 2018 that response rates to sunitinib are higher in Favorable compared to Intermediate/Poor risk patients (52% *vs* 22%). IMDC is however lacking as a predictive biomarker for

VEGFR-TKIs, as the Favorable risk group selects only 15% of patients whereas about 35% responds to sunitinib. Moreover, IMDC is not associated with response to ICI: responses across risk groups are similar, both for the combinations nivolumab + ipilimumab and pembrolizumab + axitinib. This is despite the fact that at least four of the risk factors (anemia, neutrophilia, thrombocytosis, poor performance status) reflected an inflammatory tumor subtype in a recent xenograft model. (59)we developed an empirical approach, DisHet, to dissect the tumor microenvironment (eTME Furthermore, the prognostic value of the IMDC score is less defined in the ICI era: even though survival still decreases with increasing IMDC risk factors, the outcomes of Intermediate/Poor risk patients have improved relatively more than those of Good risk patients. (60) Therefore, a new prognostic model for patient counselling is needed.

Shortly after the predictive value of the IMDC risk groups was established, several groups have also reported the association of angiogenic gene signatures with susceptibility to VEGFR-TKIs. (16,61,62) Some reports have also suggested that *PBRM1* mutations are associated with increased angiogenic gene expression and response to VEGFR-TKIs, which is in line with preclinical studies showing that *PBRM1* inactivation further upregulates *HIF1*. (16,22)

Immune checkpoint inhibitors

Typical ICI biomarkers that are well known in other tumor types, such as PD-L1 positivity and tumor mutational burden, are not useful in RCC. Across ICI trials, PD-L1 positivity (measured with different assays and cutoffs) is consistently associated with higher RR, but PD-L1 negativity was never sufficient to exclude patients from treatment. (32,34,35) Tumor mutational burden is lower in RCC compared to other tumors that are responsive to ICI, such as melanoma or non-small cell lung carcinoma, and is not associated with response. (15,16)

On the contrary, histological features are important: RCC with sarcomatoid dedifferentiation are rare but have a very poor prognosis, and have long been known to be resistant to VEGFR-TKIs. They are however surprisingly sensitive to ICI, with response rates that seem to even surpass those of RCC without sarcomatoid features. (16,34,62,63)

Perhaps the most promising ICI biomarker results were reported by the phase 2 IMmotion150 trial, that compared 1st line atezolizumab + bevacizumab with sunitinib. (64) The PD-L1 antibody atezolizumab was superior over sunitinib in tumors with a T_{eff} cell signature. However, the addition of the VEGF-antibody bevacizumab improved outcomes only in tumors that also exhibited a myeloid cell signature. (16) These findings demonstrate the value of bevacizumab as an ICI booster in tumors with an immune suppressive microenvironment, but

also suggest that combination strategies act synergistically in a specific subset of patients, which has yet to be defined. Unfortunately, these signatures were developed specifically for the atezolizumab + bevacizumab combination, and this regimen never filed for FDA approval as it seemed less promising than concurrent ICI + VEGFR-TKI combos. Of note, the predictive impact of these signatures was not fully replicated in the JAVELIN Renal 101 trial, which compared the PD-L1 inhibitor avelumab + axitinib with sunitinib: avelumab + axitinib performed better than sunitinib in T_{eff} high tumors, but not in T_{eff} + Myeloid high tumors. (62)

Open questions

For the majority of patients, the clinically most important question at this moment, is the position of ICI + VEGFR-TKI combinations against nivolumab + ipilimumab in IMDC Intermediate and Poor risk patients. Both VEGFR-TKIs and ipilimumab are added to anti-PD1 as an immune booster, but they have an entirely different mechanism of action which is likely to be relevant in different tumors (e.g. those with a myeloid high signature will probably benefit more from VEGFR-TKI, but several other immune cell populations and pathways are involved as well).

Apart from models for patients selection, we also need new models to counsel patients on their prognosis. The current IMDC model held true for 1st line treatment with VEGFR-TKIs, but the prognosis of Intermediate/Poor risk patients and of those with sarcomatoid tumors, has improved relatively more with ICI combination therapies than the prognosis of Favorable risk patients.

Furthermore, a major problem in the metastatic setting are mixed responses to treatment. We understand little of how metastatic lesions are similar to or different from the primary tumor and how they are influenced by their host organ. As metastases are rarely resected, molecular data are very scarce. These are urgently needed to gain deeper insights in the dynamics of metastases and organ-specific metastatic niches.

In conclusion, the current crowded guidelines demonstrate the urgent need for biomarkers for patient selection. Moreover, as the standard of care is changing so rapidly, an ideal biomarker would be generic, reflecting intrinsic tumor biology, rather than be developed as a companion for a specific therapy. And most of all, a deeper molecular understanding of ccRCC and their immune environment is crucial to guide future research and trial design. After all, throughout the field of oncology there is a need for trials driven by a sound scientific rationale, as potential treatments have become too numerous to test

even a small fraction of them. Gaining these insights is particularly challenging in ccRCC, which are notoriously heterogeneous on a clinical, histological, molecular and immunological level.

ccRCC can be divided into four molecular subtypes

– Key message –

In 2015, our team has proposed four molecular subtypes of advanced ccRCC, ccrcc1 to -4, based on unsupervised clustering of whole transcriptome data. These subtypes differ not only in terms of gene expression, but also mutation and methylation profiles, immune cell infiltration, histological features, prognosis and response to sunitinib. The rare ccrcc3 subtype has the best prognosis and a gene expression profile that resembles that of normal kidney. Ccrcc2 tumors, accounting for almost half of ccRCC, have a good prognosis and are sensitive to sunitinib. Ccrcc1 tumors have an intermediate prognosis and an immune cold phenotype. Finally, ccrcc4 tumors are highly aggressive, often have sarcomatoid features, respond poorly to sunitinib and display a highly inflamed phenotype with an immunosuppressive microenvironment.

The ccrcc1 to -4 molecular subtypes

This thesis is built on earlier work by our team, which in 2015 has described four molecular subtypes of ccRCC. (65) These subtypes were discovered through unsupervised cluster analysis of microarray data of 53 fresh-frozen untreated primary ccRCC, which metastasized and were treated with 1st line sunitinib. The subtypes were subsequently validated on another 47 ccRCC and on the TCGA ccRCC cohort. These four transcriptomic groups, named ccrcc1 to -4, reflect intrinsic ccRCC tumor subtypes with different tumor biology and clinical behavior: they not only differed in terms of gene expression, but also mutation and methylation profiles, immune cell infiltration, histological features, prognosis and response to sunitinib. Their main differences are summarized in Figure 1.3 and Table 1.2.

The rare ccrcc3 subtype (11%) showed a gene expression profile that resembles that of normal kidney and an indolent clinical behavior (OS 50mo after 1st line sunitinib). It upregulated mainly metabolic pathways, but hardly expressed immune signatures and showed little infiltration by CD8+ cytotoxic

Table 1.2. *Discriminatory features of the ccrcc1 to -4 molecular subtypes as discovered on fresh-frozen ccRCC.*

Subgroup (frequency)			**ccrcc1 (33%)**	**ccrcc2 (41%)**	**ccrcc3 (11%)**	**ccrcc4 (15%)**
Outcome under sunitinib						
Early progressive disease			22%	3%	0%	27%
Partial response			41%	53%	70%	20%
Median OS (mo)			24	35	50	14
Median PFS (mo)			13	19	24	8
Clinical characteristics						
IMDC	Good		6%	21%	18%	7%
	Intermediate		69%	60%	64%	60%
	Poor		25%	18%	18%	33%
Molecular characteristics						
Pathology characteristics	Mean inflammation intensity (scale 0-3)		1.3	1.2	0.8	2.2
	Mean sarcomatoid differentiation		7.5%	3.7%	1.7%	24.6%
Mutations		VHL	47%	63%	20%	20%
		PBRM1	47%	38%	20%	0%
Upregulated pathways			MYC targets Glycolysis Hypoxia	Glycolysis Hypoxia		Immunity Apoptosis Chemotaxis MYC targets Glycolysis Hypoxia
MYC expression level			++	+	--	++
Methylation status			Hyper-methylated+			Hyper-methylated ++
Polycomb stem cell phenotype			++		--	+++
Copy number amplification						2p12 / 2p22.3/8q21.13
Proposed name			**MYC.UP**	**Classical**	**Normal like**	**Immun.UP/MYC.UP**

Adapted from Beuselinck et al, Clinical Cancer Research 2015. (65) Copyright held by AACR.

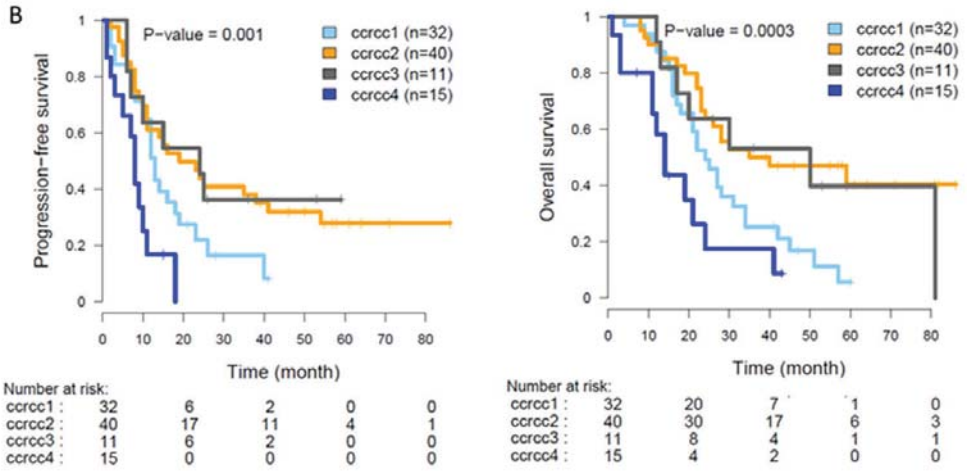

Figure 1.3. *PFS and OS on 1st line treatment with sunitinib according to molecular subtype. Adapted from Beuselinck et al, Clinical Cancer Research 2015. (65) Copyright held by AACR.*

T-cells. The ccrcc2 subtype was the most frequent one (41%), but no particular upregulated pathways were established at that time. It also showed a favorable baseline prognosis (OS 35mo) and high response rates to sunitinib (53%). Ccrcc2 tumors had intermediate infiltration by CD8+ T-cells and expression of immune signatures.

The ccrcc1 and -4 subtypes displayed a more aggressive gene expression pattern, with upregulation of *MYC* and MYC-targets. Both subtypes also showed a more undifferentiated phenotype, with hypermethylation and consequent downregulation of polycomb targets, as well as higher histological grades. The ccrcc1 subtype (33%) had an intermediate prognosis and response to sunitinib (OS 24mo, RR 41%) and showed little infiltration by CD8+ T-cells or expression of immune signatures. The ccrcc4 subtype (15%) on the other hand, had the shortest OS (8mo) and lowest RR (20%) to sunitinib. This subtype was enriched for tumors with sarcomatoid features. It showed high infiltration by CD8+ T-cells and high expression of immune signatures. These latter however also included high expression of checkpoints and immune suppressive cells such as myeloid cells, indicating an inflamed but suppressed immune response.

Importantly, a 35-gene classifier algorithm was constructed that was able to classify independent ccRCC samples into the four groups, without the need to cluster them against reference samples as is the case with the other molecular classifiers discussed below.

Other transcriptome-based ccRCC molecular subtypes

Three other teams have performed unsupervised cluster analysis of whole transcriptome ccRCC data and reported very similar results, which confirms the existence of four robust transcriptomic subtypes with different clinical behavior. (8,22,66)and to aid in predicting clinical outcomes. However, there are no current signatures for kidney cancer that are applicable in a clinical setting. Objective To generate a signature biomarker for the clear cell renal cell carcinoma (ccRCC In the adjuvant setting, the TCGA research programme identified four clusters (m1 to m4), of which m1 was the most frequent (35%) and had a favorable prognosis, whereas m2 and m3 (together 44%) had a dismall prognosis. The group of Brannon et al identified three clusters in the adjuvant setting: ccA with a favorable prognosis, ccB with a poor prognosis and cluster_3 that was rare. There is significant overlap between Brannon's ccA, TCGA's m1 and our ccrcc2 cluster, between Brannon's cluster_3 and our ccrcc3 and between Brannon's ccB, TCGA's m2+m3 and our ccrcc1+ccrcc4 clusters.

Very recently, Hakimi et al performed cluster analysis on a large cohort of primary ccRCCs that developed metastatic disease and received 1st line sunitinib or pazopanib: a clinical setting that is almost identical to the one in which the ccrcc1 to -4 subtypes were discovered. They found four clusters with a relative frequency and outcomes on VEGFR-TKIs that were very similar to the ccrcc1 to -4 clusters, and also validated their findings on our original ccrcc1 to -4 dataset. Their cluster 3 shared many characteristics with ccrcc2 tumors, whereas their cluster 4 clearly stood out as the most dismall subtype and displayed upregulation of MYC targets, proliferation markers and several immune signatures, as does our ccrcc4 subtype.

References to Chapter 1 Introduction: see Chapter 12 Concluding discussion

Chapter 2

Objectives

Because the ccrcc1 to -4 molecular subtypes reflect intrinsic differences in tumor biology, we hypothesized that they may have a prognostic and predictive value as a generic biomarker in different therapeutic settings besides 1st line sunitinib. This is in contrast to most other biomarkers, which have been optimized for their predictive power in a specific therapeutic context. Therefore these subtypes might retain their value in the swiftly changing landscape of ccRCC treatment.

More importantly, we hypothesized that a deeper molecular understanding of ccRCC and intertumor differences will be crucial to guide further research into therapeutic (combination) strategies and trial design.

However, the ccrcc1 to -4 subtypes were initially established on fresh-frozen tumor samples. This technique hampered further research, since fresh-frozen samples are not routinely collected, tedious to work with and difficult to exchange between centers.

The major aims of this thesis were therefore threefold:

1. **Assess the predictive value of the ccrcc1 to -4 molecular subtypes in different therapeutic settings**

   1.1. Association of the ccrcc1 to -4 molecular subtypes with outcome on 1st line pazopanib
   The predictive value of the subtypes in this setting described in chapter 3.

   1.2. Association of the ccrcc1 to -4 molecular subtypes with early relapse after metastasectomy
   We review the place of local ablative treatment for ccRCC metastases in chapter 4 and assess clinical outcomes after metastasectomy together with the shortcomings of current selection criteria in chapter 5. In chapter 6, we describe how the molecular subtypes can predict early relapse after metastasectomy, even after rigorous patient selection.

2. **Gain a deeper molecular understanding of the ccrcc1 to -4 molecular subtypes**

   2.1. Association of the molecular subtypes with angiogenic gene expression, IMDC risk groups, and sarcomatoid dedifferentiation
   In chapter 7 we demonstrate the association of proangiogenic gene

expression with favorable outcomes on VEGFR-TKI. In chapter 8, we show how different molecular subtypes underlie three recently established biomarkers (IMDC, angio signature, sarcomatoid features) and combine the information they provide in one generic marker.

2.2. microRNA (miRNA) expression profiles underlying the ccrcc1 to -4 subtypes

As miRNA are master regulators of mRNA, we hypothesized that the transcriptomic ccrcc1 to -4 subtypes would be at least partially driven by differences in miRNA expression. In chapter 9 we assess subtype-specific miRNA expression patterns and how these correlate with prognosis.

2.3. Gain a deeper understanding of the ccrcc1 to -4 molecular subtype's immune phenotypes

Since the ideal biomarker for immunotherapies will consist of a combination of different markers, and since the differences in immune phenotype of the molecular subtypes are incompletely understood, we assess their enrichment for specific immune signatures and immune cell infiltration in chapter 10.

2.4. Assess intra- and interpatient heterogeneity

It is unclear to what extent metastases are similar or different from the primary tumor, and to what extent they are influenced by the host organ. In chapter 10, we compare transcriptomic data of a large set of metastases and primary tumors.

3. **Validate the classification of the ccrcc1 to -4 molecular subtypes based on formalin-fixed paraffin-embedded (FFPE) samples**

In chapter 10, we establish a robust and user-friendly protocol that allows the classification of FFPE samples. In contrast to the fresh-frozen protocol, the small amount of tissue provided by one core needle biopsy is sufficient, which is important as the indications for cytoreductive nephrectomy have narrowed.

Chapter 3

Molecular subtypes of clear-cell renal cell carcinoma are associated with outcome during pazopanib therapy in the metastatic setting

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Clinical genitourinary cancer. 2018;16(3):e605-e612

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Molecular Subtypes of Clear Cell Renal Cell Carcinoma Are Associated With Outcome During Pazopanib Therapy in the Metastatic Setting

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Abstract

We previously defined 4 molecular subtypes of metastatic clear cell renal cell carcinoma, ccrcc1-4, with different response rates, progression-free survival (PFS), and overall survival (OS) during first-line treatment with sunitinib. Expecting a drug class effect, we assessed the outcomes of 28 patients treated with pazopanib as first-line therapy. The outcomes were similar, with significantly different tumor shrinkage, PFS, and OS rates among the 4 subtypes. The capability of the ccrcc1-4 molecular classification to select patients for treatment requires validation in prospective trials.

Background: We previously described 4 molecular subtypes of metastatic clear cell renal cell carcinoma (mccRCC), named ccrcc1-4 (Beuselinck et al, 2015). These have both prognostic and predictive value for patients treated with first-line sunitinib, with distinctive objective response rate (ORR), progression-free survival (PFS), and overall survival (OS). The ccrcc2 and ccrcc3 tumors have the best outcomes, followed by ccrcc1 and then ccrcc4. We hypothesized that these molecular subtypes would show similar outcomes with first-line pazopanib treatment. **Patients and Methods:** We classified 28 mccRCC tumors treated with pazopanib as first-line therapy, as described previously. The primary endpoints were PFS and OS from the start of pazopanib. A secondary endpoint was ORR. Because there were only 2 ccrcc3 tumors, they were pooled with the ccrcc2 tumors for outcome analysis. **Results:** PFS was 9 months for the ccrcc2 and ccrcc3 tumors, 5 months for ccrcc1 tumors, and 3 months for the ccrcc4 tumors ($P = .011$). The corresponding OS duration was 69, 19, and 5 months ($P = .003$). The corresponding ORR was 50%, 33%, and 0%. The corresponding mean tumor size decreased by 34%, 6%, and 2% ($P = .032$). The ccrcc1-4 classification was a stronger predictor of outcome than the International Metastatic Renal Cell Carcinoma Database Consortium score on univariate analysis ($P = .011$ vs. $P = .094$ for PFS and $P = .003$ vs. .013 for OS). Both remained independent on bivariate analysis. **Conclusion:** The molecular subtypes of mccRCC are associated with outcome on pazopanib as first-line therapy. The prognostic and predictive value of the ccrcc1-4 molecular classification requires validation in prospective trials.

Clinical Genitourinary Cancer, Vol. 16, No. 3, e605-12 © 2017 Elsevier Inc. All rights reserved.

Keywords: Biomarker, Gene expression profiling, Molecular classification, Predictive, Vascular endothelial growth factor receptor tyrosine kinase inhibitor

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Submitted: Aug 21, 2017; Revised: Oct 25, 2017; Accepted: Oct 30, 2017; Epub: Nov 7, 2017

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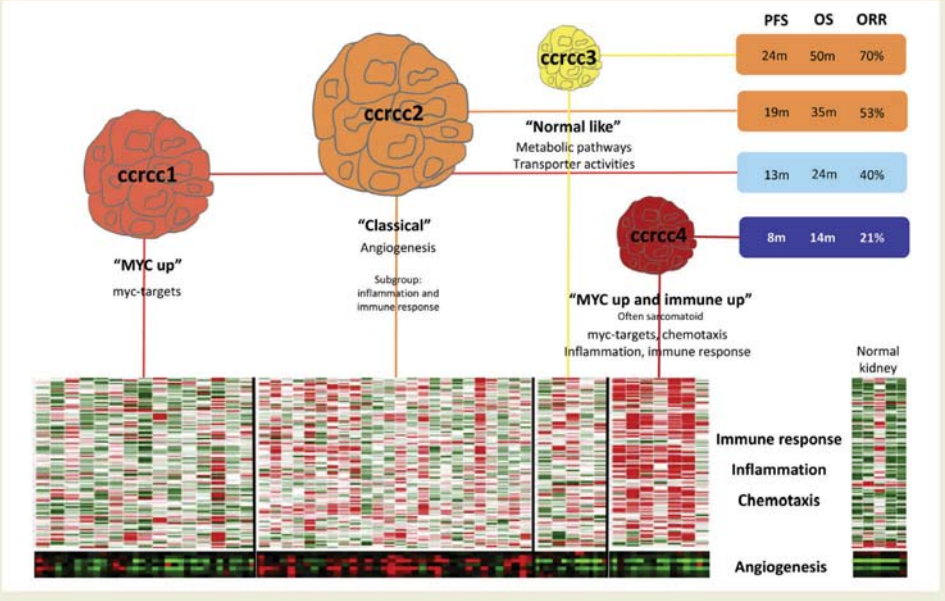
https://doi.org/10.1016/j.clgc.2017.10.017

Figure 1 Classification of Metastatic Clear Cell Renal Cell Carcinoma (mccRCC) Into 4 Molecular Subtypes, Determined by Unsupervised Clustering of Whole Genome Expression Data, With Summary of Subtype Characteristics From Untreated Primary Tumors of 98 mccRCC Patients Ultimately Treated With Sunitinib as First-line Targeted Therapy in the Metastatic Setting. Tumor Size Represents Relative Frequency (ccrcc3, 11%; ccrcc2, 41%; ccrcc1, 33%; ccrcc4, 15%). Subtypes Ranked Top to Bottom From Best to Worst Prognosis, With Corresponding Outcomes on Sunitinib on the Right and Gene Expression Profiles of Most Important Genes Implicated in Immune Response, Inflammation, Chemotaxis, and Angiogenesis on the Bottom. Tumors Classified as ccrcc2 Showed Relative Upregulation in Genes Implicated Angiogenesis; ccrcc4 and a Subgroup of ccrcc2 Tumors Showed Relative Upregulation of Genes Implicated in Immune Response, Inflammation, and Chemotaxis



Abbreviations: m = months; PFS = progression-free survival; OS = overall survival; ORR = objective response rate.

Introduction

Kidney cancer ranks in the top 10 of most frequent cancers in both men and women. Clear cell renal cell carcinoma (ccRCC) is the most frequent type, representing ~80% of cases.[1] Although localized ccRCC can often be cured with surgical resection, metastatic disease (mccRCC) is treated with systemic therapy with or without cytoreductive surgery. The standard first-line systemic treatment for mccRCC is a vascular endothelial growth factor receptor tyrosine kinase inhibitor (VEGFR-TKI; eg, sunitinib or pazopanib).[2,3] Although the use of VEGFR-TKIs has substantially improved progression-free survival (PFS), many tumors are primarily resistant to therapy, with progressive disease as the best response (7% and 18% in phase III trials of sunitinib and pazopanib, respectively).[4,5] Moreover, patients with a response to therapy will develop secondary resistance after variable intervals.[6]

For second-line therapy, many targeted agents are available, with different mechanisms of action, including other angiogenesis inhibitors, immune checkpoint inhibitors, the c-MET and VEGFR inhibitor cabozantinib, and mammalian target of rapamycin inhibitors. The optimal sequence, combination, and patient selection for these therapies, however, remains unknown and is the subject of many ongoing trials. Predictive biomarkers are, therefore, urgently needed. To predict overall survival (OS) on VEGFR-TKIs, the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) prognostic score is mostly used.[7] This score, however, is based on clinical parameters and does not address intrinsic tumor biology, leaving a knowledge gap that biomarkers can improve on.

We recently proposed a division of mccRCC into 4 molecular subtypes.[8] To this end, we performed an unsupervised cluster analysis of whole genome mRNA expression data from mccRCC patients receiving sunitinib as first-line targeted therapy. That analysis revealed 4 robust gene expression clusters, named ccrcc1-4. These subtypes showed both prognostic and predictive value for

Table 1 Patient and Tumor Characteristics (n = 28)

Characteristic	n (%)
Gender	
Female	12 (43)
Male	16 (57)
Age at diagnosis, y	
Median	67
Range	45-82
Synchronous metastases	8 (32)
Histologic type	
Fuhrman grade 2-3	14 (50)
Fuhrman grade 4	14 (50)
Sarcomatoid differentiation ≥25%	2 (7)
Characteristics at start of pazopanib	
Karnofsky performance status <80%	1 (4)
Neutrophils >7.800/mm^3	2 (7)
Platelets >450.000/mm^3	5 (18)
Low hemoglobin (<12 g/dL, women; <14 g/dL, men)	17 (60)
Lactate dehydrogenase >1.5 × upper limit of normal	0 (0)
Corrected calcium >10 mg/dL	2 (7)
Baseline C-reactive protein >5 mg/dL	13/23 (57)
Interval from nephrectomy to systemic treatment <12 mo	9 (32)
Interferon before pazopanib	1 (4)
Systemic treatment other than interferon before pazopanib	0 (0)
Site of metastasis	
Lung	22 (79)
Lymph nodes	14 (54)
Liver	7 (25)
Bone	8 (29)
Brain	3 (11)
IMDC score	
Good risk	7 (25)
Intermediate risk	17 (61)
Poor risk	4 (14)

Abbreviation: IMDC = International Metastatic Renal Cell Carcinoma Database Consortium.

treatment with first-line sunitinib, demonstrated by significant differences in PFS, OS, and the objective response rate (ORR). A 35-gene classifier is able to distinguish among the 4 subtypes.

The main characteristics of these subtypes are illustrated in Figure 1. Subtypes ccrcc2 and ccrcc3 have a favorable prognosis with first-line sunitinib treatment. The ccrcc2 tumors are marked by the relative upregulation of genes related to angiogenesis, which has been linked to improved outcome on sunitinib treatment.[9] The ccrcc3 tumors show a gene expression profile similar to that of the normal kidney and have an indolent disease course. In contrast, ccrcc1 and ccrcc4 tumors have a short PFS and OS and rarely display durable responses to sunitinib. Both show relative upregulation of c-MYC targets. Immunologically, however, ccrcc1 and ccrcc4 are very different. The ccrcc1 tumors have the lowest rate of T-cell infiltration and T-cell inhibition markers, marking them as "immune-cold" tumors. In contrast, the ccrcc4 tumors, the most aggressive subtype, display high levels of T-cell infiltration but also high expression of T-cell inhibition markers such as PD-1, PD-L1, TIM-3, and LAG-3.

We hypothesized that the differences among the molecular subtypes in the outcome on first-line sunitinib represent a class effect of VEGFR-TKIs. We therefore sought to assess the effect of the molecular subtypes in mccRCC patients treated with first-line pazopanib therapy.

Patients and Methods

Patient Population

We retrospectively searched the CIT-rein database for all mccRCC patients who had been treated with first-line pazopanib and for whom fresh-frozen primary tumor tissue was available. The French-Belgian multicentric CIT-rein kidney tumor bank contains > 400 kidney tumor samples collected at 20 academic hospitals. For inclusion in the present study, patients had to have developed synchronous or metachronous metastases, received pazopanib (800 mg/day) as first-line treatment in the metastatic setting (earlier cytokine therapy was allowed), completed at least one 28-day cycle of pazopanib, and undergone their first computed tomography assessment. The drug schedule and dose reduction policy complied with practice guidelines. Follow-up computed tomography scans were performed every 2 treatment cycles.

Sample Classification

mRNA extraction of primary tumors was performed with a Maxwell machine using the Maxwell 16 LEV simplyRNA tissue kit according to the manufacturer's protocol (Promega). Quantification was performed using a NanoDrop instrument (Thermo Fisher) and quality control using gel electrophoresis (E-gel 48.1% agarose and Mother E-base; Invitrogen). Using the High Capacity Transcription kit (Life Technologies), 500 ng of total RNA was reverse transcribed in a final volume of 50 µL. Preamplification of 6 ng cDNA/sample was performed using PreAmp Master Mix, and the specific primers of the classifier, as well as 18S. Preamplified cDNA was diluted 1:5 in DNAse/RNAse free water before quantitative reverse transcription-polymerase chain reaction (qRT-PCR). qRT-PCRs were performed using the high throughput BioMark qRT-PCR system (Fluidigm, South San Francisco, CA), according to the manufacturer's protocol. Expression data (threshold cycle values) were analyzed using the Fluidigm qRT-PCR analysis software, version 4.1.3. 18S was used for normalization. The tumors were classified into the ccrcc1-4 molecular classification using our 35-gene classifier algorithm, as described previously.[8]

Statistical Analysis

The primary endpoints were PFS and OS since the start of pazopanib. The secondary endpoints were ORR and tumor shrinkage (measured using Response Evaluation Criteria In Solid Tumors, version 1.1). However, the series was not powered to detect a significant difference. PFS and OS were estimated using the Kaplan-Meier method and compared using the log-rank test. Differences in objective response versus stable or progressive disease were assessed with Fisher's exact test. Changes in the tumor volume at the maximal response were measured, and the mean values were compared using 1-way analysis of variance. We used a Cox proportional hazard model for bivariate analysis of the ccrcc1-4 classification and IMDC score, which was retrospectively calculated for each patient. Because of the small sample size, a bivariate analysis was

Table 2 Summary of Outcomes Stratified by Molecular Subtype

Variable	Subtype			
	ccrcc1	ccrcc2	ccrcc3	ccrcc4
Frequency	**6 (21)**	**16 (57)**	**2 (7)**	**4 (14)**
Name	**MYC.UP**	**Classic**	**Normal like**	**Immune.up/MYC.up**
Outcome with pazopanib				
Early progressive disease	3 (50)	1 (6)		1 (25)
Stable disease	1 (17)	7 (44)		3 (75)
Partial response	2 (33)	8 (50)		0 (0)
Mean volume change, %	−6	−34		−2
Median OS (mo)	19	69		5
Median PFS (mo)	5	9		3
Clinical characteristic				
IMDC				
Good risk	0 (0)	7 (39)		0 (0)
Intermediate risk	6 (100)	9 (50)		2 (50)
Poor risk	0 (0)	2 (11)		2 (50)

Data presented as n (%).
Abbreviations: IMDC = International Metastatic Renal Cell Carcinoma Database Consortium; OS = overall survival; PFS = progression-free survival.

used for ccrcc2 and ccrcc3 versus ccrcc1 and ccrcc4 and IMDC good or intermediate versus IMDC poor. For the same reason, a multivariate analysis was not possible. GraphPad Prism, version 7, and XLstat 2017.4 were used for the statistical analyses.

The medical ethics review boards of the participating institutions approved the protocol, and all the patients provided informed consent in accordance with the Belgian and French legislation. Frozen biologic material from deceased patients was used when previous agreement for such use had been given by the institutional review board.

Results

A total of 28 patients met the inclusion criteria. The patient characteristics are summarized in Table 1. The median follow-up period was 14 months. Of the 28 patients, 2 had $\geq$ 25% sarcomatoid dedifferentiation, 1 with ccrcc4 and 1 with ccrcc1.

The global PFS was 7 months, and the global OS was 28 months. The response could be assessed in 26 patients, and the percentage of tumor shrinkage in 24 patients. Ten patients (36%) experienced a partial remission as the best response, 11 (39%) had stable disease, and 5 (19%) had progressive disease. One patient received interferon before the start of pazopanib.

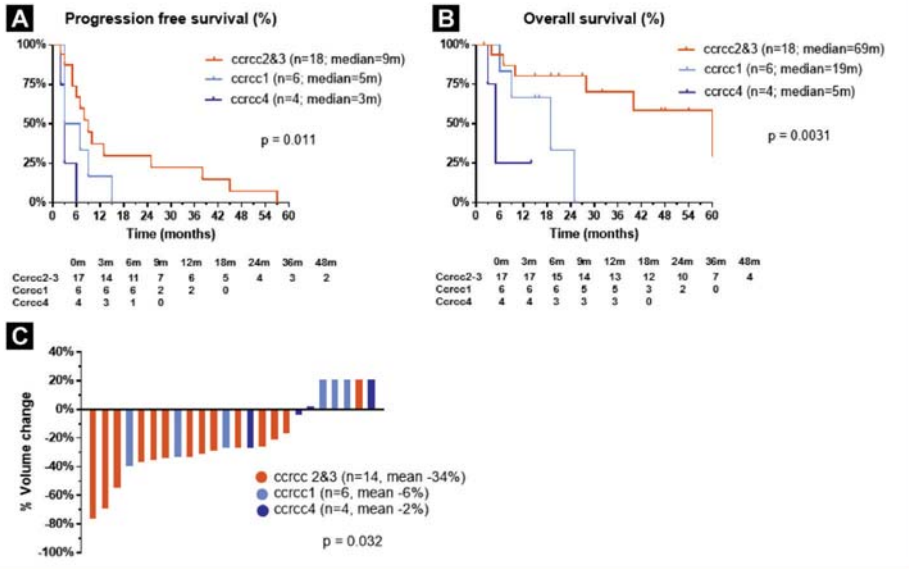
Six tumors were classified as ccrcc1 (21%), 16 as ccrcc2 (57%), 2 as ccrcc3 (7%), and 4 as ccrcc4 (14%), a distribution in line with former findings. We pooled the ccrcc3 patients with the ccrcc2 patients for the outcome analysis, because these are both good prognosis subtypes. The PFS and OS were significantly different among the subtypes. The PFS was 9 months for those in the ccrcc2 and ccrcc3 group, 5 months for those with ccrcc1 tumors, and 3 months in those with ccrcc4 tumors (P = .011). The OS was 69 months for the ccrcc2 and ccrcc3 tumor group, 19 months for those with ccrcc1 tumors, and 5 months in those with ccrcc4 tumors (P = .003). The ORR was 50%, 33%, and 0% in the ccrcc2 plus ccrcc3, ccrcc1, and ccrcc4 groups, respectively (P = NS). The corresponding mean tumor size decreased by 34%, 6%, and 2% (P = .032; Table 2 and Figure 2).

When comparing univariate analyses of common prognostic markers, the ccrcc1-4 classification showed a significant correlation with PFS and OS. The commonly used IMDC score, however, showed a significant correlation with OS but only a trend toward significance for PFS (Table 3). On bivariate analysis, both the ccrcc1-4 classification and the IMDC score were independently associated with PFS and OS (Table 4).

Discussion

The molecular ccrcc1-4 classification of mccRCC previously showed a prognostic effect in patients treated with first-line sunitinib. It also showed a predictive value, with a significantly greater PFS and ORR for the good prognosis ccrcc2 and ccrcc3 tumors. We hypothesized that these findings represent a class effect of VEGFR-TKIs. In this series of 28 patients treated with first-line pazopanib, we found a significant difference in tumor shrinkage, PFS, and OS among the subtypes. Other molecular classifications of ccRCC have been proposed.[10,11] On a genomic level, the existence of poor-risk BAP1 mutations and good-risk PBRM1 mutations is well-known, although without clinical implications.[12] Although these ccRCC driver mutations are typically subclonal, a parallel convergent evolution of subclones is often present.[13] On a transcriptomic level, other groups have demonstrated the existence of prognostic molecular subtypes of ccRCC in the postnephrectomy setting, based on cluster analysis of whole genome mRNA expression data.[14-17] Brooks et al[18] have proposed a 34-gene classifier, Clearcode34, which classifies tumors into a good-risk (ccA) or poor-risk (ccB) group. This classification assesses the risk of relapse and survival for patients undergoing curative nephrectomy. An effect of the ccA/ccB subtypes on OS with

Figure 2 Differences in Outcomes Among the ccrcc1-4 Molecular Subtypes, After Start of First-line Pazopanib. (A) Kaplan-Meier Estimate of Progression-Free Survival. (B) Kaplan-Meier Estimate of Overall Survival. (C) Waterfall Plot Showing Volume Change at Time of Best Response. Mean Values and *P* Values Were Calculated Using 1-Way Analysis of Variance



Abbreviation: m = months.

first-line systemic therapy, in general, was also shown in the data from The Cancer Genome Atlas by de Velasco et al.[19] When we applied the ccrcc1-4 classifier to the data set from Brooks et al,[18] a significant overlap between the ccA and ccrcc2 cluster and between the ccB and ccrcc1 plus ccrcc4 clusters was seen. The ccrcc1-4 classification, however, is determined on the expression profiles of primary tumors that had all reached the metastatic setting and is therefore specifically suited for that setting. It allows, for example, the subdivision of the poor prognosis cluster (ccB) into the ccrcc1 and ccrcc4 subtypes, which are immunologically very different. The ccrcc4 tumors are highly infiltrated by $CD8^+$ T-cells and display a strong inflammatory gene signature. Although T-cell infiltration has been linked to better outcomes in other tumor types, it is a poor prognostic marker in ccRCC.[20-29] This might be because of an exhausted phenotype of the T-cells present or a less organized immune response. Several studies have shown that in ccRCC, the presence of $CD8^+$ lymphocytes is accompanied by high expression of immune checkpoints, thus inhibiting T-cell function.[27,28,30] Others have shown that in ccRCC, the immune infiltrate is rarely organized into tertiary lymphoid structures, as it is in other cancer types.[29,30] In the rare ccRCC tumors with tertiary lymphoid structures and low expression of immune checkpoints, the presence of $CD8^+$ lymphocytes has been linked to a better prognosis.[30] These findings, as well as their poor response to VEGFR-TKIs, suggest ccrcc4 tumors could be good candidates for treatment with immune checkpoint inhibitors.[31] In an exploratory series of 6 patients treated with nivolumab, Senbabaoglu et al[27] reported greater baseline T-cell infiltration in the patients with a treatment response.

The main limitation of the present study was its small sample size. However, the biologic rationale for an underlying class effect of VEGFR-TKIs and the parallel findings with the sunitinib series (with crrcc2 and ccrcc3 the most favorable, ccrcc1 intermediate, and ccrcc4 poor) strengthens the validity of these results. Moreover, the strength of the biomarker is underscored by its clear effect in a small series. Other limitations were the retrospective design and the lack of a control group that was not treated with a VEGFR-TKI. The biologic rationale for the prognostic and predictive effect of the classification on outcome with VEGFR-TKI treatment, combined with the affirmative results of the present study, now allow for prospective validation in randomized trials. One phase II trial investigating the capability of the ccrcc1-4 classification to select patients for VEGFR-TKIs or immune checkpoint inhibitors has already begun enrollment (BIONIKK [a biomarker driven trial with nivolumab and ipilimumab or VEGFR TKI in treatment naive metastatic kidney cancer]; ClinicalTrials.gov identifier, NCT02960906).

Conclusion

As more and more treatment options have become available for mccRCC, the need for predictive biomarkers has increased. The

Table 3 Kaplan-Meier Estimates of Progression-free and Overall Survival

Variable	Median PFS (mo)	*P* Value	Median OS (mo)	*P* Value
Neutrophil count		.668		.616
>7.800/mm^3 (n = 2)	7		11	
≤7.800/mm^3 (n = 26)	7		40	
Platelet count		.006		<.001
>450.000/mm^3 (n = 5)	3		5	
≤450.000/mm^3 (n = 23)	8		69	
Karnofsky performance status		.365		.132
≤70 (n = 1)	5		7	
>70 (n = 27)	7		40	
Hemoglobin		.549		.013
Low[a] (n = 17)	5.5		19	
Normal (n = 11)	9		69	
Corrected calcium		.005		<.001
>10 mg/dL (n = 2)	2.5		4	
≤10 mg/dL (n = 23)	7		40	
Interval from diagnosis to systemic treatment		.023		.513
<1 y (n = 19)	5		24.5	
>1 y (n = 9)	9		40	
Bone metastases at start of pazopanib		.148		.897
Yes (n = 8)	5		19	
No (n = 20)	7		40	
Sarcomatoid dedifferentiation		.008		.008
≥25% (n = 2)	2.5		5.5	
≤24% (n = 23)	7		40	
C-reactive protein		.476		.087
>5 mg/dL (n = 13)	4		14	
≤5 mg/dL (n = 10)	7		80	
IMDC		.094		.013
Poor risk (n = 4)	4		6	
Intermediate risk (n = 17)	6.5		40	
Good risk (n = 7)	9		69	
ccrcc Classification		.011		.003
ccrcc1 (n = 6)	5		19	
ccrcc2 plus ccrcc3 (n = 18)	9		69	
ccrcc4 (n = 4)	3		5	
Factors recorded for bivariate analysis				
ccrcc Groups		.023		.004
ccrcc1 plus ccrcc4 (n = 10)	3		17.5	
ccrcc2 plus ccrcc3 (n = 18)	9		69	
IMDC risk		.031		.004
Poor (n = 4)	4		6	
Intermediate and good (n = 24)	8		69	

Local cutoffs were used for serum levels; no patient had elevated lactate dehydrogenase; and the same 2 patients had both hypercalcemia and sarcomatoid dedifferentiation.
Abbreviations: IMDC = International Metastatic Renal Cell Carcinoma Database Consortium; OS = overall survival; PFS = progression-free survival.
[a]Low hemoglobin, < 12 g/dL for women and < 14 g/dL for men.

ccrcc1-4 molecular classification was previously shown to have prognostic and predictive value for survival in mccRCC patients treated with first-line sunitinib. In the present study, we have replicated these findings in patients treated with first-line pazopanib. The classification was developed specifically for the metastatic setting and is correlated with the expression of genes related to angiogenesis and the immune response, which have been implicated in the response to different targeted therapies. It is therefore a good

Table 4 Bivariate Analysis Results for Progression-free and Overall Survival With a Cox Proportional Hazard Model

Variable	PFS			OS		
	P Value	HR	95% CI	*P* Value	HR	95% CI
IMDC intermediate/good vs. poor	.035	3.6	1.1-12.0	.003	8.7	2.1-36.02
ccrcc2 and ccrcc3 vs. ccrcc1 and ccrcc4	.026	2.8	1.1-7.0	.004	9.3	0-43.5

Abbreviations: CI = confidence interval; HR = hazard ratio; IMDC = International Metastatic Renal Cell Carcinoma Database Consortium; OS = overall survival; PFS = progression-free survival.

candidate for validation of its prognostic and predictive effect in different therapeutic settings. Moreover, because this classification was based on intrinsic tumor biology, rather than designed as a marker in a specific therapeutic context, it has the potential for it to retain its value in the rapidly changing landscape of mccRCC treatment.

Clinical Practice Points

- Many different targeted therapies are available for the treatment of mccRCC; however, each of these therapies is active in only a subgroup of patients, and no predictive biomarkers are available to select patients for the best treatment.
- The ccrcc1-4 molecular classification is prognostic and predictive for outcome with sunitinib as first-line systemic treatment; ccrcc2 and ccrcc3 tumors have favorable, ccrcc1 intermediate, and ccrcc4 poor outcomes.
- We have now shown a similar effect with the ccrcc1-4 classification for patients treated with first-line pazopanib.
- There is a biologic rationale to expect a favorable effect of immune checkpoint inhibitors in the treatment of the poor-prognosis ccrcc4 tumors.
- Randomized prospective trials are needed to assess the capability of the ccrcc1-4 molecular classification to select patients for treatment.

Acknowledgments

The original ccrcc1-4 classification project was funded by INSERM and the "Cartes d'identité des Tumeurs" program of the French National Ligue against Cancer. The present work was supported by Stichting Kom op tegen Kanker and Fonds voor Wetenschappelijk Onderzoek Vlaanderen. The funding sources were not involved in any aspect of the study design, data collection, analysis and interpretation, or writing of the report and decision to submit for publication. A. Verbiest received an Emmanuel van der Schueren grant from Stichting Kom op tegen Kanker. B. Beuselinck received a grant from Fondation Martine Midy (Paris, France) and Fonds voor Wetenschappelijk Onderzoek Vlaanderen (Belgium). E. Lerut received funding from Fonds voor Wetenschappelijk Onderzoek Vlaanderen and Stichting tegen Kanker.

Disclosure

B. Beuselinck received honoraria from Amgen, Pfizer, Janssen, and Bayer and is an investigator for the EudraCT: 2011-006085-40/MetaSun trial supported by Pfizer. B. Laguerre received honoraria for Pfizer, Novartis, BMS, and Ipsen. The remaining authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. *CA Cancer J Clin* 2017; 67:7-30.
2. Escudier B, Porta C, Schmidinger M, et al. Renal cell carcinoma: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol* 2016; 27(suppl 5):58-68.
3. Motzer RJ, Jonasch E, Agarwal N, et al. Kidney cancer, version 2.2017, NCCN clinical practice guidelines in oncology. *J Natl Compr Canc Netw* 2017; 15:804-34.
4. Sternberg CN, Davis ID, Mardiak J, et al. Pazopanib in locally advanced or metastatic renal cell carcinoma: results of a randomized phase III trial. *J Clin Oncol* 2010; 28:1061-8.
5. Motzer RJ, Hutson TE, Tomczak P, et al. Overall survival and updated results for sunitinib compared with interferon alfa in patients with metastatic renal cell carcinoma. *J Clin Oncol* 2009; 27:3584-90.
6. Rini B. Resistance to targeted therapy in renal-cell carcinoma. *Lancet Oncol* 2009; 10:992-1000.
7. Heng DY, Xie W, Regan MM, et al. Prognostic factors for overall survival in patients with metastatic renal cell carcinoma treated with vascular endothelial growth factor-targeted agents: results from a large, multicenter study. *J Clin Oncol* 2009; 27:5794-9.
8. Beuselinck B, Job S, Becht E, et al. Molecular subtypes of clear-cell renal cell carcinoma are associated with sunitinib response in the metastatic setting. *Clin Cancer Res* 2015; 21:1329-39.
9. Beuselinck B, Verbiest A, Couchy G. Pro-angiogenic gene expression is associated with better outcome on sunitinib in metastatic clear-cell renal cell carcinoma. *Acta Oncol* 2017; 2:1-11.
10. Hsieh JJ, Purdue MP, Signoretti S. Renal cell carcinoma. *Nat Rev Dis Primers* 2017; 3:17009.
11. Casuscelli J, Vano YA, Fridman WH. Molecular classification of renal cell carcinoma and its implication in future clinical practice. *Kidney Cancer* 2017; 1: 3-13.
12. Kapur P, Pena-Llopis S, Christie A. Effects on survival of BAP1 and PBRM1 mutations in sporadic clear-cell renal-cell carcinoma: a retrospective analysis with independent validation. *Lancet Oncol* 2013; 14:159-67.
13. Gerlinger M, Horswell S, Larkin J. Genomic architecture and evolution of clear cell renal cell carcinomas defined by multiregion sequencing. *Nat Genet* 2014; 46:225-33.
14. Brannon AR, Haake SM, Hacker KE, et al. Meta-analysis of clear-cell renal cell carcinoma gene expression defines a variant subtype and identifies gender influences on tumor biology. *Eur Urol* 2012; 61:258-68.
15. Choudhury Y, Wei X, Chu YH, et al. A multigene assay identifying distinct prognostic subtypes of clear-cell renal cell carcinoma with differential response to tyrosine kinase inhibition. *Eur Urol* 2015; 67:17-20.
16. Cancer Genome Atlas Research. Comprehensive molecular characterization of clear-cell renal cell carcinoma. *Nature* 2013; 499:43-9.
17. Chen F, Zhang Y, Senbabaoglu Y, et al. Multilevel genomics-based taxonomy of renal cell carcinoma. *Cell Rep* 2016; 14:2476-89.
18. Brooks SA, Brannon AR, Parker JS, et al. ClearCode34: a prognostic risk predictor for localized clear-cell renal cell carcinoma. *Eur Urol* 2014; 66:77-84.
19. de Velasco G, Culhane AC, Fay AP, et al. Molecular subtypes improve prognostic value of International Metastatic Renal Cell Carcinoma Database Consortium prognostic model. *Oncologist* 2017; 22:286-92.
20. Pages F, Kirilovsky A, Mlecnik B. In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. *J Clin Oncol* 2009; 27:5944-51.
21. Al-Shibli KI, Donnem T, Al-Saad S. Prognostic effect of epithelial and stromal lymphocyte infiltration in non-small cell lung cancer. *Clin Cancer Res* 2008; 14: 5220-7.
22. Mahmoud SM, Paish EC, Powe EG. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. *J Clin Oncol* 2011; 29:1949-55.
23. Ubukata H, Motohashi G, Tabuchi T. Evaluations of interferon-gamma/interleukin-4 ratio and neutrophil/lymphocyte ratio as prognostic indicators in gastric cancer patients. *J Surg Oncol* 2010; 102:742-7.
24. Sharma P, Shen Y, Wen S. CD8 tumor-infiltrating lymphocytes are predictive of survival in muscle-invasive urothelial carcinoma. *Proc Natl Acad Sci U S A* 2007; 104:3967-72.
25. Gao Q, Qiu SJ, Fan J. Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. *J Clin Oncol* 2007; 25:2586-93.
26. Zhang L, Conejo-Garcia JR, Katsaros D. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* 2003; 348:203-13.

27. Senbabaoglu Y, Gejman RS, Winer AG. Tumor immune microenvironment characterization in clear cell renal cell carcinoma identifies prognostic and immunotherapeutically relevant messenger RNA signatures. *Genome Biol* 2016; 17:231.
28. Becht E, Giraldo NA, Beuselinck B. Prognostic and theranostic impact of molecular subtypes and immune classifications in renal cell cancer (RCC) and colorectal cancer (CRC). *Oncoimmunology* 2015; 4:e1049804.
29. Remark R, Alifano M, Cremer I. Characteristics and clinical impacts of the immune environments in colorectal and renal cell carcinoma lung metastases: influence of tumor origin. *Clin Cancer Res* 2013; 19: 4079-91.
30. Giraldo NA, Becht E, Pages F. Orchestration and prognostic significance of immune checkpoints in the microenvironment of primary and metastatic renal cell cancer. *Clin Cancer Res* 2015; 21:3031-40.
31. Topalian SL, Taube JM, Anders RA, et al. Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. *Nat Rev Cancer* 2016; 16:275-87.

Chapter 4

Non-surgical ablative treatment of distant extracranial metastases for renal cell carcinoma:

a systematic review

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Kidney Cancer. 2018;2:57-67

Kidney Cancer 2 (2018) 57–67
DOI 10.3233/KCA-170023
IOS Press

Research Report

Non-Surgical Ablative Treatment of Distant Extracranial Metastases for Renal Cell Carcinoma: A Systematic Review

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Abstract.
Background: Local ablative treatments of extracranial metastases are increasingly used in renal cell carcinoma (RCC), but their impact on outcome and toxicity remains unclear.
Objectives: To perform a systematic review on the efficacy and toxicity of stereotactic body radiotherapy (SBRT) and radiofrequency ablation (RFA) for the treatment of distant extracranial RCC-metastases.
Methods: Search strategy: Pubmed, Embase and the national trial register were searched for the combination of metastatic RCC and SBRT or RFA. Eligible studies were original comparative studies with at least 10 patients per treatment arm, published since 2000 and reporting on at least one of: local control, overall or cancer specific survival, symptom control, toxicity. When possible, hazard ratios were calculated from survival curves.
Results and limitations: No eligible studies on RFA were found. Eight studies on SBRT were included, all were non-randomized and seven were observational. Six included only bone metastases. Radiation regime, treatment site and measured outcomes were heterogeneous and intrinsic risks of bias were high. SBRT, especially single dose 24Gy in the spine, seemed more effective than lower dose SBRT or conventional radiotherapy in achieving local control and pain control. Local control rates were 80–90%. The risk of vertebral compression fracture after SBRT for spinal metastases might be as high as 10–25% or more, especially at higher doses. Other toxicities were mild.
Conclusion: Due to the poor quality of the existing evidence, no definite recommendations on the use of SBRT in metastatic RCC can be made. SBRT, especially a single high dose, seems well tolerated and efficacious in achieving local control and pain relief, but the risk of vertebral compression fractures in spinal metastases is high. Randomized trials are needed to assess impact on local control, symptom control, survival and toxicity, to determine optimal dosing and assess potential synergistic efficacy or toxicity upon combination with systemic treatment.

Keywords: Renal cell carcinoma, metastatic, stereotactic body radiotherapy, systematic review

MeSH headings: Carcinoma, renal cell, neoplasm metastasis, radiosurgery

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INTRODUCTION

In the treatment of renal cell carcinoma (RCC), as in other cancers, there is a trend towards a more aggressive and often ablative local treatment of metastases [1]. The rationale behind this strategy is twofold. On the one hand there is the increasingly popular hypothesis of an oligometastatic state, where ablative treatment of all metastases could result in definitive cure [2, 3]. On the other hand, the increasing life expectancy due to better systemic treatments results in the need of durable local symptom control, or potentially the treatment of oligoprogressive metastases in otherwise controlled systemic disease. Although local treatment of metastases with the aim of symptom palliation is well established, the place of local ablative treatments remains unclear. In RCC, complete metastasectomy is recommended as treatment of choice in patients presenting with limited and resectable disease after a long disease free interval [4–6]. Although metastasectomy is by now well established in the treatment of RCC, this guideline is based on observational retrospective studies with high selection bias. Other local ablative treatments, for which less evidence is available, are now being increasingly used: stereotactic body radiotherapy (SBRT), radiofrequency ablation (RFA), thermal ablation, cryoablation, laser resection... of these, SBRT and to a lesser extent RFA are the most reported.

RCCs have historically been considered to be radioresistant tumors, because of the poor response of RCC-cellines to radiation *in vitro* and the disappointing results of conventional radiotherapy (CRT) in the (neo)adjuvant setting [7–12]. However, the mechanism of cell death at high-dose per fraction as in SBRT, is different from that of cell death caused by CRT and seems independent of intrinsic radiosensitivity [13, 14]. Conventionally fractionated radiotherapy at 1.8-3Gy/fraction (fx) causes tumor cell death through oxygen-dependent DNA-damage. Meanwhile the transient waves of hypoxia result in upregulation of angiogenic factors, thus protecting tumor endothelium. Single doses of 15-20Gy/fx however, cause a rapid destruction of the microvasculature, which could cause sublethal radiation-induced damage in cancer cells to become lethal. Another rare but welcome side effect of SBRT is the abscopal effect [15]. This effect is best described as radiation causing tumor regression at non-irradiated sites, which occurs likely through enhancement of tumor-specific immunity. The incidence is unknown, but it seems more frequent in immunogenic tumors such as RCC and melanoma [16, 17].

In primary RCC, RFA can be used as an alternative to (partial) nephrectomy in patients presenting with small cortical tumors that are poor candidates for surgery, although non-inferiority to surgery has not yet been conclusively demonstrated [5, 18]. The technique is currently being used for treatment of metastases as well, both in- and outside the kidney [19].

In short, of all local ablative metastasis treatments in RCC, metastasectomy is the only with a well-established role. SBRT and to a lesser extent RFA are gaining popularity. Dabestani et al. have recently published an excellent review on RCC-metastasectomy [6, 20]. Intracranial metastases in RCC are usually treated according to tumor-agnostic guidelines [21]. Stereotactic radiotherapy for intra- and extracranial metastases in RCC has been systematically reviewed by Kothari et al in 2015 [22]. However only one comparative study was included for extracranial metastases [23].

We wished to systematically review the efficacy and toxicity of the treatment of distant extracranial metastases in RCC, with SBRT or RFA, considering only comparative studies.

METHODS

The review was done according to the preferred reporting items of systematic reviews and meta-analyses (PRISMA) guidelines [24].

We searched Pubmed and Embase for articles published from 2000 till August 2017, without language restrictions. As search terms, we used the combination of “renal cell carcinoma”, “metastatic” and “stereotactic radiotherapy or radiofrequency ablation”. For all three terms, both MeSH/emtree terms and free text words were used. Alternative search terms for RCC included the different histological variants as described in the 2016 ESMO guideline [5]. The exact search strategy is provided in the supplementary materials. Reference lists of selected studies were screened for extra publications. www.clinicaltrials.gov was searched for the combination of “renal cell carcinoma, metastatic” and “radiotherapy”.

Eligibility criteria for studies to be included were: RCC with distant extracranial metastases, treated with SBRT or RFA and compared with other local therapy, systemic therapy or no therapy. At least

one of the following outcomes had to be reported: local control rate (LCR), overall or cancer specific survival, symptom control or toxicity. The scheme of SBRT and control therapy had to be reported. Only original studies with at least ten patients per treatment arm were accepted. Studies with mixed tumor types, intracranial metastases or local relapse could be included if separate results were reported for extracranial RCC-metastases. If studies reported on overlapping patient cohorts, the most recent one was selected.

The first author was responsible for the search, study selection and data extraction. In cases of doubt, a second author (BB) reviewed abstract and full text and decisions were made by consensus. Data were extracted using a pre-designed form, provided in the supplementary materials. We extracted data on study design, time period, center, funding, participants, intervention and comparator, outcome (LCR, overall or cancer specific survival, symptom control, toxicity) and systematic differences between treatment groups that could influence outcome. If hazard ratios were not reported, they were calculated wherever possible using the spreadsheet developed by Matthew Sydes [25]. This was done using presented Kaplan Meier curves, presuming a constant hazard ratio and censoring rate.

The risk of bias in randomized controlled trials was assessed with the Cochrane Collaboration risk of bias instrument and that of non-randomized comparative studies with the Cochrane Non-Randomized studies group [26]. We predefined and screened for other possible sources of bias between treatment groups that are relevant for this setting: histology, performance status, Fuhrman grade, tumor volume, treatment site, previous, concomitant or later therapy and variable fractionation regimens within the same treatment arm.

RESULTS

The selection process of eligible studies is shown in Fig. 1. We identified no studies on RFA that met the eligibility criteria. Eight studies on SBRT were included in the final review [23, 27–33]. Results are summarized in Table 1. One study was an abstract only, one was a poster [27, 29]. Six considered only bone metastases, mostly spine, two included mixed locations [27, 32]. One study was prospective [28]. All studies included RCC only. Reporting on systematic differences between treatment groups was limited: four studies reported performance status, four age, four gender, four histology, five previous therapy, two concomitant therapy, two subsequent therapy, five target volume and three biological equivalent dose. No study reported metastatic load outside the treated region, Fuhrman grade or TNM-stage.

Metastatic location, SBRT regimen, comparator intervention and measured outcomes were heterogeneous between studies. In several cases, different SBRT or CRT regimens were used within the same treatment arm. It was therefore not possible to perform a meta-analysis or even to construct a forest plot summarizing the results. Only three studies

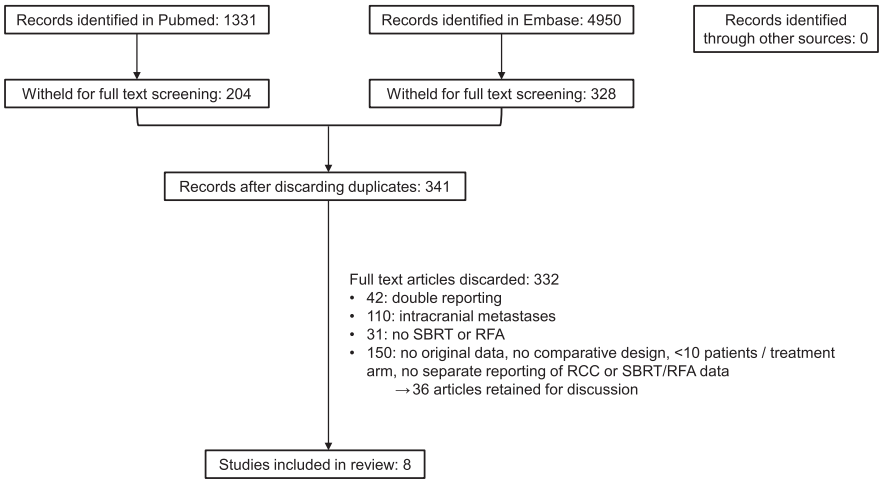


Fig. 1. Flow chart of study selection after literature search.

Table 1
Summary of included comparative studies after literature search

Author (year) Study period Type	Site	Intervention Comparator	patients /lesions	Actuarial LCR 1-year -2-year	p-value Hazard ratio (95% CI)	Overall survival (median)	p-value Hazard ratio (95% CI)	Pain relief	Toxicity
Altoos (2015) 2004-2014 [31] *Retrospective single center*	Thoracic, abdominal, soft tissue	SBRT, median 5x10Gy CRT, median 10x4Gy	?/36 ?/17	100% - 93% 62% - 32%	<0,001 0.05* (0.01-0.19)	33mo* 25mo*	0.831 1.08* (0.61-1.63)	nr	1 grade 3 of all patients
Amini (2015) 2004-2014 [30] *Retrospective single center*	Bone (36% spine)	SBRT, median 3x9Gy CRT, median 5x4Gy	?/50 ?/45	74% - 45% 61% - 23%	0,042 0.33* (0.16-0.69)	nr	-	1 yr 75% - 2yr 75% 1yr 40% - 2yr 36%	4VCF; 1 grade 3 (skin) 2 VCF
Ghia (2016) 2000-2011 [27] *Comparison of prospective phase I and II trial*	Spine	SBRT, 1x24Gy SBRT, multifraction, mostly 3x9Gy	?/21 ?/26	95% - 86% 71% - 55%	0,009 0.29* (0.10-0.86)	nr	0,409 Not calculable	nr	VCF 6/13; 1 grade 3 VCF 1/11
Hunter (2012) 2000-2010 [28] *Retrospective single center** Abstract only*	Spine (painfull)	SBRT, median 1x15Gy CRT, median 5x4Gy	76/? 34/?	nr	-	nr	-	62% (33% complete), med 4,8mo 68% (12% complete), med 1,7mo	nr
Miller (2016) 2006-2015 [32] *Retrospective single center ***	Spine	SBRT 1x16Gy (A) with first line TKI (B) alone (C) with further line TKI (D) alone after failure of TKI. (E) TKI only	 18/25 30/37 32/45 28/44 ?/43	 96% - 90%* 80% - 68%* 73% - 49%* 81% - 58%* 43% - 18%*	 0,04 (A vs B) <0,01 (E vs B) Not calculable	 18mo 21mo 16mo 8mo 26mo	 0,02 (E vs A-D) Not calculable	nr	21% VCF in all SBRT No grade 3-4 in any
Sohn (2014) 2005-2012 [29] *Retrospective multicenter matched pair*	Spine	SBRT, median 4x5Gy CRT	13/? 13/?	86% - 86%* 29% - 29%*	Nr Not calculable	15mo 7mo	0,08 Not calculable	4,4 points decrease VAS 2,8 decrease in VAS	2 VCF; 39% grade 1&2 0 VCF; 54% grade 1&2
Staehler (2015) 2007-2013 [26] *Retrospective single center poster only*	nr	Systemic therapy plus radiation: SBRT, mean 8x5Gy CRT, mean 12x3Gy	 83/? 14/?	nr	-	9,4mo 4,5mo	0,01 Not calculable	nr	23% grade 3-4 No grade 3-4
Zelefsky (2012) 2004-2010 [22] *Retrospective multicenter*	Bone (56% spine)	SBRT, 1x24Gy SBRT, 1x 18 to 23gy SBRT, multifraction, med 3x10Gy	45/? 14/? 46/?	88%* - 88%* 65%* - 21%* 63%* - 34%*	0,001 Not calculable	nr	-	nr	2 VCF; 1 grade 4 (skin) 0 VCF 2 VCF

*=as estimated from Kaplan-Meier curve. LCR = local control rated. CI = confidence interval. Med = median. Mo = months. NR = not reported. VAS = visual analog scale (pain score). VCF = vertebral compression fracture.

** same center, cohorts could be partly overlapping

presented sufficient data to calculate hazard ratios. As knowing the hazard ratios would still not allow us to perform a meta-analysis, the study authors were not contacted. Instead, we provide a narrative synthesis of the results.

Local control

Spinal metastases

Two studies reported superior local control of spine metastases with single dose 24Gy versus SBRT with multiple fractions or lower dose single fraction. Zelefsky et al. reported an 88% 1-year LCR after single dose 24Gy, versus 65% and 63% with single dose 18-23Gy or 3x10Gy/fx [23]. Interestingly, the Kaplan Meier curves for time to relapse were overlapping for the low single dose and hypofractionated cohorts, whereas the relapse curve for the high single dose was clearly superior. Ghia et al. found a 1-year LCR of 95% after single dose 24Gy, versus 71% after a hypofractionated SBRT regimen (mostly 3x9Gy) [28]. In this study, single dose SBRT was the only significant predictor for superior local control on multivariate analysis.

Two studies reported superior local control of painful spinal metastases for treatment with hypofractionated SBRT versus conventionally fractionated radiotherapy regimens. SBRT doses were lower in these studies. The first found a 1-year LCR of 86% versus 29% in patients treated with median 4x5Gy SBRT versus a conventionally fractionated regime [30]. The second reported 74% versus 61% 1-year LCR after median 3x9Gy SBRT versus median 5x4Gy CRT [31].

One study, by Miller et al., compared single fraction 16Gy with or without a tyrosine kinase inhibitor (TKI). In first line, the combination was better than SBRT alone (1-year LCR 96% versus 80%) [33]. In any line, SBRT with or without TKI was better than TKI alone.

Non-osseous metastases

One study included thoracic, abdominal and soft tissue metastases. It reported 100% versus 62% 1-year LCR for median 5x10Gy/fx SBRT ($n = 36$) versus median 10x4Gy/fx CRT ($n = 17$) [32]. As it reported, almost half of the CRT patients received only a low irradiation dose with palliative intent.

Overall survival

Five studies reported overall survival data. However, none of these had curative treatment of all metastases as main goal and none reported on metastatic load outside the treated location. Three studies did not find a significant difference in overall survival [28, 30, 32]. Two reported higher overall survival for SBRT compared with no or conventional radiotherapy respectively [27, 33]. In both, patients also received variable systemic therapies.

Symptom control

Three studies reported on the efficacy of SBRT versus CBRT on symptomatic bone metastases (mostly spine). The first showed more patients experiencing pain control with median 3x9Gy/fx SBRT versus median 5x4Gy/fx CRT: 75% versus 36% after two years [31]. The second reported al longer duration of pain relief in patients treated with single dose 15Gy versus median 5x4Gy/fx CBRT (4.8 versus 1.7 months, $p = 0.095$) [29]. There was a trend towards more complete pain relief (33% versus 12%). The third study reported a significantly larger drop in pain scores of patients treated with SBRT at median 4x5Gy/fx versus conventionally fractionated CBRT, without a significant difference in the number of patients experiencing pain relief and a similar number of patients requiring new local intervention later on (2 and 3 of 13) [30].

Adverse events

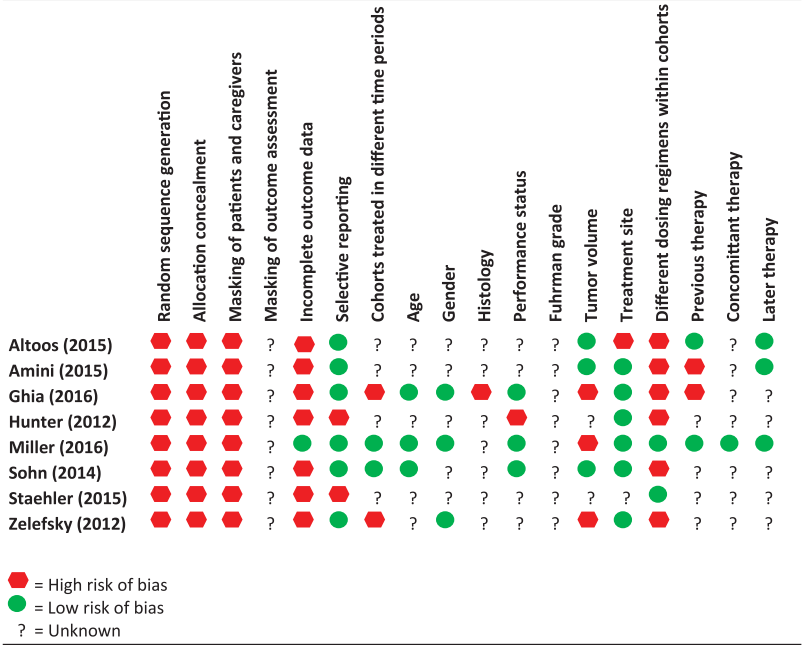
Six studies reported on adverse events

The most notable adverse event was the occurrence of vertebral compression fractures (VCF) in patients with spinal metastases. Due to different dosing regimens, the risk is difficult to estimate. One study reported however an incidence of 21% in 108 patients treated with single dose 16Gy [33], another an incidence of 46% in 13 patients treated with single dose 24Gy [28] and a third 5% in patients treated with single dose 18-24Gy [23]. In different hypofractionated SBRT regimens, the weighed incidence was 13% [23, 28, 30]. In two studies explicitly reporting on compression fractures in patients treated with CRT, the weighed incidence was 6.5%. No data on the use of bone protective agents (zolendronic acid, denosumab) were available.

Other toxicities were generally mild. In patients treated with single dose 24Gy, respectively 1/13 and 1/45 grade 3-4 toxicities were reported. Three studies comparing SBRT with CRT reported no difference in toxicities and no grade 4 toxicities [30–32].

Two studies reported on concomitant systemic therapy and SBRT. In one, irradiating spine

Table 2
Potential biases in the included studies



metastases, only grade 1-2 toxicities occurred, both in the groups treated with SBRT alone and SBRT with TKI (tyrosine kinase inhibitor) [33]. In the other study however, that did not provide location, 23% grade 3 toxicities occurred in the group treated with SBRT and systemic therapy, compared to none in the CRT with systemic therapy group [27]. It was not clear which systemic treatments were used in both groups.

Risk of bias

Potential sources of bias for each study are shown in Table 2. As all but one study were retrospective observational studies, the intrinsic risk of bias is very high. Moreover, as mentioned above, reporting of important clinical variables was often incomplete and radiation regimens often differed within the same treatment arm of a study. As SBRT is a relatively new technique, we can also assume a time bias with SBRT-patients treated more recently, when both radiation techniques and systemic therapy had improved. For an example, Ghia found better local control after single dose 24Gy than after hypofractionated SBRT, but also reported that the more recently treated single dose cohort achieved better coverage of the planned target volume [28].

DISCUSSION

We aimed to assess the impact on outcome and toxicity of SBRT for extracranial RCC-metastases. However, we identified only eight eligible comparative studies, that were all but one retrospective and observational in nature. Sample sizes were small and used treatment regimens differed substantially between and within studies. All results should therefore be interpreted with utmost caution. Moreover, all but two studies reported only on bone metastases so results may not be generalizable to other locations. The results are discussed in the context of other published literature.

Overall survival

Although locally ablative techniques are hypothesized to prolong survival, none of the studies aimed explicitly to treat all metastases or oligoprogressive disease. Therefore, even when neglecting other biases, the effect on overall survival cannot be estimated. In one non-comparative RCC-study, 12 patients had all metastases treated with SBRT. After two years their LCR was 100%, but 64% had progressed at other sites [34]. Another large study

reported significantly better local control in patients treated with curative versus palliative intent, but the numerical difference was not large (95% versus 88%) and overall survival data were not provided [35]. There are no comparative studies on SBRT versus metastasectomy, the latter being considered standard therapy though without a proven survival advantage [6]. One randomized trial exists, comparing local consolidative therapy with maintenance therapy in oligometastatic non-small cell lung carcinoma after first-line systemic therapy [36]. The investigators found a significantly longer progression free survival in patients that received local treatment, which could be SBRT, intermediate hypofractionated RT, radiochemotherapy or metastasectomy.

Local control

The most robust outcome data were on local control. All six studies reporting on local control were in favor of SBRT versus CRT or TKI and of high dose SBRT versus low dose SBRT. Most included only bone metastases. One year LCRs varied between 63% and 100%. Importantly, there seems to be a steep dose-response curve after SBRT. Zelefky reported the best LCR for treatment with single dose 24Gy and inferior, overlapping relapse curves for treatment with single dose 18 to 23Gy or 3x10Gy/fx [23]. Ghia also reported better outcome after single dose 24Gy versus 3x9Gy/fx [28]. These findings are consistent with other non-comparative studies. Greco et al noted a steep dose response curve for RCC-metastases at different locations, treated with single-dose 18-24Gy: LCR was 80% after 23-24Gy and 37% with less [37]. Recent reviews including mixed histologies, have suggested SBRT could result in 90% local control in spinal metastases treated with high dose single fraction, and in about 80% local control in liver and lung metastases treated with hypofractionated regimens [3, 38, 39]. Non-comparative retrospective series on SBRT for RCC-bone metastases report local control rates of 70–90% at one or two years, using different fractionation regimens [40–45]. Other series including non-bone metastases find similar LCRs [34, 35, 46–50]. In a recent systematic review of mainly non-comparative series, the weighted LCR for extracranial RCC-metastases was 89% [22]. Two series that included both bone and non-bone lesions, explicitly reported a superior LCR in non-bone metastases. One found a median progression free survival of 15 versus 42 months in 89 bone and 48 non-bone lesions [46]. The other found a 1-year LCR of 86% versus 93% for spine versus non-spine (total 175 lesions) [35]. Patients with spinal metastases and instability or neurological dysfunction were typically excluded, no conclusions can be drawn for this population. In one non-comparative study observational study, 55 patients with painful, instable or threatening spinal metastases were treated with single fraction 19-20Gy and TKI. One and two-year local control rate were 94% and 90% [51].

Symptom control

There was evidence of superior symptom control in bone metastases for relatively low dose SBRT compared to hyper- or hypofractionated CRT. Three studies showed respectively more patients experiencing pain relief, more patients experiencing complete pain relief and a higher drop in pain score. Other series on spinal RCC-metastases have reported 46% to 100% improvement in pain; one found 52% to be completely pain free after one year versus 23% before start of treatment [40, 42–45, 51]. A review on SBRT for spine metastases of mixed histology found an average complete response rate of 54% [39]. A randomized phase III trial, RTOG 0631, is currently evaluating the effect of single dose 16Gy SBRT versus single dose 8Gy CRT in painful spinal metastases of mixed histology.

Adverse events

SBRT was well tolerated according to all studies. This is in line with other, non-comparative studies all reporting limited toxicity [34, 35, 40–44, 46, 47, 52, 53]. However, for spinal metastases the risk of VCF was substantial (incidence 0–46%), with the largest study in 151 lesions reporting 21% VCF after single dose 16Gy [33]. Thibault et al. assessed the risk of VCF in 227 RCC-lesions treated with median 1x19Gy/fx. They found and incidence of 16%: the risk of a de novo VCF was 7%, that of progression of a preexisting VCF 51%. Importantly, there was a strong relationship with dose per fraction, that remained highly significant after multivariate analysis: the risk was 43% after 24Gy/fx, 24% after 20-23Gy/fx and 12% after $\leq$19Gy/fx [52]. In another large series that included tumors of mixed histology, the incidence was 11%, with 2/3 being de novo VCF. The risk was linked to dose per fraction $\geq$20Gy and osteolytic lesions, as are typically found in RCC [54, 55]. A systematic review of mixed histologies found a weighted incidence of 9.4% [39]. Another feared complication

of spinal radiotherapy is myelopathy, which is often the dose-limiting factor for the less accurate CRT. In the included series, only one case of grade 3 radiculopathy was mentioned [28]. For mixed histologies and different dosing regimens, the risk may be as low as 0.2% [39].

Combination therapy

A recent area of active research is the combination of SBRT with systemic therapies. One hypothesis is that in oligoprogressive disease, local treatment of resistant lesions could allow continuation of a systemic treatment that is still effective for other metastatic sites. Another rationale is that of expected synergy between SBRT and systemic therapies. As SBRT has shown to stimulate tumor-specific immunity, testified by the rare abscopal effect, it might enhance the effect of other immunomodulatory drugs [56, 57]. A phase I and II trial combining high dose interleukin 2 (IL2) with SBRT showed increased response rates to IL2 compared with historical data [58, 59]. Numerous other trials are currently ongoing [60–65]. The safety of combining SBRT with systemic therapies remains unclear [66]. In the IL2-studies "only expected toxicities" were reported. Miller reported similar, limited toxicity in 101 RCC-patients treated with single fraction SBRT with or without TKI [33]. In a phase I dose escalation trial, no dose limiting toxicities occurred in 13 RCC-patients treated with hypofractionated SBRT and pazopanib [67]. Several case reports have however been published concerning toxicities of combination strategies [68–70]. The risk is probably site dependent, as illustrated by a case of cardiac failure on sunitinib after previous irradiation of the chest wall and a case of bowel perforation on sorafenib after irradiation of L3-L5 [70, 71].

CONCLUSION

We aimed to systematically review the efficacy and toxicity of SBRT and RFA for treatment of extracranial RCC-metastases. No studies on RFA that met the inclusion criterion were found. As for SBRT, due to the sparsity, heterogeneity and low quality of available data, it is not possible to make definite recommendations on its use. SBRT seems superior over CRT for local control and may result in better pain control. As it is generally well tolerated and more patient-friendly than conventionally fractionated schemes, SBRT is a good alternative to CRT in these settings. Optimal dosing needs to be determined, but in spine a single fraction of 24Gy seems superior over other SBRT schemes. As every fraction of SBRT requires the same accuracy and surpasses the safety threshold for critical normal organs according to the AAPM guidelines, there seems to be no clear advantage of multiple fraction schemes. However, in spinal metastases the risk of VCF is significant, especially at the more effective dose of 1x24Gy. Its incidence, clinical impact and optimal dosing to reduce the risk need further investigation.

There are no comparative data on SBRT versus surgery in the settings of curative intent or spinal metastases that are unstable or cause neurological deficit. In the latter case, surgery has priority if feasible.

CONFLICT OF INTEREST STATEMENT

Benoit Beuselinck received honoraria from Amgen, Pfizer, Janssen and Bayer and is an investigator of the EudraCT: 2011-006085-40/MetaSun trial supported by Pfizer. The other authors have no conflict of interest to report.

ACKNOWLEDGMENTS

Annelies Verbiest has received a grant from Kom op tegen Kanker (Stand up to Cancer), the Flemish cancer society.

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: http://dx.doi.org/10.3233/KCA-170023.

REFERENCES

[1] Meyer C, Trinh Q-D, Vetterlein M, Löppenberg B, Adollah F, Seisen T, et al. Trends of Metastasectomy for Metastatic Renal Cell Carcinoma and Their Impact on Overall Survival. The Journal of Urology 2016;195(4).

[2] Palma DA, Salama JK, Lo SS, Senan S, Treasure T, Govindan R, et al. The oligometastatic state - separating truth from wishful thinking. Nat Rev Clin Oncol 2014;11(9):549-57.

[3] Tree AC, Khoo VS, Eeles RA, Ahmed M, Dearnaley DP, Hawkins MA, et al. Stereotactic body radiotherapy for oligometastases. The Lancet Oncology 2013;14(1):e28-e37.

[4] Ljungberg B, Bensalah K, Canfield S, Dabestani S, Hofmann F, Hora M, et al. EAU guidelines on renal cell carcinoma: 2014 update. Eur Urol 2015;67(5):913-24.

[5] Escudier B, Porta C, Schmidinger M, Rioux-Leclercq N, Bex A, Khoo V, et al. Renal cell carcinoma: ESMO Clinical

Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2016;27(suppl 5):v58-v68.
[6] Dabestani S, Marconi L, Bex A. Metastasis therapies for renal cancer. Curr Opin Urol 2016;26(6):566-72.
[7] Deschavanne PJ, Fertil B. A review of human cell radiosensitivity in vitro. Int J Radiat Oncol Biol Phys 1996;34(1):251-66.
[8] Van Der Werf-Messing B. Proceedings: Carcinoma of the kidney. Cancer 1973;32(5):1056-61.
[9] Juusela H, Malmio K, Alfthan O, Oravisto KJ. Preoperative Irradiation in the Treatment of Renal Adenocarcinoma. Scandinavian Journal of Urology and Nephrology 2010;11(3):277-81.
[10] Tunio MA, Hashmi A, Rafi M. Need for a new trial to evaluate postoperative radiotherapy in renal cell carcinoma: A meta-analysis of randomized controlled trials. Ann Oncol 2010;21(9):1839-45.
[11] Kjaer M, Frederiksen PL, Engelholm SA. Postoperative radiotherapy in stage II and III renal adenocarcinoma. A randomized trial by the Copenhagen Renal Cancer Study Group. International Journal of Radiation Oncology, Biology, Physics 1987;13(5):665-72.
[12] Finney R. An evaluation of postoperative radiotherapy in hypernephroma treatment–a clinical trial. Cancer 1973;32(6):1332-40.
[13] De Meerleer G, Khoo V, Escudier B, Joniau S, Bossi A, Ost P, et al. Radiotherapy for renal-cell carcinoma. The Lancet Oncology 2014;15(4):e170-e7.
[14] Siva S, Kothari G, Muacevic A, Louie AV, Slotman BJ, Teh BS, et al. Radiotherapy for renal cell carcinoma: Renaissance of an overlooked approach. Nat Rev Urol 2017;14(9):549-63.
[15] Reynders K, Illidge I, Siva S, Chang J, De Ruysscher D. The abscopal effect of local radiotherapy in the clinic: A systematic review on its occurrence. Radiotherapy and Oncology 2015;115:S583-S4.
[16] Wersall PJ, Blomgren H, Pisa P, Lax I, Kalkner KM, Svedman C. Regression of non-irradiated metastases after extracranial stereotactic radiotherapy in metastatic renal cell carcinoma. Acta Oncologica 2006;45(4): 493-7.
[17] Ribeiro Gomes J, Schmerling RA, Haddad CK, Racy DJ, Ferrigno R, Gil E, et al. Analysis of the Abscopal Effect With Anti-PD1 Therapy in Patients With Metastatic Solid Tumors. Journal of immunotherapy (Hagerstown, Md: 1997) 2016;39(9):367-72.
[18] Ljungberg B, Albiges L, Bensalah K, Bex A, Giles RH, Hora M, et al. Members of the RCC guidelines panel. EAU guidelines on renal cell carcinoma. Retrieved from: http://uroweborg/guideline/renal-cell-carcinoma/2017-11-01.
[19] Hiraki T, Kanazawa S. Lung radiofrequency ablation: Potential as a therapy to oligometastasis and oligorecurrence. Pulmonary Medicine 2012;2012:196173.
[20] Dabestani S, Marconi L, Hofmann F, Stewart F, Lam TBL, Canfield SE, et al. Local treatments for metastases of renal cell carcinoma: A systematic review. The Lancet Oncology 2014;15(12):e549-e61.
[21] Soffietti R, Abacioglu U, Baumert B, Combs SE, Kinhult S, Kros JM, et al. Diagnosis and treatment of brain metastases from solid tumors: Guidelines from the European Association of Neuro-Oncology (EANO). Neuro Oncol 2017;19(2):162-74.
[22] Kothari G, Foroudi F, Gill S, Corcoran NM, Siva S. Outcomes of stereotactic radiotherapy for cranial and extracranial metastatic renal cell carcinoma: A systematic review. Acta Oncol 2015;54(2):148-57.
[23] Zelefsky MJ, Greco C, Motzer R, Magsanoc JM, Pei X, Lovelock M, et al. Tumor control outcomes after hypofractionated and single-dose stereotactic image-guided intensity-modulated radiotherapy for extracranial metastases from renal cell carcinoma. Int J Radiat Oncol Biol Phys 2012;82(5):1744-8.
[24] Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gotzsche PC, Ioannidis JP, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: Explanation and elaboration. PLoS Med 2009;6(7):e1000100.
[25] Tierney JF, Stewart LA, Ghersi D, Burdett S, Sydes MR. Practical methods for incorporating summary time-to-event data into meta-analysis. Trials 2007;8:16.
[26] Sterne JA, Hernan MA, Reeves BC, Savovic J, Berkman ND, Viswanathan M, et al. ROBINS-I: A tool for assessing risk of bias in non-randomised studies of interventions. BMJ 2016;355:i4919.
[27] Staehler MD, Lisa B, Nuhn P, Schlenker B, Wilkowski R. Comparison of simultaneous high dose stereotactic radiotherapy with normofractionated radiotherapy in patients with metastatic renal cell carcinoma under systemic therapy. Journal of Clinical Oncology 2015;33(7).
[28] Ghia AJ, Chang EL, Bishop AJ, Pan HY, Boehling NS, Amini B, et al. Single-fraction versus multifraction spinal stereotactic radiosurgery for spinal metastases from renal cell carcinoma: Secondary analysis of Phase I/II trials. J Neurosurg Spine 2016;24(5):829-36.
[29] Hunter GK, Balagamwala EH, Koyfman SA, Bledsoe T, Sheplan LJ, Reddy CA, et al. The efficacy of external beam radiotherapy and stereotactic body radiotherapy for painful spinal metastases from renal cell carcinoma. Pract Radiat Oncol 2012;2(4):e95-e100.
[30] Sohn S, Chung CK, Sohn MJ, Chang UK, Kim SH, Kim J, et al. Stereotactic radiosurgery compared with external radiation therapy as a primary treatment in spine metastasis from renal cell carcinoma: A multicenter, matched-pair study. J Neurooncol 2014;119(1):121-8.
[31] Amini A, Altoos B, Bourlon MT, Bedrick E, Bhatia S, Kessler ER, et al. Local control rates of metastatic renal cell carcinoma (RCC) to the bone using stereotactic body radiation therapy: Is RCC truly radioresistant? Pract Radiat Oncol 2015;5(6):e589-e96.
[32] Altoos B, Amini A, Yacoub M, Bourlon MT, Kessler EE, Flaig TW, et al. Local Control Rates of Metastatic Renal Cell Carcinoma (RCC) to Thoracic, Abdominal, and Soft Tissue Lesions Using Stereotactic Body Radiotherapy (SBRT). Radiat Oncol 2015;10:218.
[33] Miller JA, Balagamwala EH, Angelov L, Suh JH, Rini B, Garcia JA, et al. Spine stereotactic radiosurgery with concurrent tyrosine kinase inhibitors for metastatic renal cell carcinoma. J Neurosurg Spine 2016;25(6):766-74.
[34] Ranck MC, Golden DW, Corbin KS, Hasselle MD, Liauw SL, Stadler WM, et al. Stereotactic body radiotherapy for the treatment of oligometastatic renal cell carcinoma. American Journal of Clinical Oncology 2013;36(6):589-95.
[35] Wang CJ, Christie A, Lin MH, Jung M, Weix D, Huelsmann L, et al. Safety and Efficacy of Stereotactic Ablative Radiation Therapy for Renal Cell Carcinoma Extracranial Metastases. Int J Radiat Oncol Biol Phys 2017;98(1):91-100.
[36] Gomez D, Blumenschein G, Lee J, Hernandez M, Ross Camidge D, Doebele R, et al. Local consolidative ther-

apy versus maintenance therapy or observation for patients with oligometastatic non-small-cell lung cancer without progression after first-line systemic therapy: A multicentre, randomised, controlled, phase 2 study. Lancet Oncol 2016;17(12):1672-82.

[37] Greco C, Zelefsky MJ, Lovelock M, Fuks Z, Hunt M, Rosenzweig K, et al. Predictors of local control after single-dose stereotactic image-guided intensity-modulated radiotherapy for extracranial metastases. Int J Radiat Oncol Biol Phys 2011;79(4):1151-7.

[38] Greco C, Pares O, Pimentel N, Moser E, Louro V, Morales X, et al. Spinal metastases: From conventional fractionated radiotherapy to single-dose SBRT. Rep Pract Oncol Radiother 2015;20(6):454-63.

[39] Husain ZA, Sahgal A, De Salles A, Funaro M, Glover J, Hayashi M, et al. Stereotactic body radiotherapy for de novo spinal metastases: Systematic review. J Neurosurg Spine 2017;27(3):295-302.

[40] Gerszten PC, Burton SA, Ozhasoglu C, Vogel W. Stereotactic radiosurgery for spinal metastases from renal cell carcinoma. J Neurosurg Spine 2005;3:288-95.

[41] Balagamwala EH, Angelov L, Koyfman SA, Suh JH, Reddy CA, Djemil T, et al. Single-fraction stereotactic body radiotherapy for spinal metastases from renal cell carcinoma. J Neurosurg Spine 2012;17(6):556-64.

[42] Jhaveri PM, Teh BS, Paulino AC, Blanco AI, Lo SS, Butler EB, et al. A dose-response relationship for time to bone pain resolution after stereotactic body radiotherapy (SBRT) for renal cell carcinoma (RCC) bony metastases. Acta Oncol 2012;51(5):584-8.

[43] Nguyen QN, Shiu AS, Rhines LD, Wang H, Allen PK, Wang XS, et al. Management of spinal metastases from renal cell carcinoma using stereotactic body radiotherapy. Int J Radiat Oncol Biol Phys 2010;76(4):1185-92.

[44] Sellin JN, Reichardt W, Bishop AJ, Suki D, Rhines LD, Settle SH, et al. Factors affecting survival in 37 consecutive patients undergoing de novo stereotactic radiosurgery for contiguous sites of vertebral body metastasis from renal cell carcinoma. J Neurosurg Spine 2015;22(1):52-9.

[45] Serrand J, Sunyach M, Negrier S, Carrie C. Stereotactic body radiation therapy (SBRT) on spine metastases from renal cell carcinoma (RCC): Clinical results and impact of upfront vs salvage SBRT. JCO (supp) 2016:e16073.

[46] Meyer E, Stefan D, Pasquier D, Bernadou G, Calais G, Carrie C, et al. Stereotactic radiotherapy (SRT) for oligometastatic (OM) relapse and metastatic oligoprogression (OP) in renal cell carcinoma (RCC) patients (pts): A study of the GETUG group. Journal of Clinical Oncology 2016;34.

[47] Teh B, Bloch C, Galli-Guevara M, Doh L, Richardson S, Chiang S, et al. The treatment of primary and metastatic renal cell carcinoma (RCC) with image-guided stereotactic body radiation therapy (SBRT). Biomed Imaging Interv J 2007;3(1):e6.

[48] Grossman CE, Okunieff P, Brasacchio RA, Katz AW, Singh DP, Usuki KY, et al. Stereotactic body radiation therapy for oligometastatic renal cell carcinoma or melanoma: Prognostic factors and outcomes. International Journal of Radiation Oncology Biology Physics 2015;93(3):E202.

[49] Rieber J, Andratschke N, Blanck O, Duma M, Ganswindt U, Imhoff D, et al. SBRT for lung metastases: Detailed subgroup analysis of 700 patients diagnosed with 963 lung metastases. Radiotherapy and Oncology 2015;115:S27-S8.

[50] Stinauer MA, Kavanagh B, Schefter T, Gonzalez R, Flaig T, Lewis K, et al. Stereotactic body radiation therapy for melanoma and renal cell carcinoma: Impact of single fraction equivalent dose on local control. International Journal of Radiation Oncology Biology Physics 2010;78(3):S190-S1.

[51] Staehler M, Haseke N, Nuhn P, Tullmann C, Karl A, Siebels M, et al. Simultaneous anti-angiogenic therapy and single-fraction radiosurgery in clinically relevant metastases from renal cell carcinoma. BJU Int 2011;108(5):673-8.

[52] Thibault I, Atenafu EG, Chang E, Chao S, Al-Omair A, Boehling N, et al. Factors Influencing Vertebral Compression Fracture Specific to Renal Cell Carcinoma Spinal Metastases After Stereotactic Body Radiation Therapy: A Multi-institutional Study. International Journal of Radiation Oncology Biology Physics 2014;90(1).

[53] Svedman C, Sandstrom P, Pisa P, Blomgren H, Lax I, Kalkner KM, et al. A prospective Phase II trial of using extracranial stereotactic radiotherapy in primary and metastatic renal cell carcinoma. Acta Oncol 2006;45(7):870-5.

[54] Cunha MV, Al-Omair A, Atenafu EG, Masucci GL, Letourneau D, Korol R, et al. Vertebral compression fracture (VCF) after spine stereotactic body radiation therapy (SBRT): Analysis of predictive factors. International Journal of Radiation Oncology, Biology, Physics 2012;84(3):e343-9.

[55] Zekri J, Ahmed N, Coleman RE, Hancock BW. The skeletal metastatic complications of renal cell carcinoma. Int J Oncol 2001;19(2):379-82.

[56] De Wolf K, Vermaelen K, De Meerleer G, Lambrecht BN, Ost P. The potential of radiotherapy to enhance the efficacy of renal cell carcinoma therapy. Oncoimmunology 2015;4(10):e1042198.

[57] Singh AK, Winslow TB, Kermany MH, Goritz V, Heit L, Miller A, et al. A Pilot Study of Stereotactic Body Radiation Therapy Combined with Cytoreductive Nephrectomy for Metastatic Renal Cell Carcinoma. Clinical cancer research: An official journal of the American Association for Cancer Research 2017;23(17):5055-65.

[58] Seung SK, Curti BD, Crittenden M, Walker E, Coffey T. Phase 1 study of stereotactic body radiotherapy and Internleuking-2: tumor and immunological responses. Science Translational Medicine 2012;4(137):1-7.

[59] Hannan R, Ishibara D, Louder K, Ahn C. Phase II trial of high-dose Interleukin-2 (IL-2) and stereotactic radiation therapy (SABR) for metastatic clear cell renal cell carcinoma (ccRCC): interim analysis. JCO (supp) 2016:abstract 532.

[60] Medicine UNLo. ClinicalTrials.gov. https://clinicaltrialsgov/ct2/show/NCT02019576. 2017.

[61] Medicine UNLo. ClinicalTrials.gov. https://clinicaltrialsgov/ct2/show/NCT01896271. 2017.

[62] Medicine UNLo. ClinicalTrials.gov. https://clinicaltrialsgov/ct2/show/NCT01884961. 2017.

[63] Medicine UNLo. ClinicalTrials.gov. https://clinicaltrialsgov/ct2/show/NCT03065179. 2017.

[64] Medicine UNLo. ClinicalTrials.gov. https://clinicaltrialsgov/ct2/show/NCT02781506. 2017.

[65] Medicine UNLo. ClinicalTrials.gov. https://clinicaltrialsgov/ct2/show/NCT02864615. 2017.

[66] Kroeze SG, Fritz C, Hoyer M, Lo SS, Ricardi U, Sahgal A, et al. Toxicity of concurrent stereotactic radiotherapy and targeted therapy or immunotherapy: A systematic review. Cancer Treat Rev 2017;53:25-37.

[67] De Wolf K, Rottey S, Vermaelen K, Decaestecker K, Sundahl N, De Lobel L, et al. Combined high dose radiation

and pazopanib in metastatic renal cell carcinoma: A phase I dose escalation trial. Radiat Oncol 2017;12(1):157.
[68] Yuasa T, Kitsukawa S, Sukegawa G, Yamamoto S, Kudo K, Miyazawa K, et al. Early onset recall pneumonitis during targeted therapy with sunitinib. BMC Cancer 2013;13:3.
[69] Lordick F, Geinitz H, Theisen J, Sendler A, Sarbia M. Increased risk of ischemic bowel complications during treatment with bevacizumab after pelvic irradiation: Report of three cases. Int J Radiat Oncol Biol Phys 2006;64(5):1295-8.
[70] Peters NA, Richel DJ, Verhoeff JJ, Stalpers LJ. Bowel perforation after radiotherapy in a patient receiving sorafenib. J Clin Oncol 2008;26(14):2405-6.
[71] Van Keerberghen CA, Van Wambeke S, Wolter P, Schoffski P, Van Cleemput J, Van Limbergen E, et al. A Case of Severe Acute Cardiac Failure on Sunitinib After Left-Sided Thoracal Radiation Therapy. Clin Genitourin Cancer 2017;15(1):e155-e8.

Chapter 5

Long-term outcomes in clear-cell renal cell carcinoma patients treated with complete metastasectomy

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Manuscript in preparation

Chapter 5: Contents

Abstract 61

Introduction 62

Methods 63
- Statistical analysis 63

Results 64
- Outcomes after first metastasectomy 65
- Outcomes after repeat metastasectomy 65
- Systemic therapy after metastasectomy 66
- Comparison of outcomes of patients with and without metastasectomy 66

Discussion 68

References 70

Abstract

Background: Complete metastasectomy is routinely performed in selected patients with metastatic clear-cell renal cell carcinoma (ccRCC).

Objectives: To assess (1) outcomes after first and repeat metastasectomy, (2) outcomes on targeted therapy in patients who underwent previous metastasectomy and (3) compare outcomes with and without metastasectomy after correction for selection bias.

Methods: Metastatic ccRCC patients treated with or without metastasectomy at University Hospitals Leuven were included from prospective databases. We calculated disease-free survival (DFS), time to systemic therapy and cancer-specific survival (CSS) after metastasectomy, and progression-free survival (PFS) and CSS on 1st line sunitinib/pazopanib. We calculated propensity scores to estimate a patient's likelihood to undergo metastasectomy.

Results: We included 113 patients who underwent complete metastasectomy and 139 who did not. (1) Median DFS after complete metastasectomy was 18mo, time to systemic therapy was 73mo and CSS was 101mo. 20% did not relapse during long-term follow-up. Outcomes remained favorable after repeat metastasectomy. (2) PFS and CSS on 1st line sunitinib/pazopanib were 15mo and 35mo. (3) The propensity scores of patients who did and did not undergo metastasectomy showed no overlap, indicating that correction for selection bias is impossible and comparison of outcomes unreliable.

Conclusions: Complete metastasectomy and repeat metastasectomy can result in excellent outcomes in highly selected patients, even when its causal benefit cannot be formally assessed. Previous metastasectomy does not impair outcomes on targeted therapies.

Introduction

The contemporary treatment of metastatic clear-cell renal cell carcinoma (ccRCC) consists of 1st line systemic therapy with an immune checkpoint inhibitor (ICI), combined with another ICI or an angiogenesis inhibitor. (1) Local treatments have historically played an important role, but their current position is ill-defined. Cytoreductive nephrectomy, the long-standing standard of care, did not improve overall survival (OS) in patients treated with 1st line sunitinib that were Intermediate or Poor risk according to the International Metastatic ccRCC Database Consortium (IMDC) criteria. (2) Yet its role in the ICI era is unclear. The radical local treatment of metastases has been associated with long-term OS and deferral of systemic therapy in retrospective series of patients with favorable prognostic features, such as long disease-free interval, limited number of metastases, good performance status, lung metastases, low T-stage, low Fuhrman grade and absence of sarcomatoid features. (3,4) Radical local treatment is therefore recommended in selected patients, although its exact benefit remains unclear in the absence of randomized trials. The rapid improvement of systemic treatment options for ccRCC now poses new questions. Will metastasectomy be abandoned in favor of ICI combinations? Or will we increase its use in combination with ICI? The latter option is currently being investigated in several randomized trials (NCT03024996, NCT03055013, NCT03142334). Therefore, it is important to establish a baseline for outcomes after complete metastasectomy without systemic therapy. With this study, we contribute to establishing this baseline in a large patient series from a tertiary referral center, with long-term follow-up. Furthermore, we aimed to fill the current gaps in the literature on ccRCC metastasectomy: outcomes after repeat metastasectomy, outcomes on antiangiogenic therapy after metastasectomy and comparison of outcomes without metastasectomy, after correction for selection bias through propensity score analysis. Of note, other series that compare outcomes of patients with and without metastasectomy typically do so through multivariable Cox regression, which can take into account much less variables than a propensity score.

Methods

Patient population

After approval by the medical ethics review board, we selected patients from two prospective records at University Hospitals Leuven.

The first record was of RCC patients undergoing metastasectomy since 1995. Inclusion criteria were: clear-cell histology, resection of the primary tumor, metastasectomy with aim of complete resection, no second cancer and no prior or adjuvant systemic therapy. Ipsilateral adrenal lesions that were spatially separated from the primary tumor were considered metastases, continuous growth into the adrenal gland was not. Patients who relapsed were treated in accordance with local practice guidelines: second metastasectomy, active surveillance or systemic therapy.

As a control group, we selected patients from another prospective record of metastatic RCC patients treated with systemic therapy, kept since 2005. Inclusion criteria were: clear-cell histology, resection of the primary tumor, no second cancer, systemic treatment started for metastatic disease and not in the (neo-)adjuvant setting. Local treatment for symptomatic reasons was allowed if it concerned <30% of metastatic tumor volume.

Statistical analysis

Survival outcomes were estimated with the Kaplan-Meier method. Disease-free survival (DFS) was calculated as time from surgery to relapse or death from any cause. Progression-free survival (PFS) on 1st line sunitinib/pazopanib was calculated as time from start of therapy and to radiological progression or death from any cause.

We calculated propensity scores to estimate the probability of patients to undergo metastasectomy. Propensity scores can take into account much more variables than classic multivariate models. They are therefore more reliable to correct for selection bias when comparing outcomes of patients who did and did not undergo metastasectomy. We used a multivariable logistic regression model to calculate propensity scores, including patient characteristics that correlated significantly with treatment assignment on univariable analysis ($p < 0.05$). As potential predictors at time of nephrectomy, we tested: T-stage ≥3, Fuhrman grade ≥3, lymph node positivity, synchronous metastases, sarcomatoid component and gender. As predictors at time of diagnosis of metastasis: age, multiple metastases, multiorgan metastases, disease-free interval >1 year,

Karnofsky performance status <80%, anemia (hemoglobin <14g/dl in men, <12g/dl in women), neutrophils >4,500/µl, platelets >450,000/µl, corrected calcium >2.55mmol/l, C-reactive protein (CRP) >5mg/l, lactate dehydrogenase above 1.5 upper limit of normal and location (including sites with at least 10 affected patients: lung, bone, adrenal, liver, lymph node, pleura, pancreas, local relapse).

Graphpad Prism 8.3.0 and XLstat 2020.1.3 were used for statistical analysis.

Results

We included 113 patients who underwent complete metastasectomy and 139 patients who were treated with systemic therapy. In the metastasectomy patients, median follow-up after first metastasectomy was 78mo (interquartile range 42-177mo) and 53 CSS events occurred (47%). Resection margins were negative in 91% of cases. Patient characteristics are summarized in Table 5.1.

Table 5.1. *Characteristics of 113 patients that underwent complete metastasectomy.*

At diagnosis of primary RCC			
Male		79/113	*70%*
Age (median, IQ range)		60	*52-69*
T-stage	T1	21/94	*22%*
	T2	28/94	*30%*
	T3	42/94	*45%*
	T4	3/94	*3%*
Lymph node positive		8/55	*15%*
Synchronous metastases		26/113	*23%*
Fuhrman grade	1	0	
	2	22/89	*25%*
	3	34/89	*38%*
	4	33/89	*37%*
Sarcomatoid component		6/35	*17%*

KPS = Karnofsky performance status; Anemia = hemoglobin < 14g/dl (men) or 12.5g/dl (women); ULN neutrophils = 4.500/µl; ULN platelets = 450.000/µl; ULN albumin-corrected calcium = 2.55mmol/l; CRP = c-reactive protein; ULN CRP= 5mg/l; LDH = lactate dehydrogenase; ULN = upper limit of normal.

At first metastasectomy			
Number of metastases	1	76/113	*67%*
	2	12/113	*11%*
	≥3	25/113	*22%*
Organs affected	1	98/113	*87%*
	2	10/113	*9%*
	≥3	5/113	*4%*
Site	lymph nodes	10/113	*9%*
	lung	40/113	*35%*
	liver	3/113	*3%*
	brain	2/113	*2%*
	kidney/local	18/113	*16%*
	adrenal	22/113	*19%*
	pancreas	5/113	*4%*
	bone	13/113	*12%*
	other	13/113	*12%*
KPS < 80%		6/75	*8%*
Anemia		42/107	*39%*
Elevated neutrophils		29/89	*33%*
Elevated platelets		1/106	*1%*
Hypercalcemia		5/86	*6%*
Elevated CRP		29/94	*31%*
LDH > 1.5 ULN		2/36	*6%*
After first metasasectomy			
Negative resection margins		95/104	*91%*

Outcomes after first metastasectomy

Median DFS was 18mo (Table 5.2, Figure 5.1). A substantial number of patients achieved long-term DFS, with actuarial 2-year DFS of 41% and 10-year DFS 13%. Of note, this number is negatively influenced by patients who deceased without relapse: 23 patients (=20%) did not relapse during follow-up, of whom five died from other causes. The median follow-up of those who did not relapse was 131mo, whereas the latest recorded relapse occurred at 107mo.

The median time to systemic therapy was 73mo, and the median CSS 124mo.

Outcomes after repeat metastasectomy

One third of patients (n=39) was eligible for second metastasectomy and another 41% of these for third metastasectomy (n=16). DFS, time to systemic therapy and CSS were numerically shorter compared to first metastasectomy, but still favorable and survival curves were largely overlapping (Table 5.2, Figure 5.1). A substantial number of these patients did not relapse during follow-up: 5 of 39 after second metastasectomy (follow-up 28 to 70mo) and 4 of 16 after third metastasectomy (follow-up 27 to 220mo).

Table 5.2. *Outcomes after metastasectomy.*

	Nephrectomy	Complete metastasectomy		
		1st	2nd	3rd
Number	113	113	39	16
Disease-free survival				
median	33mo	18mo	15mo	18mo
2 years	66%	41%	30%	40%
5 years	32%	20%	13%	25%
10 years	5%	13%	undefined	25%
Time to systemic therapy				
median	135mo	73mo	56mo	43mo
2 years	90%	78%	77%	74%
5 years	79%	51%	39%	26%
10 years	58%	39%	20%	26%
Cancer-specific survival				
median	182mo	124mo	127mo	90mo
2 years	97%	89%	93%	90%
5 years	81%	67%	68%	69%
10 years	66%	52%	49%	42%

Systemic therapy after metastasectomy

The median time to systemic therapy was much longer than the DFS after metastasectomy (73 *vs* 18mo), indicating that most relapsing patients were eligible for a period of active surveillance or repeat metastasectomy. The 59 patients who started systemic therapy during follow-up, did so after a median of 32mo after relapse. This is again an indication of the overall indolent disease course in patients who are eligible for metastasectomy. 41 patients were treated with 1st line sunitinib or pazopanib. Their PFS and CSS were 18 and 35mo, with a response rate of 50%. These outcomes are excellent compared to historical trial results, indicating that patients do not miss a window of opportunity by deferring antiangiogenic therapy in favor of metastasectomy.

Comparison of outcomes of patients with and without metastasectomy

We aimed to compare outcomes in patients who did and did not undergo metastasectomy after correction for propensity score. This score indicates a patient's probability to undergo metastasectomy and can therefore address selection bias. On univariable logistic regression, following predictors were significantly associated with higher probability of metastasectomy: T-stage ≤2, Fuhrman grade ≤2, metachronous metastases, disease-free interval >12mo, single metastasis, single organ, adrenal or liver metastasis. The following predictors were associated with lower probability: bone, lymph node or pleural metastasis, anemia, elevated neutrophils, elevated platelets, elevated CRP and hypercalcemia. After selective bivariable analyses, following predictors were selected for inclusion in the propensity model: T-stage, Fuhrman grade, synchronous metastases, multiple metastases, multiple organs, elevated CRP, hypercalcemia and four sites (bone, lymph node, adrenal gland, pleura). However, the area under the curve of the resulting propensity model was 0.98. Accordingly, the histograms of the propensity scores of the two populations were barely overlapping (Figure 5.2). This implies that baseline prognostic factors determine treatment choice and that the characteristics of the patients who undergo metastasectomy *vs* those who do not were so different, that a meaningful comparison of outcomes is not plausible. It was therefore not possible to analyze the causal effect of metastasectomy on outcome.

A more positive score indicates a higher probability to receive metastasectomy, a more negative score a lower probability. In case of missing data for one or more predictors in a patient, the known predictors were used in the multivariable

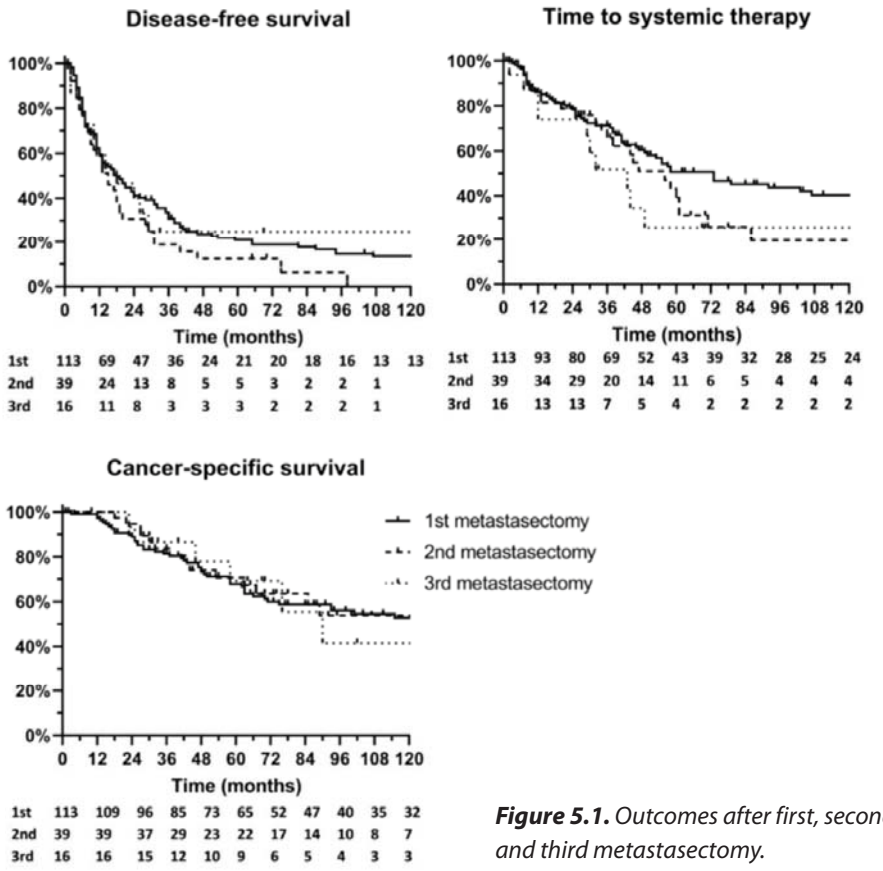

Figure 5.1. *Outcomes after first, second and third metastasectomy.*

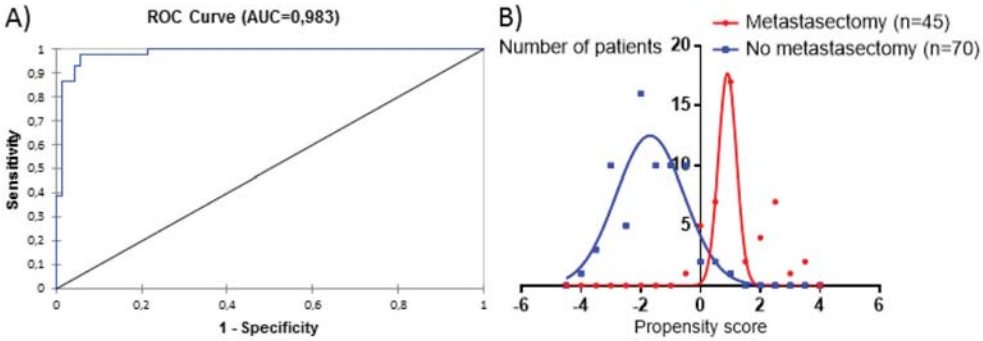

Figure 5.2*. Propensity model that estimates a patient's probability to undergo metastasectomy.* **(A)** *over 98% of the probability is determined by baseline characteristics.* **(B)** *There is almost no overlap between the propensity scores of patients who undergo metastasectomy and those who do not. Therefore these populations cannot be compared.*

logistic regression model calculating the AUC, but the patients' propensity score was not calculated.

Discussion

In this large and homogeneous case series with long-term follow-up, we show that ccRCC patients can achieve excellent long-term outcomes after complete metastasectomy. The 10-year DFS was 13% and is probably even underestimated, as several patients were censored because of death from other cause. As the latest relapse on our series occurred at 8 years, this high probability of 10-year DFS indicates potential curative treatment for a subset of patients. The current recommendation to consider radical local treatment in selected patients remains therefore unchallenged, even in the absence of randomized trials. This makes sense, as it has been shown that metastatic lesions can metastasize themselves. (5) Indeed, a randomized trial in metastatic non-small cell lung carcinoma showed that radical local treatment could delay the onset of new metastatic lesions. (6) A novel finding we report are the favorable outcomes in patients who are eligible for repeat metastasectomy: this supports the practice of offering surgery to patients that maintain favorable features.

This series offers some interesting insights on the decision between metastasectomy or systemic therapy. First, it is often assumed that metastasectomy can defer systemic therapy and the associated toxicity, and is therefore a good option if expected morbidity is low. In our series, we report indeed a very long time to systemic therapy after metastasectomy (median 73mo). But even after relapse, the median time before start was 32mo: this is an effect of indolent disease course, and not metastasectomy itself. Nonetheless, metastasectomy probably contributes to the delay, as was also shown in a randomized trial setting in prostate cancer. (7) Importantly, outcomes on 1st line sunitinib/pazopanib were superior to those in historical trials (PFS 15mo, CSS 35mo, response rate 50%). This reflects the selection of indolent tumors for metastasectomy, but importantly also indicates that patients do not miss a window of opportunity for systemic therapy by opting for metastasectomy.

The calculation of propensity scores that estimate a patient's likelihood to undergo metastasectomy, showed very explicitly that the populations that are selected for metastasectomy and those that are not, are entirely different. Therefore, outcomes in these populations cannot be compared to estimate the causal benefit of metastasectomy, not even by multivariate regression as

is done in several series. (8–13) One could argue that we should have selected a more similar control group. However, if such a control group existed, it would have been apparent by overlap of the propensity score curves, which was not the case. This has the reassuring implication that patient selection is based on rigorous clinical criteria, and not patient or physician's preference. Indeed, the strict selection at our center is also apparent from the 91% negative resection margins and outcomes that are better than most reported series: the favorable reported outcomes should therefore be carefully interpreted in light of this stringent selection.

While the long-term follow-up and rigorous patient selection are strong points of this study, it is important to note its limitations. There is a certain time bias, as we included patients since 1995. Yet, as patient outcomes have only improved over time, this will not result in overestimation of the outcomes after metastasectomy. Of note, only 5 of 113 patients died before sunitinib became available, which will therefore not have a major influence on the results. More importantly, 1st line treatment with sunitinib or pazopanib is by now outdated and the place of metastasectomy in the ICI era is unclear. Whereas several trials testing adjuvant angiogenesis inhibitors were strictly negative, the (neo-)adjuvant use of ICI is now being investigated. (14,15) In node-positive melanomas, early results of neo-adjuvant ipilimumab-nivolumab followed by radical surgery are extremely encouraging. (16) This might be a future paradigm for oligometastastic ccRCC, rather than complete resection followed by systemic therapy at relapse. Yet this gives even more importance to the development of predictive tools that can select the population in whom metastasectomy alone may be curative. In this context, molecular subtypes may prove useful: recent work showed that among highly selected patients, primary tumors with a favorable subtype (ccrcc2 or -3) have a high probility of long DFS, whereas those with an unfavorable subtype (ccrcc1 or -4) are at risk of early relapse. (17)

In conclusion, complete metastasectomy and repeat metastasectomy can result in excellent outcomes in highly selected patients, even when its causal benefit cannot be formally assessed. Previous metastasectomy does not impair outcomes on targeted therapies. In light of upcoming combination strategies of metastasectomy with immunotherapies, it is important to identify the populations who will need immunotherapy and those for whom surgery alone may suffice.

References

1. Escudier B, Porta C, Schmidinger M, Rioux-Leclercq N, Bex A, Khoo V, et al. Renal cell carcinoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up†. Ann Oncol. 2019;30(5):706–20.
2. Méjean A, Ravaud A, Thezenas S, Colas S, Beauval J-B, Bensalah K, et al. Sunitinib alone or after nephrectomy in metastatic renal-cell carcinoma. N Engl J Med. 2018;379(5):417–27.
3. Dabestani S, Marconi L, Bex A. Metastasis therapies for renal cancer. Curr Opin Urol. 2016;26(6):566–72.
4. Zaid HB, Parker WP, Safdar NS, Gershman B, Erwin PJ, Murad MH, et al. Outcomes following complete surgical metastasectomy for patients with metastatic renal cell carcinoma: a systematic review and meta-analysis. J Urol. 2017;197(1):44–9.
5. Gundem G, Van Loo P, Kremeyer B, Alexandrov LB, Tubio JMC, Papaemmanuil E, et al. The evolutionary history of lethal metastatic prostate cancer. Nature. 2015;520(7547):353–7.
6. Gomez DR, Blumenschein Jr GR, Lee JJ, Hernandez M, Ye R, Camidge DR, et al. Local consolidative therapy versus maintenance therapy or observation for patients with oligometastatic non-small-cell lung cancer without progression after first line systemic therapy: a multicentre, randomised, controlled, phase 2 study. Lancet Oncol. 2016;17(12):1672–82.
7. Phillips R, Shi WY, Deek M, Radwan N, Lim SJ, Antonarakis E. Outcomes of observation vs stereotactic ablative radiation for oligometastatic prostatce cancer: the ORIOLE phase 2 randomized clinical trial. JAMA oncology. 2020. In press. doi:10.1001/jamaoncol.2020.0147.
8. Eggener SE, Yossepowitch O, Kundu S, Motzer RJ, Russo P. Risk score and metastasectomy independently impact prognosis of patients with recurrent renal cell carcinoma. J Urol. 2008;180(3):873–8.
9. Alt AL, Boorjian SA, Lohse CM, Costello BA, Leibovich BC, Blute ML. Survival after complete surgical resection of multiple metastases from renal cell carcinoma. Cancer. 2011;117(13):2873–82.
10. Yu X, Wang B, Li X, Lin G, Zhang C, Yang Y, et al. The significance of metastasectomy in patients with metastatic renal cell carcinoma in the era of targeted therapy. Biomed Res Int. 2015:ID176373.
11. Staehler MD, Kruse J, Haseke N, Stadler T, Roosen A, Karl A, et al. Liver resection for metastatic disease prolongs survival in renal cell carcinoma: 12-year results from a retrospective comparative analysis. World J Urol. 2010;28(4):543–7.
12. Petralia G, Roscigno M, Zigeuner R, Strada E, Sozzi F, Da Pozzo L, et al. 450 Complete metastasectomy is an independent predictor of cancer-specific survival in patients with clinically metastatic renal cell carcinoma. Eur Urol Suppl. 2010;9(2):162.
13. Kwak C, Park YH, Jeong CW, Lee SE, Ku JH. Metastasectomy without systemic therapy in metastatic renal cell carcinoma: comparison with conservative treatment. Urol Int. 2007;79(2):145–51.

14. Procopio G, Cognetti F, Miceli R, Milella M, Mosca A, Chiuri VE, et al. A randomized, open label, multicenter phase 2 study, to evaluate the efficacy of sorafenib (So) in patients (pts) with metastatic renal cell carcinoma (mRCC) after a radical resection of the metastases: RESORT trial. J Clin Oncol. 2018;36(15_suppl:4502.

15. Appleman LJ, Puligandla M, Pal SK, Harris W, Agarwal N, Costello BA, et al. Randomized, double-blind phase III study of pazopanib versus placebo in patients with metastatic renal cell carcinoma who have no evidence of disease following metastasectomy: A trial of the ECOG-ACRIN cancer research group (E2810). J Clin Oncol. 2019;37(15_suppl:4502.

16. Rozeman EA, Menzies AM, van Akkooi ACJ, Adhikari C, Bierman C, van de Wiel BA, et al. Identification of the optimal combination dosing schedule of neoadjuvant ipilimumab plus nivolumab in macroscopic stage III melanoma (OpACIN-neo): a multicentre, phase 2, randomised, controlled trial. Lancet Oncol. 2019;20(7):948–60.

17. Verbiest A, Couchy G, Job S, Caruana L, Lerut E, Oyen R, et al. Molecular Subtypes of Clear-cell Renal Cell Carcinoma are Prognostic for Outcome After Complete Metastasectomy. Eur Urol. 2018;74(4).

Chapter 6

Molecular subtypes of clear-cell renal cell carcinoma are prognostic for outcome after complete metastasectomy

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European urology. 2018;74(4):474-480

EUROPEAN UROLOGY 74 (2018) 481–482

available at www.sciencedirect.com
journal homepage: www.europeanurology.com

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Platinum Priority – Editorial

Referring to the article published on pp. 474–480 of this issue

"To Cut or Not to Cut", a Biomolecular Approach to Metastasectomy in Metastatic Clear Cell Renal Cell Carcinoma

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In recent years, guidelines for the optimal management of metastatic clear cell renal cell carcinoma (mccRCC) have been changed continuously [1]. In 2005, we moved from the old immunotherapy approaches to the antiangiogenic era, with a major increase in terms of disease control rate and progression-free survival (PFS). More recently, immunotherapy came back into clinical practice with the new checkpoint inhibitors, which provide marked advantages in overall survival (OS) [2,3].

Moreover, the ongoing revolution in the treatment paradigm for patients with mccRCC comprises some other important aspects. Indeed, our understanding of renal carcinoma has evolved over the past few years. ccRCC is no longer considered a single disease entity, but rather a heterogeneous disease, consisting of many different subtypes, each one with distinctive genetic and molecular alterations, a different clinical course, and potentially, different responses to therapy. For instance, poor-risk patients or cases with sarcomatoid features usually present poor outcome and derive limited benefits from antiangiogenic agents, but may have important benefits from checkpoint inhibitors. On the other hand, antiangiogenic agents have been also recently confirmed as the best option for good-risk patients [2,4].

New evidence from recent trials comparing different and innovative drug combinations (ipilimumab plus nivolumab or bevacizumab plus atezolizumab) with sunitinib, and also evaluating the prognostic and possibly predictive role of the PD-L1 status and prognostic risk classification (mainly good-risk versus intermediate/poor-risk cases) will likely change clinical practice, leading to improved patient selection and quality of care [3,4]. Despite these major improvements in systemic treatment, in most cases cure remains a challenge and only a minority of patients, usually treated by multimodality approaches, may achieve a complete and sustained remission.

Metastasectomy could represent, in selected cases, an important option to further increase the possibility of a cure. However, only limited data from retrospective studies are available to date on this issue, as stated from Dabestani et al [5] in their recent systematic review for RCC. As an example, Alt et al [6] reported the results of a large study including 125 patients who underwent radical resection of all metastases: a non-evident disease status was associated with improved OS compared with those who did not undergo radical resection (5-yr OS: 44% vs 13%, $p = 0.001$). However, all these data present high risks of bias since they were collected in positively selected patients. In fact, surgical resection is mainly offered to fit patients who present a limited tumor load (mainly oligometastatic cases), resectable metastasis, and often a long disease-free interval [5,6]. Moreover, no clear guidelines exist to aid in patient selection for metastasectomy.

In this issue of *European Urology*, Verbiest et al [7] published an interesting paper about the possibility of identifying subgroups of patients with mccRCC (ccrcc1–4) who can get the most evident benefit from metastasectomy in terms of disease-free survival, time to systemic treatment, and OS. Interestingly, the genomic approach proposed in this paper, based on difference in mRNA,

DOI of original article: https://doi.org/10.1016/j.eururo.2018.01.042.
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https://doi.org/10.1016/j.eururo.2018.06.039
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expression, methylation status, mutational profile, cytogenetic anomalies, and immune infiltrate, provides a prognostic tool, with a potential predictive relevance in this setting. The probability of remaining free from disease after metastasectomy was 85% for patients classified as molecular subtypes ccRCC 2&3 but only 19% for ccRCC1&4.

How to approach mccRCC patients with oligometastatic disease is widely debated, but no randomized trials on this issue have been published. Verbiest et al, in their cutting-edge work, address this issue and considerably contribute to our knowledge in this area by adding some clues to proper patients' selection, not only in the oligometastatic setting but also in every phase of the decision-making process. In fact, the same group has previously demonstrated that in the metastatic setting patients with ccrcc2&3 subtypes are not only characterized by improved OS but also by different response rates and PFS to the tyrosine kinase inhibitors sunitinib and pazopanib; on the other hand, ccrcc1&4 subtypes are associated with intermediate and poor prognosis, respectively, and rarely show durable responses with the same agents [8,9].

Of course, these data should be interpreted with respect to the results of the recent first-line studies Checkmate 214 and IMmotion 150, providing innovative information in terms of OS and possible identification of optimal candidates to different treatments [3,4]. This integration may be possible since it is not in contrast with the identification of optimal candidates to modern immunotherapy (ccrcc4 tumors) or antiangiogenic approaches (ccrcc 2&3). Indication to treatment for patients classified as group ccrcc1 (negative prognosis) remains unclear, with the possible suggestion of enrolment in clinical trials or more detailed genomic analyses. If validated in larger and prospective studies, comprehensive also of the new immunological agents and combinations, the ccrcc1–4 molecular classification may become an important predictive tool for a more individualized treatment selection.

In 2005, mRCC was defined as a "good in vivo model for developing new drugs" from both an ethical and therapeutic perspective since it was considered an orphan disease strongly dependent from the angiogenic pathway. At present, we expect to cure about 25–30% of the advanced cases, mainly by the modern immunological approaches (single agents or combination strategies also with anti-angiogenic agents), while the aim remains to prolong OS in most patients. A biomolecular identification of optimal candidates to different systemic options, a concept that may be also comprehensive of a planned radical metastasectomy or focal treatments, may significantly increase the number of potentially curable patients [3,4,7].

All these possible treatment options or treatment sequences should be discussed and evaluated within multidisciplinary tumor boards. Given the impressive and continuous improvement in our knowledge of the disease biology, the availability of several effective new drugs and modern techniques can overcome the classical mechanisms of resistance of this disease.

In the modern mccRCC scenario, metastasectomy has moved from a condition of "desperate surgery" to a possibly planned approach also because of an observed increase in OS, the availability of innovative prognostic tools, and further effective treatment options in the case of relapse. The paper by Verbiest et al represents further, elegant evidence in this new scenario.

Conflicts of interest: SB: Advisory Board Member for Janssen, Astellas, Pfizer, MSD, BMS, Ipsen, Roche. Honoraria from: Roche, Janssen, Novartis, BMS, Astellas, Pfizer. Travel support: Novartis, Bayer, BMS, Astellas, Roche, Ipsen, Exelixis, Pfizer, Janssen, AstraZeneca.
AG: Travel support: Ipsen, BMS, Astellas. No other conflicts of interest.
CC: Advisory Board Member for Ipsen, Novartis, BMS, Pfizer. Travel support: Novartis, BMS, Janssen, Astellas, Pfizer, AstraZeneca.

Acknowledgments: We are grateful to Luca Giacomelli, PhD (University of Genoa, Genoa, Italy), for useful discussion.

References

[1] Escudier B, Porta C, Schmidinger M, et al. ESMO Guidelines Committee. Renal cell carcinoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2016;27:v58–68.
[2] Motzer RJ, Escudier B, McDermott DF, et al. CheckMate 025 Investigators. Nivolumab versus Everolimus in Advanced Renal-Cell Carcinoma. N Engl J Med 2015;373:1803–13.
[3] Motzer RJ, Tannir NM, McDermott DF, et al. CheckMate 214 Investigators. Nivolumab plus Ipilimumab versus Sunitinib in Advanced Renal-Cell Carcinoma. N Engl J Med 2018;378:1277–90.
[4] McDermott DF, Huseni MA, Atkins MB, et al. Clinical activity and molecular correlates of response to atezolizumab alone or in combination with bevacizumab versus sunitinib in renal cell carcinoma. Nat Med 2018;24:749–57.
[5] Dabestani S, Marconi L, Hofmann F, et al. Local treatments for metastases of renal cell carcinoma: a systematic review. Lancet Oncol 2014;15:e549–61.
[6] Alt AL, Boorjian SA, Lohse CM, Costello BA, Leibovich BC, Blute ML. Survival after complete surgical resection of multiple metastases from renal cell carcinoma. Cancer 2011;117:2873–82.
[7] Verbiest A, Couchy G, Job S, et al. Molecular subtypes of clear-cell renal cell carcinoma are prognostic for outcome after complete metastasectom. Eur Urol 2018;74:474–80.
[8] Beuselinck B, Job S, Becht E, et al. Molecular subtypes of clear cell renal cell carcinoma are associated with sunitinib response in the metastatic setting. Clin Cancer Res 2015;21:1329–39.
[9] Verbiest A, Couchy G, Job S, et al. Molecular subtypes of clear cell renal cell carcinoma are associated with outcome during pazopanib therapy in the metastatic setting. Clin Genitourin Cancer 2018;16:e605–12.

EUROPEAN UROLOGY 74 (2018) 474–480

available at www.sciencedirect.com
journal homepage: www.europeanurology.com

European Association of Urology



Platinum Priority – Kidney Cancer

Editorial by Sergio Bracarda, Annalisa Guida and Claudia Caserta on pp. 481–482 of this issue

Molecular Subtypes of Clear-cell Renal Cell Carcinoma are Prognostic for Outcome After Complete Metastasectomy

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Article info

Article history:
Accepted January 30, 2018

Associate Editor:
James Catto

Keywords:
Biomarker
Clear-cell renal cell carcinoma
Gene expression profile
Metastasectomy
Metastatic
Molecular subtypes
Prognosis

Abstract

Background: Metastasectomy is routinely performed in selected patients with metastatic clear-cell renal cell carcinoma (ccRCC) as an alternative to systemic therapy. In the absence of randomized trials, the benefit and best way of patient selection remain unclear. Earlier, we described four molecular ccRCC-subtypes (ccrcc1–4) that have a prognostic and predictive value upon first-line sunitinib or pazopanib.
Objective: Assess the prognostic value of ccrcc1–4 subtypes after complete metastasectomy. (1) Compare outcomes of good-prognosis ccrccc2&3-tumors with intermediate/poor-prognosis ccrcc1&4-tumors. (2) Compare outcomes of the four subtypes separately.
Design, setting, and participants: Single-center retrospective study (1995–2017), assessing 43 ccRCC patients undergoing complete metastasectomy without systemic treatment.
Intervention: Molecular subtype determined with established 35-gene expression classifier.
Outcome measurements and statistical analysis: Median disease-free survival (DFS), time to systemic therapy, cancer-specific (CSS) and overall survival (OS) from metastasectomy, estimated with Kaplan-Meier method and tested against other predictors with multivariable Cox regression.
Results and limitations: Median DFS was 23 mo for ccrcc2&3-tumors versus 9 mo for ccrcc1&4-tumors ($p = 0.011$, hazard ratio [HR] = 2.6). Median time to systemic therapy was 92 mo versus 28 mo ($p = 0.003$, HR = 3.3). Median CSS was 133 mo versus 50 mo ($p < 0.001$, HR = 2.7). Median OS was 127 mo versus 50 mo ($p = 0.011$, HR = 2.5). The classification remained independent upon multivariable analysis. Outcomes remained significantly different when comparing four subtypes separately. The intrinsic heterogeneity of expression profiles is a limitation of this approach.
Conclusion: Even after clinical patient selection, patients with a ccrcc1- or ccrcc4-tumor are at a higher risk of relapse after complete metastasectomy. Patients with a ccrcc2- or ccrcc3-tumor usually experience a long DFS. These results need validation in a larger cohort to establish the subtypes as prognostic marker.
Patient summary: Metastasectomy is recommended for some patients with metastatic clear-cell kidney cancer; however, we do not know who will benefit the most. We show that molecular subtypes increase the possibility to predict which patients are at risk for early relapse after metastasectomy and who may benefit more from other treatment options.
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https://doi.org/10.1016/j.eururo.2018.01.042
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1. Introduction

Clear-cell renal cell carcinoma (ccRCC) accounts for the majority of primary kidney cancers, which are responsible for about 35 000 deaths per year in Europe [1]. Metastatic ccRCC is preferentially treated in first-line with an angiogenesis inhibitor (sunitinib or pazopanib) in International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) good risk patients, or immune checkpoint inhibitors (nivolumab-ipilimumab) in IMDC intermediate/poor risk patients [2]. However, in selected patients, complete metastasectomy is recommended with the aim of prolonging survival or at least postponing systemic therapy [3]. The intervention is mainly offered to fit patients presenting with a limited tumor load after a long disease-free interval [4].

Although retrospective series typically show favorable outcomes after metastasectomy, it remains an open question to what extent surgery provides actual benefit, rather than the indolent tumor biology in these highly selected patients. In non-small-cell lung carcinoma, a recent randomized controlled trial pointed toward a progression-free survival benefit in patients who received local treatment of metastases (mostly stereotactic body radiotherapy) [5]. In other tumor types, randomized trials are lacking. A recent systematic review on metastasectomy in RCC identified only 15 comparative studies; however, all were retrospective in nature and with a high risk of bias [4,6]. Although most studies pointed toward the benefit of complete metastasectomy, a multivariable analysis was performed in only eight to correct for a limited set of confounders. Moreover, in a large patient series at our own center, we found that the baseline prognostic characteristics of patients who undergo metastasectomy are too favorable to allow for a liable comparison of outcomes with patients who do not undergo metastasectomy, even by multivariable analysis (A. Verbiest, unpublished data, 2017). Importantly, no clear guidelines exist to aid patient selection for metastasectomy.

Briefly, an important knowledge gap remains to the extent to which intrinsic tumor biology explains the favorable outcomes after metastasectomy and to the selection of patients that will benefit from it. This study aimed to resolve the second question.

Earlier, unsupervised cluster analysis of whole genome mRNA expression data has revealed distinct molecular subtypes of ccRCC. These subtypes, based on intrinsic tumor biology, are correlated with prognosis. In the post-nephrectomy setting, Brannon et al. [7] and the Cancer Genome Atlas project (TCGA) [8] have identified two (ccA and ccB) and four (m1–m4) subtypes, respectively, that show different survival after nephrectomy. In the metastatic setting, we have identified four subtypes, named ccrcc1–4, that differ in terms of mRNA expression, methylation status, mutation profile, cytogenetic anomalies, and immune infiltrate [9]. These subtypes are not only correlated with overall survival (OS) but also with response rate and progression-free survival on the angiogenesis inhibitors sunitinib and pazopanib. Subtypes ccrcc2&3 have a favorable prognosis and high response rate to sunitinib and pazopanib [9,10]. Ccrcc2-tumors display relative upregulation of genes implicated in angiogenesis, whereas ccrcc3-tumors exhibit an expression profile similar to that of the normal kidney tissue [9,11]. In contrast, subtypes ccrcc1&4 have an intermediate and poor prognosis, respectively, and rarely show durable responses to sunitinib or pazopanib [9,10]. Both show relative upregulation of Myc-targets. However, ccrcc1-tumors have an immune-cold phenotype with virtual absence of tumor-infiltrating lymphocytes, whereas ccrcc4-tumors show an immune-inflamed phenotype, with an abundant but exhausted immune infiltrate and relative upregulation of genes involved in inflammation, migration, and immune response [9,12].

The molecular subtypes as described by Brannon et al [7], TCGA [8], and Beuselinck et al [9] are overlapping. As they have a prognostic value in the post-nephrectomy and metastatic setting, we hypothesized that they would also be able to predict outcome after metastasectomy.

2. Materials and methods

2.1. Patient population

At University Hospitals Leuven, a systematic record from 1995 to present is maintained of RCC patients who undergo metastasectomy. We selected patients who met the following inclusion criteria: clear-cell histology, resection of the primary tumor, complete metastasectomy, no prior or adjuvant systemic treatment, no other metastatic cancer, availability of fresh frozen primary tumor tissue, and at least one follow-up computed tomography (CT) scan after metastasectomy. Clear-cell histology was confirmed by an expert genitourinary pathologist. Ipsilateral adrenal lesions that were spatially separated from the primary tumor were considered metastases, whereas continuous growth into the adrenal gland was not. Follow-up policy was left to the discretion of the treating physician.

The protocol was approved by the local medical ethics review boards, and signed consent was obtained from all patients in accordance with Belgian legislation. Frozen biological material from deceased patients was used when prior agreement for such use was given by the institutional review board.

2.2. Sample classification

Extraction of mRNA from fresh frozen primary tumor tissue was performed using the Maxwell 16 LEV simplyRNA tissue kit according to the manufacturer's protocol (Promega). Quantification was performed using the NanoDrop spectrophotometer (ThermoFisher) and quality control by gel electrophoresis (E-gel 48 1% agarose and Mother E-base by Invitrogen). Using the High-Capacity Transcription kit from ThermoFisher, 500 ng of total RNA was reverse transcribed in a final volume of 50 μl. Preamplification of 6 ng cDNA/sample was performed using PreAmp Master Mix and the specific primers of the classifier as well as 18s. Preamplified cDNA were diluted 1:5 in DNAse/RNAse free water before qRT-PCR. qRT-PCR reactions were performed using the high throughput BioMark qRT-PCR system (Fluidigm) according to the manufacturer's protocol. Expression data (CT values) were analyzed using the Fluidigm qRT-PCR Analysis software version 4.1.3. 18S was used for normalization. The tumors were classified into the ccrcc1–4 molecular classification by our 35-gene classifier algorithm as described earlier [9].

2.3. Statistical analysis

Primary endpoints were median disease-free survival (DFS), time to systemic therapy, cancer-specific survival (CSS), and OS after complete metastasectomy. Sample size was limited due to the restricted availability of fresh frozen tumor tissue. Therefore, we focused on pooled analysis, comparing the good-prognosis ccrcc2&3-subtypes with the intermediate/poor-prognosis ccrcc1&4-subtypes. As secondary endpoints, we calculated outcomes for the four subtypes separately. Survival and hazard ratios (HR) were estimated using the Kaplan-Meier method and log-rank test. Kaplan-Meier analysis with log-rank test was also performed to assess the impact of baseline characteristics on DFS and CSS: T-, N- and M-stage, Fuhrman grade ≥3, DFS after nephrectomy (continuous), sex, age at metastasectomy, anemia, hypercalcemia, thrombocytosis, elevated neutrophils, Karnofsky performance status, presence of lung metastases, multiple metastases, multiorgan metastases, and Leuven-Udine prognostic score. The Leuven-Udine prognostic score predicts CSS after metastasectomy based on five risk factors (Fuhrman grade, T-stage, disease-free interval, lung metastases, multiorgan metastases) [13]. The IMDC score could not be calculated as it includes the interval between nephrectomy and start of systemic therapy, whereas patients had not received systemic therapy at the time of metastasectomy [14]. Multivariable Cox regression was performed for variables with $p < 0.2$ on Kaplan-Meier analysis. Graphpad was used for Kaplan-Meier analysis and XLstat for multivariable Cox regression (GraphPad Prism 7.00 For Windows, GraphPad.Software, La Jolla, California, USA and XLSTAT, Data Analysis and Statistical Solution for Microsoft Excel, Addinsoft, Paris, France).

3. Results

Forty-three patients met the inclusion criteria. Patient characteristics are summarized in Table 1. There were 33 (77%) good-prognosis tumors (28 ccrcc2; 5 ccrcc3) and 10 (23%) intermediate/poor-prognosis tumors (5 ccrcc1; 5 ccrcc4). The median follow-up of patients who were alive at the time of analysis was 56 mo (quartiles: 30–98 mo, $n = 25$), all others were followed until death ($n = 18$).

In the global series, median DFS after metastasectomy was 17 months (mo) with estimated 5-yr DFS 23%. Median time to systemic therapy was 79 mo, median CSS was

Table 1 – Characteristics of 43 patients in the study: 33 ccrcc2 and 3, 10 ccrcc1 and 4 [a,b]

	ccrcc2	ccrcc3	ccrcc1	ccrcc4
	(%)		(%)	
Patient number	77		23	
Characteristics at time of nephrectomy				
Male	73		80	
Lymph node positive	5		0	
Synchronous metastasis	24		20	
T stage:				
T1	27		22	
T2	30		44	
T3	39		33	
T4	3		0	
Fuhrman grade:				
Grade 2	19		0	
Grade 3	34		33	
Grade 4	47		66	
Characteristics at time of metastasectomy				
Median age (yr)	62 (49–74)*		62 (57–67)*	
Median disease-free interval since nephrectomy (mo)	35 (10–62)*		22 (5–52)*	
Karnofsky performance status <80	10		0	
Anemia	33		30	
Hypercalcemia	0		0	
Thrombocytosis	9		0	
Elevated neutrophils	7		14	
Multiple lesions	27		30	
Site of metastasis:				
Lung	33		20	
Adrenal	15		30	
Bone	12		0	
Other	24		40	
Multiorgan	15		10	
Leuven-Udine prognostic group**:				
A	28		12	
B	22		25	
C	41		38	
D	9		25	
Negative resection margins	94		100	

* Quartiles.
** Five-yr cancer-specific survival after metastasectomy for groups A, B, C, D is estimated to be 83%, 53%, 24%, 0%, respectively [13].
[a] Absolute numbers per subtype are provided in Supplementary Table S1.
[b] Local cut-offs were used for laboratory values: hemoglobin 14 g/dl (men) or 12.5 g/dl (women), calcium 2.55 mg/dl, neutrophils 7.500/l, thrombocytes 450.000/l.

Table 2 – Survival outcomes after metastasectomy as estimated with the Kaplan-Meier method, reported as median in months. ccrcc2- and 3-subtypes are compared with ccrcc1- and 4-subtypes using the log-rank test. Outcomes of the four separate subtypes are reported in grey

	All	ccrcc2	ccrcc3	ccrcc1	ccrcc4	Hazard ratio	*p* value
	n = 43	*n* = 28	*n* = 5	*n* = 5	*n* = 5		
Disease-free survival	17	23		9		2.56	0.011
		18.5	36	7	13		0.039
1 yr, *n* (%)	63	71		40			
2 yr, *n* (%)	39	48		10			
5 yr, *n* (%)	23	27		10			
10 yr, *n* (%)	15	17		Not estimable			
Time to systemic therapy	79	92		28		3.29	0.003
		79	107	25	33		0.029
Cancer-specific survival	127	133		50		2.66	<0.001
		133	163	56.5	48		<0.001
5 yr, *n* (%)	80	89		40			
Overall survival	121	127		50		2.54	0.011
		127	163	56.5	48		0.004
5 yr, *n* (%)	73	80		40			

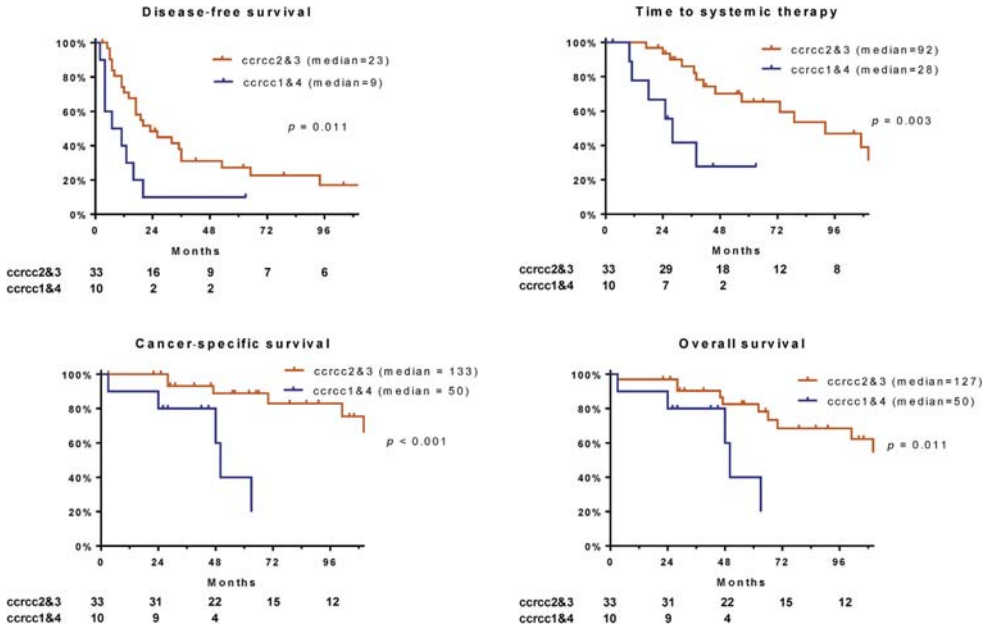

Fig. 1 – Kaplan-Meier curves showing outcomes of ccrcc2&3-tumors and ccrcc1&4-tumors after complete metastasectomy.

127 mo with estimated 5-yr CSS 80%, and median OS was 121 mo.

3.1. *Impact of molecular subtype on outcome*

Outcomes are summarized in Table 2 and Figure 1. Median DFS after metastasectomy was 23 mo for ccrcc2&3-patients versus 9 mo for ccrcc1&4-patients (HR = 2.6, $p = 0.011$). Estimated1-yr DFS was 71% versus 40%, 2-yr DFS was 48% versus 10%, and 5-yr DFS was 27% versus 10%, respectively. Estimated10-yr DFS was 17% for ccrcc2&3-patients.

Median time from metastasectomy to start of systemic therapy was 92 mo for ccrcc2&3-patients versus 28 mo for ccrcc1&4-patients (HR = 3.3, $p = 0.003$). At the time of analysis, 33 patients had relapsed, of which 21 had started systemic therapy. In these patients, median time from relapse to start was 34 mo in ccrcc2&3-patients and 11 mo in ccrcc1&4-patients.

Table 3 – Multivariable Cox regression models for cancer-specific survival, testing the ccrcc1–4 molecular classification against other predictors with p 0.2 on Kaplan-Meier analysis. Two different Cox models were used because multiorgan disease is itself a parameter of the Leuven-Udine score

	p value	Hazard ratio	95% Confidence interval
ccrcc1&4	0.001	16	2.9–88
Anemia	0.020	6.1	1.3–27
Multiorgan disease	0.051	4.8	0.99–23
ccrcc1&4	0.002	12	2.4–59
Anemia	0.012	6.7	1.5–29
Leuven-Udine C or D*	0.35	1.7	0.6–5.2

* The Leuven-Udine prognostic score was recoded as A or B versus C or D to limit the number of variables.

Median CSS was 133 mo for ccrcc2&3-tumors versus 50 mo for ccrcc1&4-tumors (HR = 2.7, $p < 0.001$). Estimated 5-yr CSS was 89% versus 40%, respectively.

Median OS was 127 mo for ccrcc2&3-tumors versus 50 mo for ccrcc1&4-tumors (HR = 2.5, $p = 0.011$). Estimated 5-yr OS was 80% versus 40%, respectively.

Survival differences remained significant when the four separate subtypes were compared.

3.2. *Multivariable survival analysis*

According to log-rank tests, there was no significant impact of baseline parameters on DFS. Three variables were related with CSS with $p < 0.2$: anemia ($p = 0.007$), multiorgan metastases ($p = 0.056$), and Leuven-Udine prognostic score ($p = 0.13$). The molecular classification was tested against these factors in a multivariable regression analysis. Two models were used as the presence of multiorgan metastases is already included in the Leuven-Udine prognostic score. The ccrcc1–4 classification and anemia remained independent, with the ccrcc1–4 classification being the strongest predictor (Table 3).

4. Discussion

The aim of this study was to assess the prognostic value of the ccrcc1–4 molecular subtypes of ccRCC in the setting of complete metastasectomy. Indeed, in this series of 43 patients, we show an important difference in DFS, time to systemic therapy, CSS, and OS according to subtype.

When comparing with the literature, we report a global 5-yr OS of 73% versus mostly 30–50% in other series. These good results can be explained by stringent patient selection, certainty of complete metastasectomy, and exclusive clear-cell histology in our series. However, the good-prognosis ccrcc2&3-subtypes show a 5-yr OS of 80% (CSS 89%), far better than the published results. On the other hand, the intermediate/poor-prognosis ccrccc1&4-subtypes show a 5-yr OS of only 40%. The 5-yr DFS is more difficult to compare as this was only reported for mixed metastasectomy sites in the series by Daliani et al [15]. Our 5-yr DFS was comparable with their study in ccrcc2&3-patients (27%) but was only 10% in ccrcc1&4-patients (Table 4).

Importantly, all patients were deemed to have a good prognosis on clinical grounds when they were offered metastasectomy. The molecular classification also remained a stronger and independent predictor for survival when tested against established prognostic factors like the Leuven-Udine prognostic score. Moreover, the good-prognosis ccrcc2&3-tumors were overrepresented in this series compared with our previous sunitinib-treated series (77% vs 53%). This probably reflects the more aggressive disease course of patients with ccrcc1&4-tumors who are less often considered eligible for metastasectomy. The ccrcc1&4-tumors in this series were probably those with, for their molecular subtype, a rather indolent behavior.

The finding that common prognostic markers such as TNM-stage and Fuhrman grade were not correlated with outcome in this series is probably explained by the fact that our patients were highly selected: to be considered eligible for metastasectomy, patients with some unfavorable clinical characteristics must have had others that were favorable.

Consequently, our findings strongly support an important role for intrinsic tumor biology as explanation for the generally favorable outcomes after metastasectomy. The ccrcc1–4 classification has the potential to become a tool for individualized treatment selection. Considering the 2-yr DFS of only 10%, metastasectomy might best be avoided in ccrcc1&4-patients even if they seem good candidates on a clinical basis. This is especially the case when the envisaged surgery could cause considerable morbidity. Moreover, the immune-inflamed phenotype of the poor-prognosis ccrcc4-tumors suggests them as good candidates for treatment with immune checkpoint inhibitors instead of surgery [12,16]. Assessing the response of these tumors to immune checkpoint blockade is indeed one of our future goals. The alternative is less clear for ccrcc1-patients who have a non-inflamed phenotype and have only intermediate response rates to angiogenesis inhibitors. In the future, these patients might benefit from therapies, now still investigational, which alter the immune phenotype. For example, one could hypothesize that local treatment of ccrcc1 metastases with stereotactic body radiotherapy instead of surgery could induce immunogenic cell death, thus enhancing the intrinsic immune response and increasing sensitivity to immune modulating systemic treatments [17].

As to the good-prognosis ccrcc2&3-tumors, the fact that they usually relapse as well and yet still take years before needing systemic therapy even after relapse, might also raise the question whether surgery is overly aggressive in these patients. However, the 17% 10-yr DFS that might reflect curative treatment and the psychological relief of being disease-free for several years are strong arguments in favor of metastasectomy in selected patients.

We recognize the limitations of this study. First, the sample size is modest; therefore, these findings need validation in a larger series. Yet the fact that an important effect can be shown in only 43 patients also illustrates the robustness of the ccrcc1–4 classification as a prognostic

Table 4 – Outcomes of renal cell carcinoma patients undergoing complete metastasectomy as reported in literature

Author (yr)	*N*	Location	DFS		OS		CSS	
			5 yr, *n* (%)	Median	5 yr, *n* (%)	Median	5 yr, *n* (%)	Median
Verbiest et al (2017)	43	Mixed (30% lung)	23	17	73	121	80	127
ccrcc2&3	33	Mixed (33% lung)	27	23	80	127	89	133
ccrcc1&4	10	Mixed (20% lung)	10	9	40	50	40	50
Thomas et al (2016) [19]	102	Local recurrence	33*	23	–	–	52	66
Yu et al (2015) [20]	31	Mixed	–	–	43*	52	–	–
Alt et al (2011) [21]	125	Mixed (39% lung)	–	–	45	48	–	–
Meimarakis et al (2011) [22]	202	Lung	–	–	39	40	–	–
Naito et al (2011) [23]	357	Mixed	–	–	–	110	–	–
Petralia et al (2010) [24]	35	Mixed	–	–	–	–	–	30
Daliani et al (2009) [15]	38	Mixed (20% lung)	27*	21,6	47*	55	–	–
Staehler et al (2009) [25]	183	Mixed (66% lung)	–	–	58	–	–	–
Zerbi et al (2008) [26]	23	Pancreas	34*	44	88	–	–	–
Kwak et al (2007) [27]	21	Mixed (33% lung)	–	–	48	37	–	–
Russo et al (2007) [28]	61	Mixed (72% lung)	–	–	30*	30	–	–
Thelen et al (2007) [29]	31	Liver	26	27	39	48	–	–
Hofmann et al (2005) [30]	64	Lung	–	–	40	47	–	–
Eggener et al (2005) [31]	44	Mixed (43% lung)	–	–	49	45	–	–
Murthy et al (2005) [32]	92	Lung	–	–	31	–	–	–
Kavolius et al (1998) [33]	141	Mixed	–	–	44	–	–	–

CSS = cancer-specific survival; DFS = disease-free survival; OS = overall survival.
* As estimated from the Kaplan-Meier survival curve.

marker. Second, as with other prognostic expression profiles, intratumor heterogeneity of mRNA expression remains a concern needing further investigation [18]. We are working to quantify intratumor heterogeneity of the ccrcc1–4 subtypes and optimize sampling. Third, like other metastasectomy series, this study was retrospective and observational in nature. However, this approach was unavoidable and did allow for long follow-up times. It was not possible to select matched controls from patients who did not undergo metastasectomy since their clinical characteristics were too unfavorable to allow for comparison. Finally, our current classification technique is based on Fluidigm qRT-PCR of mRNA extracted from fresh frozen tumor tissue, which cannot be easily implemented in the clinic. However, efforts are underway to validate the ccrcc1–4 classification on paraffin-embedded tissue using a Nanostring nCounter panel, which is more readily available.

5. Conclusions

In this series, molecular subtypes of metastatic ccRCC were prognostic for outcome after complete metastasectomy, independent of other patient and tumor characteristics, and even in a highly selected patient population. Ccrcc1&4-tumors are at a risk for early relapse after metastasectomy, whereas ccrcc2&3-tumors have very favorable outcomes superior to those reported in literature. If validated on in larger cohort, the ccrcc1–4 molecular classification has the potential to become a tool for a more proper individualized treatment selection.

Author contributions: Benoit Beuselinck had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Beuselinck, Verbiest, Zucman-Rossi
Acquisition of data: Verbiest, Couchy, Joniau
Analysis and interpretation of data: Verbiest
Drafting of the manuscript: Verbiest
Critical revision of the manuscript for important intellectual content: Van Poppel, Wozniak
Statistical analysis: Job, de Reyniès
Obtaining funding: Beuselinck
Administrative, technical, or material support: Caruana, Lerut, Oyen, Tosco, Van Den Eynde, Van Raemdonck
Supervision: Beuselinck
Other: None

Financial disclosures: Benoit Beuselinck certifies that all conflicts of interest, including specific financial interests and relationships and affiliations relevant to the subject matter or materials discussed in the manuscript (eg, employment/affiliation, grants or funding, consultancies, honoraria, stock ownership or options, expert testimony, royalties, or patents filed, received, or pending), are the following: Benoit Beuselinck received honoraria from Amgen, Pfizer, Janssen, and Bayer and is an investigator of the EudraCT: 2011-006085-40/MetaSun trial supported by Pfizer. The other authors have no conflicts of interest to declare.

Funding/Support and role of the sponsor: Kom op tegen Kanker (Annelies Verbiest); Fondation Martine Midy (Benoit Beuselinck); Fonds voor Wetenschappelijk Onderzoek Vlaanderen (Benoit Beuselinck and Evelyne Lerut).

Acknowledgments: The original ccrcc1–4 classification project was funded by INSERM and the "Cartes d'identité des Tumeurs" program of the French National Ligue against Cancer. Annelies Verbiest received a grant from Kom op tegen Kanker (Stand up to Cancer), the Flemish cancer society. Benoit Beuselinck received a grant from Fondation Martine Midy (Paris, France) and Fonds voor Wetenschappelijk Onderzoek Vlaanderen (Belgium). Evelyne Lerut received funding from Fonds voor Wetenschappelijk Onderzoek Vlaanderen and Stichting tegen Kanker.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.eururo.2018.01.042.

References

[1] Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. Eur J Cancer 2013;49:1374–403.

[2] Powles T, Albiges L, Staehler M, et al. Updated European Association of Urology Guidelines Recommendations for the treatment of first-line metastatic clear cell renal cancer. Eur Urol 2018;73:311–5.

[3] Ljungberg B, Bensalah K, Canfield S, et al. EAU guidelines on renal cell carcinoma: 2014 update. Eur Urol 2015;67:913–24.

[4] Dabestani S, Marconi L, Bex A. Metastasis therapies for renal cancer. Curr Opin Urol 2016;26:566–72.

[5] Gomez DR, Blumenschein GR, Lee JJ, et al. Local consolidative therapy versus maintenance therapy or observation for patients with oligometastatic non-small-cell lung cancer without progression after first-line systemic therapy: a multicentre, randomised, controlled, phase 2 study. Lancet Oncol 2016;17:1672–82.

[6] Dabestani S, Marconi L, Hofmann F, et al. Local treatments for metastases of renal cell carcinoma: a systematic review. Lancet Oncol 2014;15:e549–61.

[7] Brannon AR, Reddy A, Seiler M, et al. Molecular stratification of clear cell renal cell carcinoma by consensus clustering reveals distinct subtypes and survival patterns. Genes Cancer 2010;1:152–63.

[8] Cancer Genome Atlas Research N. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature 2013;499:43–9.

[9] Beuselinck B, Job S, Becht E, et al. Molecular subtypes of clear cell renal cell carcinoma are associated with sunitinib response in the metastatic setting. Clin Cancer Res 2015;21:1329–39.

[10] Verbiest A, Couchy G, Job S, et al. Molecular subtypes of clear cell renal cell carcinoma are associated with outcome during pazopanib therapy in the metastatic setting. Clin Genitourin Cancer. In press. doi:10.1016/j.clgc.2017.10.017.

[11] Beuselinck B, Verbiest A, Couchy G, et al. Pro-angiogenic gene expression is associated with better outcome on sunitinib in metastatic clear-cell renal cell carcinoma. Acta Oncol 2017;1–11.

[12] Chen DS, Mellman I. Elements of cancer immunity and the cancer-immune set point. Nature 2017;541:321–30.

[13] Tosco L, Van Poppel H, Frea B, Gregoraci G, Joniau S. Survival and impact of clinical prognostic factors in surgically treated metastatic renal cell carcinoma. Eur Urol 2013;63:646–52.

[14] Heng DY, Xie W, Regan MM, et al. Prognostic factors for overall survival in patients with metastatic renal cell carcinoma treated with vascular endothelial growth factor-targeted agents: results from a large, multicenter study. J Clin Oncol 2009;27:5794–9.

[15] Daliani DD, Tannir NM, Papandreou CN, et al. Prospective assessment of systemic therapy followed by surgical removal of metastases in selected patients with renal cell carcinoma. BJU Int 2009;104:456–60.

[16] McDermott D, Huseni M, Rini B, et al. Abstract CT081: Molecular correlates of differential response to Atezolizumab+/-Bevacizumab vs Sunitnib in a Phase II study in untreated metastatic renal cell carcinoma (RCC) patients. Cancer Res. In press. doi:10.1158/1538-7445.AM2017-CT081.

[17] De Wolf K, Vermaelen K, De Meerleer G, Lambrecht BN, Ost P. The potential of radiotherapy to enhance the efficacy of renal cell carcinoma therapy. Oncoimmunology 2015;4:e1042198.

[18] Gulati S, Martinez P, Joshi T, et al. Systematic evaluation of the prognostic impact and intratumour heterogeneity of clear cell renal cell carcinoma biomarkers. Eur Urol 2014;66:936–48.

[19] Thomas AZ, Adibi M, Borregales LD, et al. Surgical Management of Local Retroperitoneal Recurrence of Renal Cell Carcinoma after Radical Nephrectomy. J Urol 2015;194:316–22.

[20] Yu X, Wang B, Li X, et al. The Significance of Metastasectomy in Patients with Metastatic Renal Cell Carcinoma in the Era of Targeted Therapy. Biomed Res Int 2015;2015:176373.

[21] Alt AL, Boorjian SA, Lohse CM, Costello BA, Leibovich BC, Blute ML. Survival after complete surgical resection of multiple metastases from renal cell carcinoma. Cancer 2011;117:2873–82.

[22] Meimarakis G, Angele M, Staehler M, et al. Evaluation of a new prognostic score (Munich score) to predict long-term survival after resection of pulmonary renal cell carcinoma metastases. Am J Surg 2011;202:158–67.

[23] Naito S, Kinoshita H, Kondo T, et al. Prognostic factors of patients with metastatic renal cell carcinoma with removed metastases: a multicenter study of 556 patients. Urology 2013;82:846–51.

[24] Petralia G, Roscigno M, Zigeuner R. Complete metastasectomy is an independent predictor of cancer-specific survival in patients with clinically metastatic renal cell carcinoma. Eur Urol Suppl 2010;9:1.

[25] Staehler MD, Kruse J, Haseke N. Metastasectomy significantly prolongs survival in patients with metastatic renal cell cancer. Eur Urol Suppl 2009;181:2.

[26] Zerbi A, Ortolano E, Balzano G, Borri A, Beneduce AA, Di Carlo V. Pancreatic metastasis from renal cell carcinoma: which patients benefit from surgical resection? Ann Surg Oncol 2008;15:1161–8.

[27] Kwak C, Park YH, Jeong CW, Lee SE, Ku JH. Metastasectomy without systemic therapy in metastatic renal cell carcinoma: comparison with conservative treatment. Urol Int 2007;79:145–51.

[28] Russo P, Synder M, Vickers A, Kondagunta V, Motzer R. Cytoreductive nephrectomy and nephrectomy/complete metastasectomy for metastatic renal cancer. Sci World J 2007;7:768–78.

[29] Thelen A, Jonas S, Benckert C, et al. Liver resection for metastases from renal cell carcinoma. World J Surg 2007;31:802–7.

[30] Hofmann HS, Neef H, Krohe K, Andreev P, Silber RE. Prognostic factors and survival after pulmonary resection of metastatic renal cell carcinoma. Eur Urol 2005;48:77–81, discussion-2.

[31] Eggener SE, Yossepowitch O, Kundu S, Motzer RJ, Russo P. Risk score and metastasectomy independently impact prognosis of patients with recurrent renal cell carcinoma. J Urol 2008;180:873–8, discussion 8.

[32] Murthy SC, Kim K, Rice TW, et al. Can we predict long-term survival after pulmonary metastasectomy for renal cell carcinoma? Ann Thorac Surg 2005;79:996–1003.

[33] Kavolius JP, Mastorakos DP, Pavlovich C, Russo P, Burt ME, Brady MS. Resection of metastatic renal cell carcinoma. J Clin Oncol 1998;16:2261–6.

Chapter 7

Pro-angiogenic gene expression is associated with better outcome on sunitinib in metastatic clear-cell renal cell carcinoma

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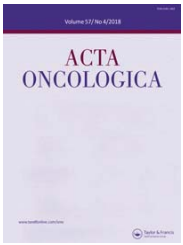
Acta oncologica. 2018;57(4):498-508.



Acta Oncologica

ISSN: 0284-186X (Print) 1651-226X (Online) Journal homepage: https://www.tandfonline.com/loi/ionc20

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To cite this article: Benoit Beuselinck, Annelies Verbiest, Gabrielle Couchy, Sylvie Job, Aurélien de Reynies, Clément Meiller, Maarten Albersen, Virginie Verkarre, Evelyne Lerut, Arnaud Méjean, Jean-Jacques Patard, Brigitte Laguerre, Nathalie Rioux-Leclercq, Patrick Schöffski, Stéphane Oudard & Jessica Zucman-Rossi (2018) Pro-angiogenic gene expression is associated with better outcome on sunitinib in metastatic clear-cell renal cell carcinoma, Acta Oncologica, 57:4, 498-508, DOI: 10.1080/0284186X.2017.1388927

To link to this article: https://doi.org/10.1080/0284186X.2017.1388927

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ACTA ONCOLOGICA, 2018
VOL. 57, NO. 4, 498–508
https://doi.org/10.1080/0284186X.2017.1388927

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ORIGINAL ARTICLE

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Pro-angiogenic gene expression is associated with better outcome on sunitinib in metastatic clear-cell renal cell carcinoma

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ABSTRACT

Objectives: Clear-cell renal cell carcinomas (ccRCC) are characterized by hyper-vascularization and can respond to vascular endothelial growth factor receptor (VEGFR) inhibitors such as sunitinib. We aimed to study the predictive value of the expression of genes in the hypoxia induced factor (HIF) – vascular endothelial growth factor (VEGF) - VEGFR-pro-angiogenic pathway in metastatic ccRCC (m-ccRCC) patients treated with sunitinib and the correlation between the expression of these genes and the molecular ccrcc-classification, the expression of genes involved in the immune-suppressive microenvironment and Von Hippel-Lindau (VHL) - and Polybromo-1 (PBRM1) - mutational status.

Material and methods: m-ccRCC patients treated with sunitinib as first-line targeted therapy were included. Gene expression was studied in the primary nephrectomy sample by qRT-PCR, VHL- and PBRM1-mutational status by sequencing. Response rate by RECIST, progression-free survival (PFS) and overall survival (OS) were study endpoints.

Results: One hundred and four patients were included. On multivariate-analysis, HIF2A-, platelet derived growth factor receptor beta (PDGFRB)-, VEGFC-, VEGFR1- and VEGFR2-expression were correlated with PFS and HIF1A-, HIF2A-, VEGFR1- and VEGFR2-expression with OS. VEGFR2-expression showed the strongest association with outcome, being significantly correlated with all outcome parameters. HIF2A, VEGFA, VEGFR1, VEGFR2 and VEGFR3 were highly expressed in the transcriptomic ccrcc2-subtype of tumors, known to be highly sensitive to sunitinib. In the total tumor series, there was no correlation nor inverse correlation between the expression of genes involved in angiogenesis and in the immune-suppressive microenvironment. In tumors with a bi-allelic PBRM1-inactivation, HIF2A-, VEGFA-, VEGFR1- and VEGFR2-expression were higher, compared to tumors with one or two functional PBRM1-alleles.

Conclusions: Intratumoral expression of genes involved in the HIF-VEGF-VEGFR-pro-angiogenic pathway, especially VEGFR2, is associated with favorable outcome on sunitinib in m-ccRCCs. Several genes involved in this pathway are upregulated in the molecular ccrcc2-subgroup, which usually responds well to sunitinib.

ARTICLE HISTORY
Received 14 July 2017
Accepted 2 October 2017

Background

Targeted therapies directed against the VEGF-pathway have significantly improved PFS of m-ccRCC-patients as compared to historical treatment options. Sunitinib is a tyrosine kinase receptor inhibitor (TKI) targeting VEGFR-1, -2 and -3. In a randomized controlled trial, sunitinib significantly prolonged PFS (11 versus 5 months, $p < .001$) as compared to interferon-α [1,2]. Median OS was 26.4 and 21.8 months ($p = .051$), respectively. Sunitinib is currently a standard treatment in m-ccRCC, but other VEGFR-TKIs like sorafenib, pazopanib and axitinib are also used in metastatic disease.

Although roughly 50% of RCC-patients receiving sunitinib experience an objective response and 43% disease stabilization, 7% will have progressive disease (PD) at first evaluation due to intrinsic resistance or due to other factors [1]. Moreover, almost all patients with clinical benefit will ultimately acquire resistance and relapse. The identification of biomarkers able to predict intrinsic resistance could guide

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Supplemental data for this article can be accessed here.

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alternative treatment decisions for these patients avoiding unnecessary costs and adverse events. The identification of biomarkers for acquired resistance could provide novel directions to develop therapies that block resistance pathways. Although different mechanisms of resistance have been proposed, reliable biomarkers predictive of sunitinib sensitivity or primary/secondary resistance are still lacking.

Since ccRCCs are characterized by hyper-vascularization and respond to inhibition of the HIF-VEGF-VEGFR-pathway, it seems plausible that the expression level of genes involved in this pathway could be predictive for outcome on targeted therapies like sunitinib. Indeed, four studies were recently published including between 40 and 67 m-ccRCC patients treated with sunitinib, that showed correlations between the expression of these genes and treatment outcome [3–6]. The primary objective of this study was to assess the impact of the expression of genes in the HIF-VEGF-VEGFR pro-angiogenic pathway on outcome in our own series of m-ccRCC patients treated with sunitinib.

Recently, we described a molecular classification of ccRCCs into four robust subgroups with distinctive characteristics on multi-omics analysis. Based on these molecular characteristics, we named our subtypes as follows: ccrcc1='c-myc-up' (33% of tumors), ccrcc2='classical' (41%), ccrcc3='normal-like' (11%) and ccrcc4='c-myc-up and immune-up' (15%). The ccrcc4-subgroup has an important immune-suppressive microenvironment. This classification was associated with outcome of m-ccRCC treated with sunitinib: ccrcc2- and ccrcc3-tumors were found to be highly sensitive to sunitinib, while ccrcc1-tumors have an intermediate and ccrcc4-tumors a poor outcome when treated with sunitinib [7]. Therefore, the secondary objectives of this research project were to study (A) the correlation between the expression of the genes in the HIF-VEGF-VEGFR-pathway and the new molecular classification of ccRCCs, (B) the correlation between the expression of the genes involved in the HIF-VEGF-VEGFR-pathway and in the immune-suppressive microenvironment and (C) the impact of the mutational status of the two most often mutated genes in ccRCC (VHL and PBRM1) on the expression of genes involved in the HIF-VEGF-VEGFR-pathway and in the immune-suppressive microenvironment.

Material and methods

Included patients

We included all tumors in the CIT-rein tumor bank with available extracted mRNA of good quality from patients treated in the metastatic setting with sunitinib as first-line targeted agent. The French-Belgian multicenter CIT-rein kidney tumor bank contains more than 400 frozen primary kidney tumor samples collected at nephrectomy in 20 academic hospitals. For inclusion in the study, patients had to have developed synchronous or metachronic metastases, received sunitinib (50 mg/day, four weeks-on/two weeks-off) as first-line treatment in the metastatic setting, and completed at least one 28-day cycle of sunitinib. Eligible patients could have received cytokines as systemic treatment for kidney tumors before starting sunitinib as a monotherapy, but they could not have received any other anti-VEGFR-TKI or mammalian target of rapamycin inhibitors before starting sunitinib. Most of the patients were treated in routine clinical practice, some were included in clinical trials. All patients had PD when sunitinib was started. Drug schedules and dose-reduction policy were left to the discretion of the treating physicians in accordance with current local practice guidelines. Follow-up chest/abdomen CT-scans were performed every 2 cycles of treatment. The study protocol was approved by the medical ethics review boards of all participating institutions, and signed consent for the use of the biologic material and the clinical data was obtained from all patients. Frozen biological material from deceased patients was used when prior agreement for such use had been given by the institutional review board.

Objectives of the study

The primary objective was to study the impact of the expression of the HIF-VEGF-VEGFR-pathway on outcome in m-ccRCC patients treated with sunitinib. Therefore, we focused on the following genes: HIF1A, HIF2A, PDGFRA, PDGFRB, VEGFA, VEGFC, VEGFR1, VEGFR2 and VEGFR3. Secondary objectives were to study (A) the expression of the HIF-VEGF-VEGFR-pathway in the molecular ccrcc-subgroups, (B) the correlation of the expression of genes in the HIF-VEGF-VEGFR-pathway and in the immune-suppressive microenvironment and (C) the impact of VHL and PBRM1-mutational status on the expression of genes involved in the HIF-VEGF-VEGFR-pathway and in the immune-suppressive microenvironment.

mRNA-expression and mutation analysis in the nephrectomy specimen

Using the High Capacity Transcription kit from Lifetechnologies, 500 ng of total RNA was reverse transcribed in a final volume of 50 µl. qRT-PCR reactions were performed using the high throughput BioMark qRT-PCR system (Fluidigm, South San Francisco, CA). Preamplifications of 6ng cDNA were performed using PreAmp Master Mix. A primer mix was prepared combining each primer used in the present study except the RNA actine-B (ACTB) probe. 1 µl of TaqMan PreAmp Master Mix was mixed with 1µl of the pooled primer mix and 3µl of diluted cDNA (2 ng/µl), and incubated at 95 °C for 2 min and 14 cycles of 95 °C for 15 sec and 60 °C for 4 min. Preamplified cDNA were diluted 1:5 in DNAse/RNAse free water before qRT-PCR. Gene expression mRNA was analyzed by qRT-PCR performed in Dynamic Array Integrated Fluidic Circuits on the Biomark HD system. We combined 3.5µl of Taqman Gene Expression Master Mix (Applied Biosystems, Foster City, CA, USA) and 0.35µl 20X GE Sample Loading Reagent with 3.15µl of diluted PreAmplified cDNA and transferred 5µl from this previous mix to the sample inlets of a 96.96 Dynamic Arrays. qRT-PCR was performed on the BioMark HD reader (50 °C for 2 min, 70 °C for 30 min,

25 °C for 10 min, 96.5 °C for 10 min, followed by 40 cycles of 96.5 °C for 15 sec and 60 °C for 1 min). Expression data (CT values) were acquired using the Fluidigm qRT-PCR Analysis software (4.1.3). Actine-B (ACTB) was used for the normalization of expression data. For the gene symbol, gene name and Applied Biosystems Assay ID-number for the genes studied, we refer to our previous publication [7].

In our previous publication [7], qRT-PCR was done with Low Density Array technology and not with Fluidigm. From our previous patients series, we decided to include all the tumors of which mRNA was still available for qRT-PCR with Fluidigm ($n = 91$). In some tumors ($n = 7$), no extracted mRNA nor frozen tissue was left for Fluidigm. All the new tumoral samples that arrived in the CIT-tumor bank since 2013 and that were eligible for this study ($n = 13$) were directly analyzed by Fluidigm. For the VHL- and PBRM1-mutational status, we used the data that was previously generated by direct sequencing (Primers and protocols available on request) for our previous study.

Statistical analysis

Based on the expression data of 35 genes of the predictor, the tumoral samples were classified into the ccrcc1-4 molecular classification as described in [7]. Differential analysis of gene expression according to the molecular subgroups was studied with ANOVA-models for multigroup comparison.

Best response on sunitinib was assessed by RECIST and documented as PR, SD or PD. Survival curves were calculated according to the Kaplan-Meier method and differences between curves were assessed using the log-rank test. PFS was calculated from the first sunitinib treatment till documented progression. Patients who were lost to follow-up or did not reach progression at the time of the study were treated as censored events. OS was calculated from the first sunitinib treatment. Patients who were lost to follow-up or alive at the time of the study were treated as censored events.

Fisher Exact test was used to assess the correlation between gene-expression levels and best response. For the correlation of gene expression levels and PFS and OS, we used Cox regression. Univariate models were performed on all the pathological and clinical covariates in order to find clinical criteria related to PFS or OS [8–11]. Corrected calcium was used when albumin levels were available. In other cases, calcium levels were used. Covariates showing a significant association to prognosis (logrank $p < .05$) at the univariate level were selected to be analyzed in multivariate models. Correlation of gene expression levels was tested with Pearson correlation. Correlations between VHL- and PBRM1-mutational status and gene expression were assessed by Student test. For correction for multiple testing we calculated false discovery rates (FDRs).

All time-to-event analyzes were done with Δ-CT normalized expression data. All other analyzes (such as ANOVA-analyzes to compare expression levels between different tumoral samples) were analyzed with $2 - \Delta\Delta CT$ normalized expression data.

Table 1. Patient characteristics.

Patient characteristics at diagnosis ($n = 104$)	
Age, median (range) (years)	59 (38–80)
Male	70.2% (73/104)
Renal cell carcinoma at diagnosis	
Fuhrman Grade 1–2	0.0% (0/104)
Sarcomatoid dedifferentiation ≥25%	9.8% (10/102)
ccrcc classification	
ccrcc1	31.0% (31/100)
ccrcc2	39.0% (39/100)
ccrcc3	15.0% (15/100)
ccrcc4	15.0% (15/100)
Treatment before start sunitinib	
Nephrectomy	100% (104/104)
Immunotherapy prior to sunitinib	28.2% (29/103)
Patient characteristics at start of sunitinib	
Time between diagnosis and start systemic therapy <12 months	64.4% (67/104)
Site of metastasis	
Bone	30.8% (32/104)
Liver	14.4% (15/104)
Lung	80.1% (84/104)
Brain	7.7% (8/104)
Median number of metastatic sites	2 (range 1–5)
Karnofsky performance status <80	10.6% (11/104)
Serum albumin ≤3.5 g/dl	17.9% (12/67)
Neutrophils ≥7800/mm^3	11.5% (12/104)
Platelets ≥450 000/mm^3	11.5% (12/104)
LDH >1.5 × upper limit of normal	8.2% (8/98)
Corrected serum calcium >10 mg/dl	5.6% (4/71)
Low hemoglobin (<13.5 g/dl for men, <11.5 g/dl for women)	41.7% (43/103)
IMDC score	
Good	16.0% (16/100)
Intermediate	66.0% (66/100)
Poor	18.0% (18/100)

CRP: C-reactive protein; LDH: lactate dehydrogenase; IMDC: International Metastatic Renal Cell Carcinoma Database Consortium.

The database was closed in October 2016. Statistical analyzes were conducted using GraphPad Prism 5 (GraphPad Software, La Jolla, California, USA), XLSTAT software (Addinsoft, Paris, France) and SAS software (version 9.4 of the SAS System for Windows, Cary, North Carolina, United States). For the interpretation of Pearson correlation, an *r*-value of 0.5–0.7 was considered as a weak association, and an *r*-value of >0.7 as a strong correlation.

Results

One hundred and four patients were included. Patient characteristics are listed in Table 1. Among these 104 patients, 91 were already included in the analysis that led to our previous publication [7]. Thirteen new patients were available for this study. The clinical data of the 91 previously included patients were updated from September 2013 (database closure for the previous publication) to October 2016 (database closure for the present publication).

Median PFS (mPFS) was 14 months and median OS (mOS) 27 months. PR-rate was 46%, while 13% of patients had PD as best response on sunitinib. Global outcome results as well as International Metastatic Renal Cell Carcinoma Database (IMDC) prognostic score were similar to those of the patients included in larger prospective trials. The results of univariate analysis for clinical-biochemical biomarkers associated PFS and OS are shown in supplemental Table 1. mPFS and mOS

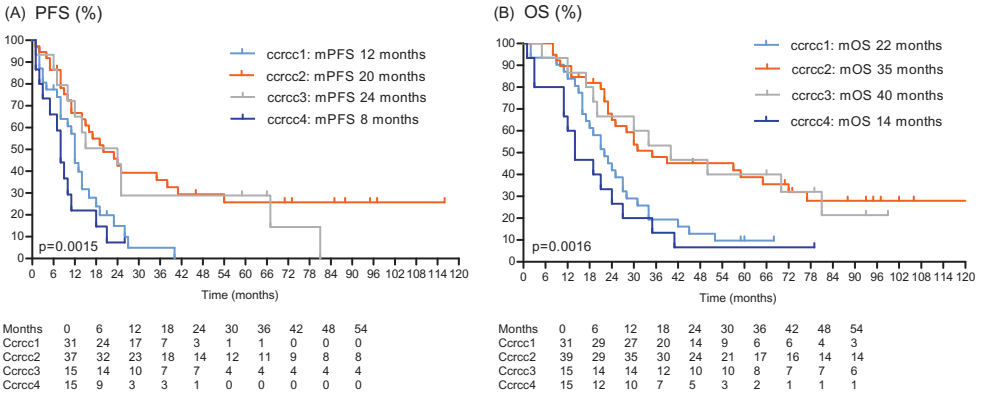

Figure 1. (A–B) Kaplan-Meier estimates of progression-free survival (A) and overall survival (B) correlated to the molecular ccrcc 1-4 classification.

according to the molecular ccrcc1-4 classification are reported in Figure 1. Patients with ccrcc2-tumors displayed long mPFS (20 months) and mOS (35 months). mPFS (24 months) and mOS (40 months) were similar in patients with ccrcc3-tumors. Patients with ccrcc1-tumors had an intermediate outcome (mPFS 12 months and mOS 22 months) and patients with ccrcc4-tumors the poorest outcome (mPFS 8 months and mOS 14 months). The tumors of all long responding patients belong to the subgroups ccrcc2 and ccrcc3.

mRNA-expression of genes associated with angiogenesis is correlated with good outcome. We analyzed 104 tumors of patients treated by sunitinib for the expression of 9 genes associated with angiogenesis. On univariate analysis, higher intratumoral PDGFRB- and VEGFR2-expression were associated with higher PR-rate. High HIF2A-, PDGFRB-, VEGFC-, VEGFR1-, VEGFR2- and VEGFR3-expression were associated with improved PFS, and high HIF2A-, VEGFR1-, VEGFR2- and VEGFR3-expression with improved OS. FDRs were between 0.05 and 0.16 (Table 2) (Figure 2). In contrast, lower VEGFA-, VEGFR1-, VEGFR2- and VEGFR3-expression were associated with more PD as best response (Table 2). On multivariate analysis, HIF2A-, PDGFRB-, VEGFC-, VEGFR1- and VEGFR2-expression were correlated with PFS and HIF1A-, HIF2A-, VEGFR1- and VEGFR2-expression with OS. Overall, VEGFR2-expression showed the strongest association with outcome, being significantly correlated with all outcome parameters. In order to see if VEGFR2-expression had a prognostic impact, we checked by Cox-regression the impact of VEGFR2-expression on time-to-metastasis, as defined by the laps of time between the initial diagnosis or nephrectomy and the diagnosis of metastases. No clear prognostic impact was observed (HR 0.84; 95% Confidence Interval 0.651–1.099; $p = .21$).

mRNA-expression of genes associated with angiogenesis correlate with the molecular ccrcc-classification (Table 3) (Figure 3). To integrate the genes related to angiogenesis in the molecular ccrcc-classification, we analyzed their expression in relation with the transcriptomic classification in four subgroups. Hundred tumors could be classified into the molecular classification. In four cases, the tumors could not clearly be classified. HIF2A, VEGFA, VEGFR1, VEGFR2 and VEGFR3 were higher expressed in the ccrcc2-subtype, previously shown to include tumors well responding in sunitinib, compared to their expression levels in the subgroups ccrcc1, -3 and -4. VEGFA, VEGFR1 and VEGFR2 were overexpressed in ccrcc2-tumors compared to normal kidney samples. HIF2A was downregulated in ccrcc4-tumors, the subgroup of tumors associated with poor prognosis in sunitinib treated patients. PDGFRA was downregulated in ccrcc1, -2 and -4 tumors compared to ccrcc3-tumors and normal kidney samples. ccrcc1-tumors had intermediate VEGFA-expression, whereas expression of the pro-angiogenic pathway was similar in the 'normal-like' ccrcc3-subgroup and in normal kidney tissue. Summarized, the ccrcc2-subgroup, known to be well responding on sunitinib, displayed the highest expression of HIF2A, VEGFA, VEGFR1, VEGFR2 and VEGFR3. On bivariate analysis (supplemental Table 2) of the molecular classification with VEGFR2-expression, the molecular classification was more strongly correlated to PFS and OS than VEGFR2-expression-levels.

Gene expression of tumoral angiogenesis did not correlate with immune-suppressive microenvironment. Several genes involved in angiogenesis (HIF2A, VEGFA, VEGFR1, VEGFR2, VEGFR3) were closely correlated with each other in terms of expression (supplemental Table 3). The same held true for several genes involved in the immune-suppressive microenvironment (CXCL9, CXCL13, IFNG, LAG3, PD1, CD3G). There was however no correlation, nor inverse correlation, between expression of genes involved in angiogenesis and in the immune-suppressive microenvironment. CX3CL1, classified among the immune genes, behaved like an angiogenesis gene. Nine of the genes involved in the immune-suppressive microenvironment (CD3G, CXCL13, CXCL9, IFNG, IL10, LAG3, PD1, PDL2 and TNF) were highly expressed in the ccrcc4-subgroup. Nevertheless, several of these genes (CD3G, CXCL9, LAG3 and PD1) were also highly expressed in the ccrcc2-subgroup compared to normal samples and ccrcc1- and ccrcc3-samples (Figure 4). On univariate analysis, we observed very few correlations between the expression of genes involved in

Table 2. Correlation between expression of genes involved in neo-angiogenesis and outcome (Progression-free survival and overall survival).

	PFS			OS				PD		PR	
Gene	HR (UV) HR (MV)	95%CI (UV) 95%CI (MV)	*p* (UV) *p* (MV) FDR	HR (UV) HR (MV1) HR (MV2)	95%CI (UV) 95%CI (MV1) 95%CI (MV2)	*p* (UV) *p* (MV1) *p* (MV2) FDR	Gene expression	%	*p* (UV)	%	*p* (UV)
HIF1A	1.123	0.924–1.365	.2441	**1.241**	**1.025–1.503**	**.0269**	<Median	11.8% (6/51)	.77	47.8% (22/46)	.76
				1.303	**1.062–1.598**	**.011**	>Median	13.7% (7/51)		44.7% (21/47)	
				1.263	**1.035–1.541**	**.021**					
						.15					
HIF2A	**1.237**	**1.028–1.490**	**.0246**	**1.262**	**1.034–1.541**	**.0222**	<Median	17.3% (9/52)	.16	37.5% (18/48)	.08
	1.225	**1.005–1.493**	**.044**	**1.244**	**1.012–1.528**	**.038**	>Median	8% (4/50)		55.5% (25/45)	
			.09	1.209	0.977–1.498	.081					
						.15					
PDGFRA	1.004	0.930–1.084	.9228	1.014	0.942–1.093	.7066	<Median	10.0% (5/50)	.41	48.9% (22/45)	.62
							>Median	15.4% (8/52)		43.8% (21/48)	
PDGFRB	**1.174**	**1.009–1.365**	**.0379**	1.136	0.967–1.335	.1208	<Median	18% (9/50)	.12	**36.2% (17/47)**	**.049**
	1.202	**1.029–1.405**	**.020**				>Median	7.7% (4/52)		**56.5% (26/46)**	
			.10								
VEGFA	1.094	0.958–1.249	.1847	1.070	0.933–1.227	.3307	<Median	**19.6% (10/51)**	**.04**	43.5% (20/46)	.53
							>Median	**5.8% (3/51)**		50.0% (23/46)	
VEGFC	**1.282**	**1.079–1.524**	**.0047**	1.182	0.990–1.411	.0647	<Median	17.6% (9/51)	.14	44.7% (21/47)	.76
	1.256	**1.067–1.478**	**.006**				>Median	7.8% (4/51)		47.8% (22/46)	
			.05								
VEGFR1	**1.148**	**1.013–1.300**	**.0304**	**1.148**	**1.009–1.307**	**.0362**	<Median	**21.6% (11/51)**	**.008**	37.5% (18/48)	.08
	1.145	**1.003–1.306**	**.045**	1.137	**0.992–1.304**	.065	>Median	**3.9% (2/51)**		55.6% (25/45)	
			.096	**1.149**	**1.003–1.315**	**.045**					
						.16					
VEGFR2	**1.183**	**1.038–1.347**	**.0114**	**1.201**	**1.053–1.371**	**.0066**	<Median	**21.6% (11/51)**	**.008**	**35.4% (17/48)**	**.03**
	1.166	**1.018–1.336**	**.027**	**1.189**	**1.036–1.364**	**.014**	>Median	**3.9% (2/51)**		**57.8% (26/45)**	
			.06	**1.189**	**1.033–1.370**	**.016**					
						.14					
VEGFR3	**1.237**	**1.050–1.458**	**.0109**	**1.220**	**1.031–1.444**	**.0208**	<Median	**19.6% (10/51)**	**.04**	42.6% (20/47)	.47
	1.181	0.989–1.412	.066	1.170	0.971–1.408	.098	>Median	**5.8% (3/51)**		50.0% (23/46)	
			.06	1.191	0.996–1.424	.055					
						.15					

HR: hazard ratio; UV: univariate analysis; MV: multivariate analysis; PFS: progression free survival; OS: overall survival; PD: progressive disease; PR: partial response; CI: confidence interval; CI: Confidence Interval; FDR: false discovery rates.

HR >(<)1 means higher (lower) risk for higher level of mRNA expression. The *p*-values for PFS and OS were calculated with the Mantel-Cox test. The *p*-values for comparison of early PD and PR rates were calculated with a Fisher Exact test. Significant results are in bold.

The multivariate analysis for PFS was done with all the parameters associated with PFS on univariate analysis with a *p*-value <.2: sarcomatoid dedifferentiation >25% of tumor volume, neutrophil count, platelet count, bone metastasis, time from initial diagnosis to systemic treatment, Karnofsky performance status.

A first multivariate analysis (MV1) for OS was done with all the parameters associated with OS on univariate analysis with a *p*-value <0.2: sarcomatoid dedifferentiation >25% of tumor volume, neutrophil count, bone metastasis, liver metastasis, Karnofsky performance status (see Supplemental Table 4). A second multivariate analysis (MV2) for OS was done with the IMDC prognostic score (Good/Intermediate versus Poor).

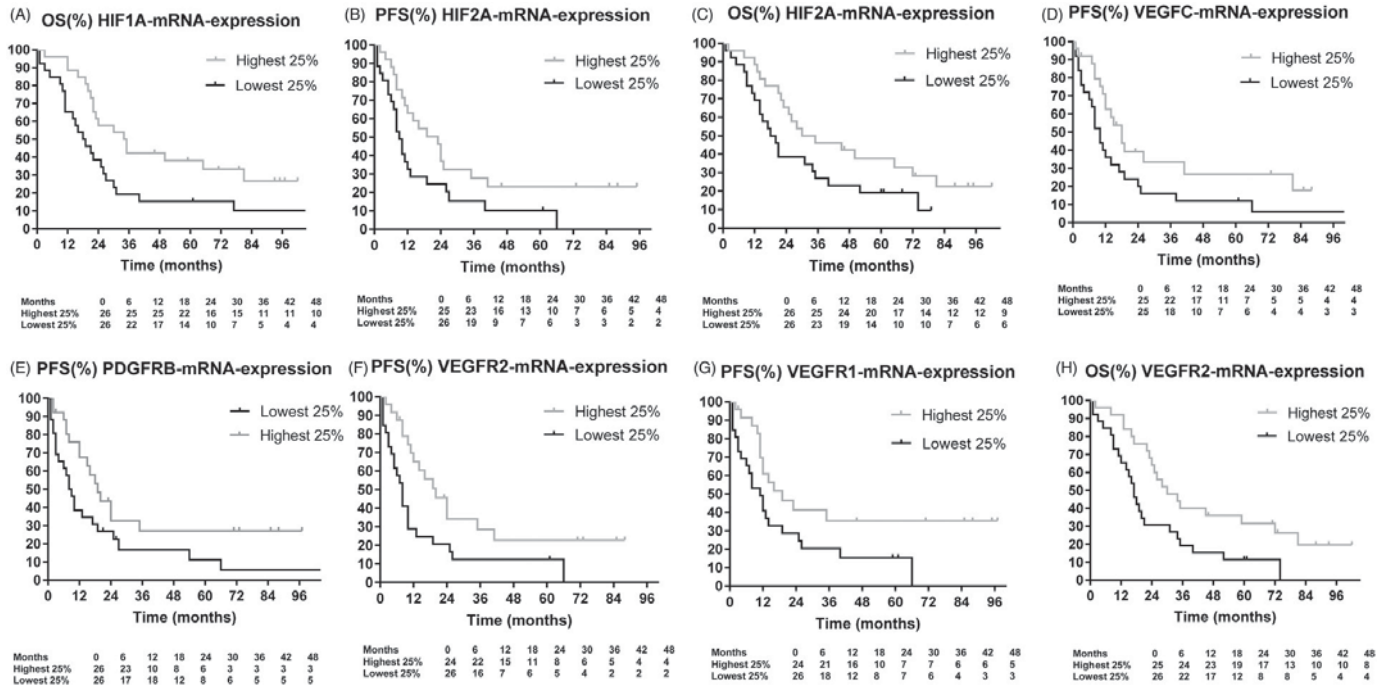

Figure 2. (A–H) Kaplan-Meier estimates: impact of mRNA-expression-levels of genes involved in the angiogenetic pathway and outcome on sunitinib (progression-free survival (PFS) and overall survival (OS)). The figures below present PFS and OS curves for patients with 25% lowest versus 25% highest gene expression levels for the genes that were withheld after multivariate analysis.

Table 3. Median mRNA-expression compared to molecular subgroups.

GENE	ccrcc1 ($n = 31$)	ccrcc2 ($n = 39$)	ccrcc3 ($n = 15$)	ccrcc4 ($n = 15$)	$p(*)$	Normal kidney samples	$p(**)$
Genes involved in the vegf-proangiogenetic pathway							
HIF1A	0.5483	0.5408	0.9463	0.5390	.0924	1.193	–
HIF2A	0.5904	**1.126**	0.6886	0.3215	**<.0001**	0.9099	.3
PDGFRA	0.1496	0.1173	1.015	**0.2810**	**.002**	1.073	–
PDGFRB	0.9202	0.9790	1.123	0.5455	.17	0.9026	–
VEGFA	1.463	**3.067**	0.6770	0.7977	**<.0001**	0.6077	**<.0001**
VEGFC	0.6733	1.120	0.7105	0.8097	.26	0.8873	–
VEGFR1	0.8520	**2.130**	0.5008	0.5855	**<.0001**	0.6254	**.0002**
VEGFR2	0.3164	**1.254**	0.4010	0.3196	**<.0001**	0.5790	**.0476**
VEGFR3	0.5487	**0.9970**	0.5250	0.3291	**<.0001**	0.7582	.16
Genes involved in immune-suppressive micro-environment							
CD3G	1.480	2.996	0.8590	**3.483**	**.0002**	0.9368	–
CSF1R	1.776	2.607	1.897	3.238	.14	0.9842	–
CXCL13	2.466	2.638	0.9635	**12.14**	**.0017**	1.137	–
CXCL9	0.5539	1.490	0.6006	**2.374**	**.0014**	1.403	–
CX3CL1	0.4044	**1.738**	0.8809	0.2483	**<.0001**	0.8961	–
IFNG	0.4510	0.5824	0.2601	**1.942**	**.03**	0.7468	–
IL10	1.478	1.462	**0.4748**	1.663	**.0019**	0.9034	–
IL12A	0.5016	0.7605	0.5443	0.4934	.08	0.8972	–
LAG3	0.7752	1.504	0.7977	**3.423**	**.0046**	0.9574	–
PD1	1.078	1.836	1.250	**2.840**	**.0322**	1.074	–
PDL1	0.6074	0.9879	0.6884	1.021	.29	1.126	–
PDL2	0.5648	1.169	0.8617	**1.742**	**<.0001**	1.060	–
TNF	0.6209	0.8434	1.148	**1.429**	**.0023**	1.101	–

The $p(*)$-value was calculated with ANOVA and compares mRNA-expression between the four molecular subgroups ccrcc1-4, but not with mRNA-expression in the normal kidney samples. The $p(**)$-value was calculated with an unpaired *t*-test and compares mRNA-expression between molecular subgroup ccrcc2 and normal kidney samples. Significant results are in bold.

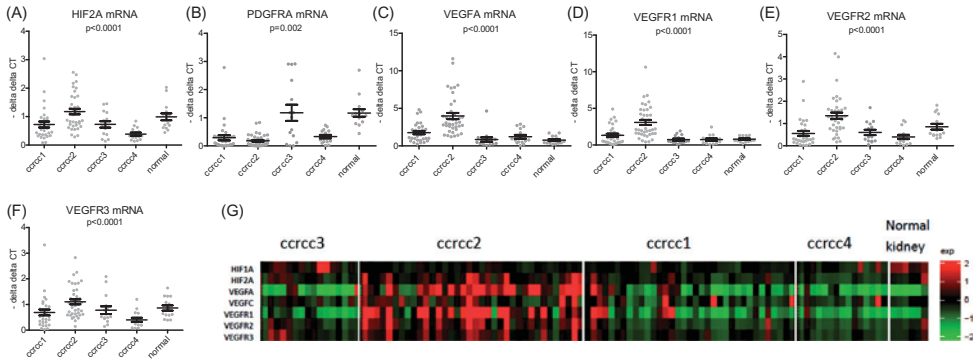

Figure 3. (A–F) Boxplots showing the expression levels of genes in the different molecular subgroups. The *p*-values, calculated with ANOVA, describe the differences between the four subgroups ccrcc1-4. (G) Heat map showing the expression of genes involved in angiogenesis in the molecular subgroups ccrcc1-4 and in the normal kidney samples.

the immune-suppressive microenvironment and outcome on sunitinib. On multivariate-analysis, only IL12A-expression was correlated with PFS (Supplemental Table 4).

Impact of VHL- and PBRM1- mutational status on the expression of genes involved in angiogenesis and in the immune-suppressive microenvironment. VHL- and PBRM1-mutational statuses were available in 93 patients. VHL-promoter hypermethylation was associated with lower CX3CL1- and higher CXCL13- and IL12A-expression. The presence of a VHL-mutation was associated with higher VEGFA- and CX3CL1-expression and lower HIF1A-expression. In tumors with a bi-allelic VHL-inactivation, VEGFA- and CX3CL1-expression were higher, compared to tumors with one or two functional VHL-alleles, but CXCL13- and CXCL9-expression were lower. The presence of a PBRM1-mutation was associated with higher VEGFR1- and CX3CL1-expression. In tumors with a bi-allelic PBRM1-inactivation, CX3CL1-, HIF2A-, VEGFA-, VEGFR1- and VEGFR2-expression were higher, compared to tumors with one or two functional PBRM1-alleles (Figure 5) (Supplemental Table 5).

Discussion

After twelve years of routine clinical use of VEGFR-TKIs in m-ccRCC, the selection of patients who might benefit from this treatment remains a challenge. We present the largest m-ccRCC patient series published until today studying the predictive value of the expression of genes involved in the

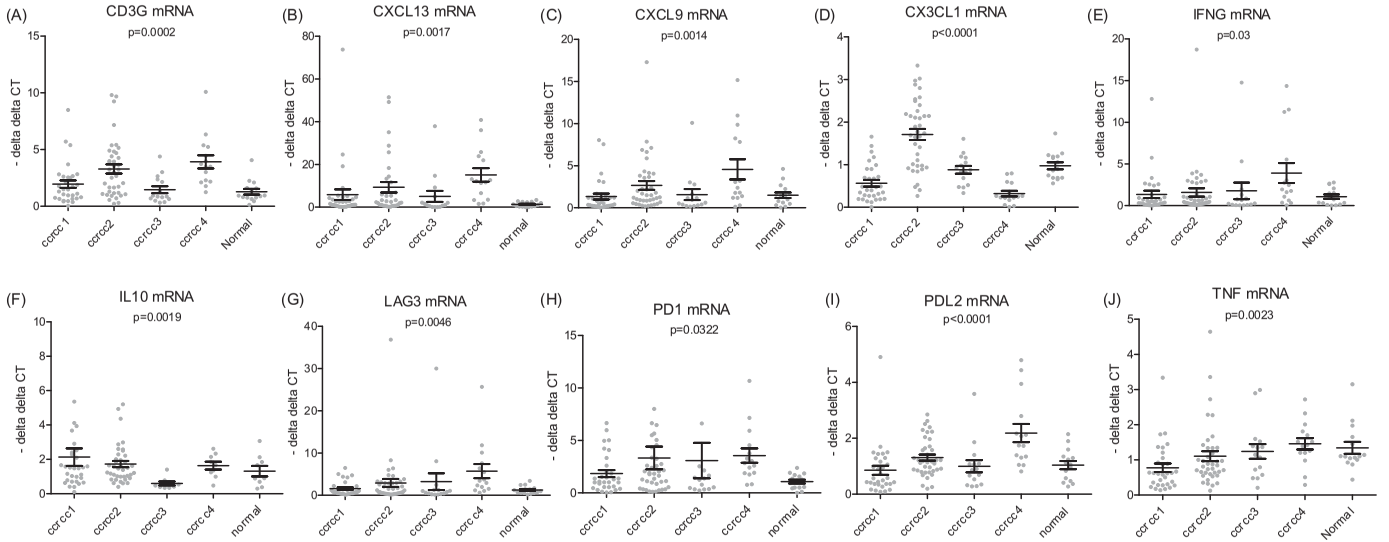

Figure 4. (A–J) Boxplots showing the expression levels of genes involved in the immune-suppressive microenvironment in the different molecular subgroups. The *p*-values, calculated with ANOVA, describe the differences between the four subgroups ccrcc1-4.

Table 4. Correlation of findings in literature.

Gene	Expression	Effect on outcome on sunitinib in present study ($n = 104$)	Dornbusch et al. [4] ($n = 42$)	Garcia-Donas et al. [5] ($n = 67$)	Terakawa et al. [6] ($n = 40$)	You et al. [7] ($n = 65$)
HIF1A	High	Longer OS	Higher RR, longer PFS			
HIF2A	High	Longer PFS and OS		Higher RR, longer OS		
PDGFRB	High	Higher RR, longer PFS	Higher RR	Higher RR		
VEGFA	High	Less patients with early PD		Shorter OS (possibly an opposite finding)		
VEGFR1	High	Less number of patients with early PD. Longer PFS. Longer OS	Higher RR, longer OS			
VEGFR2	High	Less patients with early PD, higher RR Longer PFS and OS	Higher RR		Longer PFS	Higher RR
VEGFR3	High	Less patients with early PD Longer PFS and OS (UV only)	Longer PFS	Longer PFS		

PFS: progression free survival; OS: overall survival; PD: progressive disease; RR: response rate; HIF: hypoxia inducible factor; VEGFR: vascular endothelial growth factor receptor; VEGF: vascular endothelial growth factor; PDGFR: platelet derived growth factor receptor; UV: univariate.

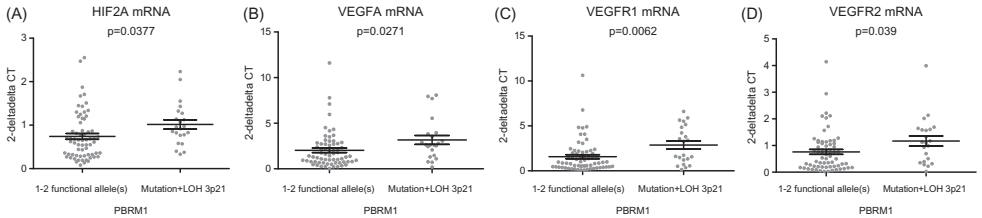

Figure 5. (A–D) Boxplot with the correlation between the number of functional PBRM1-alleles and the expression of genes involved in angiogenesis. *p*-values were calculated with an unpaired *t*-test. The boxplots show an upregulation of the pro-angiogenic HIF2A-VEGFA-VEGFR1-VEGFR2-pathway in tumors without a functional PBRM1-copy.

pro-angiogenic HIF-VEGF-VEGFR-pathway in a homogenous series of m-ccRCC patients treated with sunitinib as first-line VEGFR-TKI. Moreover, we correlated gene expression data with VHL- and PBRM1-mutational status and with the transcriptomic ccrcc-classification. Our findings underscore the important role of VEGFR2 as a target of sunitinib and the predictive value of its expression levels.

Our primary objective was to study the impact of the intratumoral expression of genes involved in the HIF-VEGF-VEGFR-pathway on outcome in m-ccRCC patients treated with sunitinib. On a series of 104 m-ccRCC patients, we observed that higher intratumoral HIF1A-, HIF2A-, PDGFRB-, VEGFC-, VEGFR1 and VEGFR2-expression were correlated with better outcome on sunitinib. VEGFR2-expression showed the strongest association with outcome, as it was correlated with PFS, OS, early-PD and PR-rate. In absence of a control group of patients treated with placebo, it is difficult to make the difference between a prognostic or a predictive impact. In fact, a longer PFS and OS and a lower rate of PD as best response, can be a consequence of an indolent disease. Nevertheless, two findings point towards a predictive value of VEGFR2-expression. First, on Cox-regression, we did not find a correlation between (higher) VEGFR2-levels and (longer) time-to-metastasis. Moreover, tumor shrinkage (as measured for instance by PR-rate), can only be induced by the therapy and will never be a spontaneous evolution of the tumor.

FDRs of our findings were not very low (between 0.05 and 0.16). However, the probability of false positive findings is rather low, given the fact that previously, smaller studies of gene-expression quantified by immunohistochemistry have shown similar results (Table 4). In 42 patients treated with sunitinib, HIF1A- and VEGFR3-staining were associated with longer PFS and VEGFR1-staining with longer OS. HIF1A-, PDGFRB-, VEGFR1- and VEGFR2-staining were higher in responders compared to non-responders [3]. In a series of 67 patients, VEGFR3-staining was associated with longer PFS, HIF2A-staining with longer OS and HIF2A- and PDGFRB-staining with better response to sunitinib. However, higher VEGFA-expression was associated with shorter OS in this patient series. This could be in opposition with our findings (we have found less patients with early PD if VEGFA-expression was high), although it is not excluded that patients with tumors with high VEGFA-expression can have more clinical benefit from sunitinib (i.e. more disease stabilization or tumor shrinkage), but that the overall prognosis in these patients remains poor [4]. In a series of 40 patients, VEGFR2-staining was associated with improved PFS on sunitinib [5]. Finally, in a series of 65 patients, VEGFR2-staining was associated with a better PR-rate [6]. All these studies are coherent in the sense that increased HIF1A-, HIF2A-, PDGFRB-, VEGFR1-, VEGFR2- and VEGFR3-expression are associated with better outcome on sunitinib.

mRNA-expression of genes associated with angiogenesis correlate with the molecular ccrcc-classification. Compared to the other molecular subgroups, the ccrcc2-subgroup, known to be sensitive to sunitinib, with a higher PR-rate and longer PFS and OS [7], displayed the highest HIF2A-, VEGFA-, VEGFR1-, VEGFR2- and VEGFR3-expression. VEGFA, VEGFR1 and VEGFR2 were overexpressed in ccrcc2-tumors compared to normal kidney tissue. As a consequence, the efficacy of sunitinib in ccrcc2-tumors can likely be explained in part by the higher expression of the specific targets of sunitinib (VEGFR1, -2 and -3) in these tumors.

Other research groups have also described correlations between the expression of genes in the HIF-VEGF-VEGFR-pathway and transcriptomic classifications of ccRCCs. Brannon et al. divided the tumors in a ccA- and ccB-subgroup. The ccA-subgroup, that corresponds to our ccrcc2-subgroup and has a better OS after nephrectomy, displayed VEGFR1-, VEGFR2- and VEGFR3-upregulation. In the ccB-subgroup, these genes were downregulated [12]. Sato et al. also identified two major clusters, ccA and ccB. The ccA-subgroup displayed an upregulation of VEGFR1, VEGFR2, VEGFR3 and VEGFA, while these genes were downregulated in the ccB-subgroup [13]. However, these studies did not report on the impact on outcome on VEGFR-TKIs in the metastatic setting.

Correlation between the expression of genes involved in the HIF-VEGF-VEGFR-pathway and in the immune-suppressive microenvironment. In the poor responding ccrcc4-subgroups, genes involved in the immune-suppressive microenvironment are highly expressed, and genes involved in neo-angiogenesis poorly expressed. However, in the total patient series, the expression of these two groups of genes was not correlated nor inversely correlated. Neither did we find any correlation between the expression of the immune-response genes and outcome on sunitinib. A possible explanation is the fact that some of the genes involved in the immune-suppressive microenvironment are also highly expressed in a subgroup of patients of the ccrcc2-subgroup. Moreover, in the ccrcc1-subgroup, both genes involved in the immune-suppressive microenvironment and in angiogenesis are poorly expressed. Therefore, we hypothesize that not only patients with ccrcc4-tumors, characterized by an important immune-infiltrate and immune-suppressive microenvironment, could be good candidates for treatment with immune checkpoint-inhibitors, but also patients with ccrcc2-tumors, or at least a yet to be defined subgroup of ccrcc2-patients [7]. Patients with ccrcc1-tumors, with intermediate outcome on sunitinib, are probably not good candidates for treatment with immune checkpoint inhibitors.

Correlation between VHL- and PBRM1-mutational status and the expression of genes involved in the HIF-VEGF-VEGFR-pathway and in the immune-suppressive microenvironment. Our principal finding is a higher expression of genes involved in angiogenesis (HIF2A, VEGFA, VEGFR1 and VEGFR2) in tumors with a bi-allelic PBRM1-inactivation, compared to tumors with one or two functional PBRM1-alleles. PBRM1-mutations are present in around 40% of all ccRCCs [14]. PBRM1-mutated tumors are known to be of a better prognosis than BAP1-mutated tumors (15% of cases and usually mutually exclusive with PBRM1-mutations) [15]. In coherence with our findings, Sato et al. showed that PBRM1-mutated tumors were enriched for upregulated expression of the gene set with a hypoxia signature [13]. As for VHL, we have found that tumors with biallelic VHL-inactivation have higher VEGFA-expression. However, we could not confirm the findings of Messai et al., showing on a series of 32 samples, a correlation between HIF2A- and PDL1-expression and higher PDL1-expression in tumors with bi-allelic VHL-inactivation [16].

Weaknesses of our study. Although we have found differences in expression of genes involved in the HIF-VEGFA-VEGFR-pathway in the different subgroups, as well as a correlation between the expression of these genes and outcome, we could not show any association with microvascular density in the tumors as we did not test this.

Conclusions

Intratumoral expression of genes involved in angiogenesis in ccRCC is associated with favorable outcome on sunitinib in the metastatic setting. As VEGFR2-expression is associated with mPFS, mOS, PR-rate and PD as best response, VEGFR2-expression might be a predictive biomarker for sunitinib efficacy in m-ccRCC.

Several genes involved in the HIF-VEGF-VEGFR-pro-angiogenic pathway are upregulated in the molecular ccrcc2-subgroup. Nevertheless, on bivariate analysis, the molecular ccrcc-classification is a better predictor of mPFS and mOS than VEGFR2-expression levels.

Disclosure statement

Benoit Beuselinck received honoraria from Amgen, Pfizer, Janssen and Bayer. Benoit Beuselinck is an investigator of the EudraCT: 2011-006085-40/MetaSun trial supported by Pfizer. Stéphane Oudard received honoraria from Novartis, Pfizer, Roche, BMS and Bayer. Patrick Schöffski received institutional honoraria from Novartis and GSK for participation in educational activities in the context or renal cell carcinoma and research support. Jean Jacques Patard is a consultant and principal investigator in Pfizer trials. The other authors have no conflicts of interest to declare.

Funding

Benoit Beuselinck received a grant from Fondation Martine Midy (Paris, France) and Fonds voor Wetenschappelijk Onderzoek Vlaanderen (Belgium) [grant number 1701412N]. Evelyne Lerut received fundings from Fonds voor Wetenschappelijk Onderzoek Vlaanderen (Belgium) [grant number G.0715.09] and Stichting tegen Kanker. Annelies Verbiest received a grant from Kom op tegen Kanker.

References

[1] Motzer RJ, Hutson TE, Tomczak P, et al. Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med. 2007;356:115–124.

[2] Motzer RJ, Hutson TE, Tomczak P, et al. Overall survival and updated results for sunitinib compared with interferon alfa in patients with metastatic renal cell carcinoma. JCO. 2009;27:3584–3590.

[3] Dornbusch J, Zacharis A, Meinhardt M, et al. Analyses of potential predictive markers and survival data for a response to sunitinib

in patients with metastatic renal cell carcinoma. PloS One. 2013;8:e76386.
[4] Garcia-Donas J, Leandro-Garcia LJ, Gonzalez Del Alba A, et al. Prospective study assessing hypoxia-related proteins as markers for the outcome of treatment with sunitinib in advanced clear-cell renal cell carcinoma. Ann Oncol. 2013;24:2409–2414.
[5] Terakawa T, Miyake H, Kusuda Y, et al. Expression level of vascular endothelial growth factor receptor-2 in radical nephrectomy specimens as a prognostic predictor in patients with metastatic renal cell carcinoma treated with sunitinib. Urol Oncol. 2013; 31:493–498.
[6] You D, Song SH, Cho YM, et al. Predictive role of tissue-based molecular markers in patients treated with sunitinib for metastatic renal cell carcinoma. World J Urol. 2015;33:111–118.
[7] Beuselinck B, Job S, Becht E, et al. Molecular subtypes of clear cell renal cell carcinoma are associated with sunitinib response in the metastatic setting. Clin Cancer Res. 2015;21:1329–1339.
[8] Heng DY, Xie W, Regan MM, et al. Prognostic factors for overall survival in patients with metastatic renal cell carcinoma treated with vascular endothelial growth factor-targeted agents: results from a large, multicenter study. JCO. 2009;27:5794–5799.
[9] Patil S, Figlin RA, Hutson TE, et al. Prognostic factors for progression-free and overall survival with sunitinib targeted therapy and with cytokine as first-line therapy in patients with metastatic renal cell carcinoma. Ann Oncol. 2011;22:295–300.
[10] Beuselinck B, Oudard S, Rixe O, et al. Negative impact of bone metastasis on outcome in clear-cell renal cell carcinoma treated with sunitinib. Ann Oncol. 2011;22:794–800.
[11] Beuselinck B, Lerut E, Wolter P, et al. Sarcomatoid dedifferentiation in metastatic clear cell renal cell carcinoma and outcome on treatment with anti-vascular endothelial growth factor receptor tyrosine kinase inhibitors: a retrospective analysis. Clin Genitourinary Cancer. 2014;12:e205–e214.
[12] Brannon AR, Reddy A, Seiler M, et al. Molecular stratification of clear cell renal cell carcinoma by consensus clustering reveals distinct subtypes and survival patterns. Genes Cancer. 2010;1: 152–163.
[13] Sato Y, Yoshizato T, Shiraishi Y, et al. Integrated molecular analysis of clear-cell renal cell carcinoma. Nat Genet. 2013;45: 860–867.
[14] Varela I, Tarpey P, Raine K, et al. Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. Nature. 2011;469:539–542.
[15] Pena-Llopis S, Vega-Rubin-de-Celis S, Liao A, et al. BAP1 loss defines a new class of renal cell carcinoma. Nat Genet. 2012;44: 751–759.
[16] Messai Y, Gad S, Noman MZ, et al. Renal cell carcinoma programmed death-ligand 1, a new direct target of hypoxia-inducible factor-2 alpha, is regulated by von Hippel-Lindau gene mutation status. Eur Urol. 2015;70:623–632.

Chapter 8

Clear-cell renal cell carcinoma: molecular characterization of IMDC risk groups and sarcomatoid tumors

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Clinical genitourinary cancer. 2019;17(5):e981-e994.

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Clear-cell Renal Cell Carcinoma: Molecular Characterization of IMDC Risk Groups and Sarcomatoid Tumors

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Abstract

In advanced clear-cell renal cell carcinoma, the Checkmate214 study showed superior outcomes with ipilimumab-nivolumab in International Metastatic RCC Database Consortium intermediate-/poor-risk patients and with sunitinib in good-risk patients. We demonstrate how underlying molecular subtypes and angiogenic gene expression can explain these differences and can characterize the heterogeneous International Metastatic RCC Database Consortium intermediate-risk group.

Introduction: Recent trials have suggested predictive biomarkers in advanced clear-cell renal cell carcinoma (accRCC): International Metastatic RCC Database Consortium (IMDC) good risk or angiogenic gene signature for sunitinib and IMDC intermediate/poor risk for ipilimumab-nivolumab and T-effector cell signature or sarcomatoid dedifferentiation for atezolizumab-bevacizumab. We hypothesized that earlier described molecular subtypes, ccrcc1 to ccrcc4, could provide similar information as a single generic biomarker and molecularly characterize the heterogeneous intermediate-risk group. **Patients and Methods:** Patients with accRCC treated with systemic therapies were included. We assessed associations between the 5 biomarkers and their impact on progression-free survival (PFS) and response rate (RR) on first-line sunitinib or pazopanib. The cutoff percentage of sarcomatoid dedifferentiation with optimal discriminative value was determined. **Results:** In total, 430 patients were included (163 with molecular data). The molecular ccrcc2 subtype identified tumors with higher angiogenic gene expression across IMDC risk groups: prevalence was high in IMDC good risk and low in IMDC poor risk ($P < .001$). Molecular subtype, IMDC, and angiogenic gene expression had comparable C-indices to predict PFS and RR (range, 60%-66%). The ccrcc2 subtype and angiogenic gene expression were positive predictors of PFS in IMDC intermediate-risk patients ($P = .006$; $P = .04$). Immune signature did not differ between IMDC groups, but was strongly correlated with molecular subtype ($P = .8$ and $P = .0007$). A cutoff value of 25% sarcomatoid differentiation discriminated tumors with distinct molecular characteristics and therapeutic sensitivity. **Conclusion:** In accRCC, molecular subtypes can explain differences in IMDC risk group, expression of angiogenesis and immune response genes, and sarcomatoid dedifferentiation. They

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Submitted: Mar 7, 2019; Revised: Apr 24, 2019; Accepted: May 20, 2019; Epub: May 25, 2019

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https://doi.org/10.1016/j.clgc.2019.05.009

can identify molecularly different patient populations within the heterogeneous IMDC intermediate group and select patients for systemic therapies.

Clinical Genitourinary Cancer, Vol. 17, No. 5, e981-94 © 2019 Elsevier Inc. All rights reserved.
Keywords: Angiogenesis, Biomarker, Immune checkpoint inhibitor, Molecular subtype, Sunitinib

Introduction

Clear-cell renal cell carcinomas (ccRCCs) are hallmarked by hypervascularity on the one hand and their capacity to elicit an immune response on the other hand.[1] Advanced disease (accRCC) may therefore be treated with angiogenesis inhibitors, immune checkpoint inhibitors (ICPIs), or a combination strategy.[2-7] Outcomes on different therapies are, however, highly variable, and in the absence of predictive biomarkers, the optimal way of patient selection is unclear.

Only very recently have some advances in the field of predictive biomarkers been made. The phase III Checkmate214 trial (NCT0221749) compared ipilimumab-nivolumab with the angiogenesis inhibitor sunitinib in first-line therapy, after stratification for International Metastatic RCC Database Consortium (IMDC) risk group.[7,8] Ipilimumab-nivolumab showed efficacy across all IMDC risk groups and was superior over sunitinib in intermediate-/poor-risk patients. It is therefore recommended in this population. Sunitinib, on the other hand, yielded favorable outcomes in good-risk patients, and sunitinib or pazopanib are recommended for these patients.[2] However, the IMDC risk group has important limitations as a biomarker. It is based on clinical risk factors that may change over time, and the majority of patients are intermediate-risk, a group that is known to be very heterogeneous in terms of prognosis and treatment outcome.[8-10]

The phase III IMmotion151 trial (NCT02420821) compared atezolizumab-bevacizumab with sunitinib in first-line treatment and validated predictive gene signatures.[4,11] A T-effector cell (T_{eff}) signature and programmed death ligand 1 (PD-L1) expression were associated with favorable outcomes on atezolizumab-bevacizumab and were similar across risk groups. An angiogenic gene signature was associated with favorable outcomes on sunitinib, and was higher in good-risk patients. Patients with sarcomatoid tumors responded poorly to sunitinib and had lower angiogenic signatures, but higher T_{eff}-signatures and PD-L1 expression. Yet, although these signatures are very promising, they have been developed specifically for sunitinib and atezolizumab-bevacizumab. Therefore, they only partly reflect intrinsic tumor biology and cannot be extrapolated to other treatments.

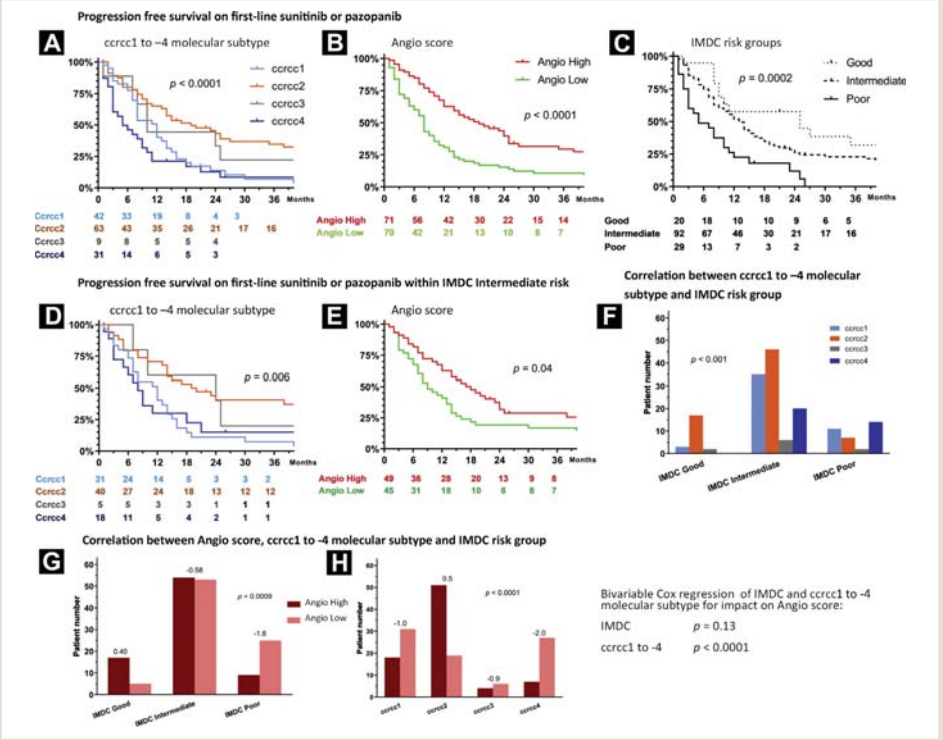
Another predictive marker that has been described in accRCC are 4 molecular subtypes, ccrcc1 to ccrcc4.[12] These subtypes were established based on unsupervised clustering of whole genome mRNA-expression data, and therefore reflect intrinsic differences in

Table 1 Correlation of Different Biomarkers With Outcome on First-line Sunitinib or Pazopanib

	N	PFS, mos	*P*	C-index (95% CI)	RR, %	*P*	C-index (95% CI)	OS, mos	*P*
IMDC	145								
Good	21	25	.0002	0.60 (0.55-0.65)	63	.01	0.62 (0.54-0.70)	72	<.0001
Intermediate	95	13			46			24	
Poor	29	5			19			12	
ccrcc1-4 subtype	145								
ccrcc1	42	12	<.0001	0.60 (0.57-0.68)	34	.01	0.66 (0.57-0.75)	23	.0002
ccrcc2	63	19			57			31	
ccrcc3	9	10			63			50	
ccrcc4	31	5			25			11	
Angio score	145								
High	74	19	<.0001	0.64 (0.58-0.69)	56	.01	0.60 (0.50-0.70)	28	.005
Low	71	8			33			19	
Sarcomatoid	366								
<25%	344	11	<.0001		46	.001		24	<.0001
≥25%	22	3			10			8	

The discriminative power of predictors of RR and PFS was quantified with Harrells C-index (Harrell et al,[18] JAMA 1982).
Favorable IMDC risk group, ccrcc2 or ccrcc3 molecular subtype, High Angio score, and < 25% sarcomatoid dedifferentation are predictors of favorable outcome.
Abbreviations: CI = Confidence interval; IMDC = International Metastatic RCC Database Consortium; N = number; OS = overall survival; PFS = median progression-free survival; RR = response rate.

Figure 1 **Impact on PFS and Mutual Correlations Between IMDC Risk Group, ccrcc1 to ccrcc4 Molecular Subtype, Angio Score, and Immune Score. A-C, ccrcc2 or ccrcc3 Subtype, High Angio Score, and More Favorable IMDC Risk Group Were Correlated With Longer PFS on First-line Sunitinib or Pazopanib. D, E, Within the IMDC Intermediate Risk Group, Molecular Subtype and Angio Score Were Predictive for PFS. F, The IMDC Good Risk Group Was Enriched for the ccrcc2 Subtype, and the IMDC Poor Risk Group Was Enriched for the ccrcc4 Subtype. G-I, The ccrcc2 Subtype Identified Tumors With Higher Angio Scores Across IMDC Subgroups. J-M, Immune Score did Not Differ Between IMDC Risk Groups, but was Different Between the ccrcc1 to ccrcc4 Molecular Subtypes. The ccrcc4 Subtype and a Subgroup of the ccrcc2 Subtype had High Immune Scores. The Median Angio and Immune Scores are Reported in Graphs G, H, J, and K. The Expression of Individual Angiogenesis and Immune Genes According to IMDC Risk Group and Molecular Subtype Is Shown in Supplemental Tables 2 and 3 (in the Online Version)**

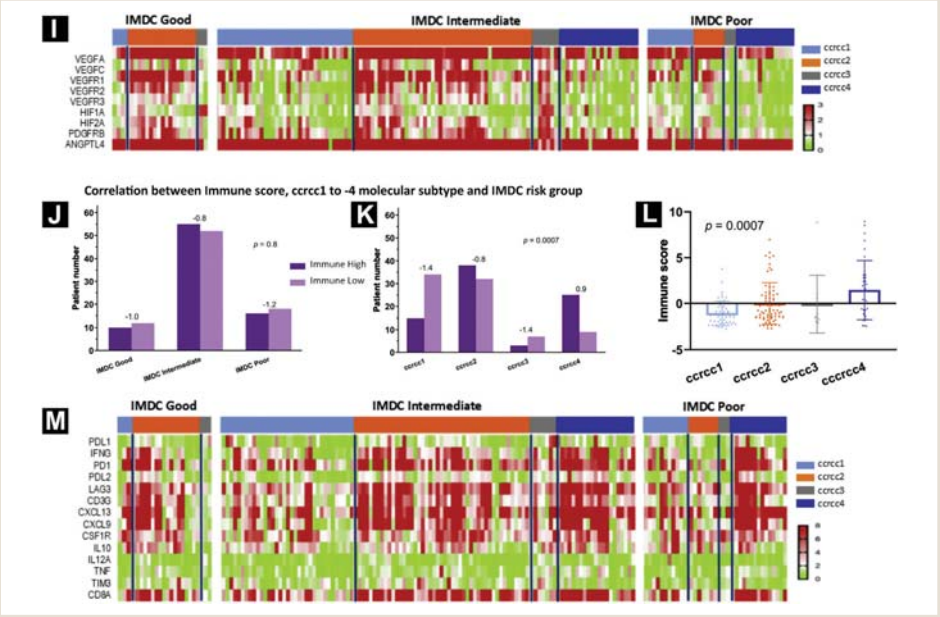


Abbreviations: IMDC = International Metastatic RCC Database Consortium; PFS = progression-free survival.

tumor biology. They also differ in terms of immune infiltrate, cytogenetic anomalies, sarcomatoid dedifferentiation, methylation, and mutation profile in the tumor. The subtypes are prognostic for overall survival (OS) after nephrectomy and after metastasectomy with curative intent.[12,13] The prognostic value of similar subtypes has been described by others in the setting of nephrectomy with curative intent.[14,15] However, the ccrcc1 to ccrcc4 subtypes are also predictive for outcome on first-line sunitinib or pazopanib.[12,16] ccrcc2 tumors are characterized by pro-angiogenic gene expression and ccrcc3 tumors by a normal-kidney-like gene expression pattern.[12,17] Both ccrcc2 and ccrcc3 have a good prognosis and favorable outcomes with sunitinib or pazopanib. A subset of ccrcc2 tumors also exhibit an active immune response. The 2 other subtypes, ccrcc1 and ccrcc4, are marked by upregulation of MYC-targets. ccrcc1 tumors have an intermediate prognosis and outcome on sunitinib or pazopanib and an immune cold phenotype, with little T_{eff}-cell infiltration. On the other hand, ccrcc4 tumors have a very poor prognosis and outcome on sunitinib or pazopanib, but are marked by a strongly inflamed, yet exhausted immune phenotype and are often sarcomatoid.

We hypothesized that the ccrcc1 to ccrcc4 molecular subtypes can explain differences in IMDC risk group, angiogenic signature, T_{eff}-signature, and sarcomatoid dedifferentiation, thus providing a maximum of information as a single biomarker. Therefore, the

Figure 1 Continued



major aims of this study were to assess molecular differences between tumors in function of IMDC risk group and sarcomatoid dedifferentiation and correlate these with outcome on first-line sunitinib or pazopanib. A secondary aim was to characterize the heterogeneous IMDC intermediate-risk group on a molecular level.

Patients and Methods

Patients and Tissue

The CIT-rein database was searched for patients of whom the clinical and molecular data addressed in this study were available. The CIT-rein database is a French-Belgian multicenter database that includes patients with accRCC treated with systemic therapy or metastasectomy, containing over 600 cases. Patients are included after informed consent, and both clinical data and tumoral tissue are collected in a prospective way. Primary tumor tissue is collected at time of nephrectomy, before the start of any systemic therapy. Clear-cell histology and sarcomatoid dedifferentiation are confirmed by an expert uro-pathologist (N.R.L., E.L., V.V.). Outcome is systematically measured with Response Evaluation Criteria In Solid Tumors (RECIST) by one of the authors (B.B.). Drugs are dosed according to their manufacturer's label, with dose reductions as needed. Timing of assessments is at the discretion of the treating physician, usually once every 3 months. The study was approved by the ethics committees of the partaking centers.

Molecular Profiling

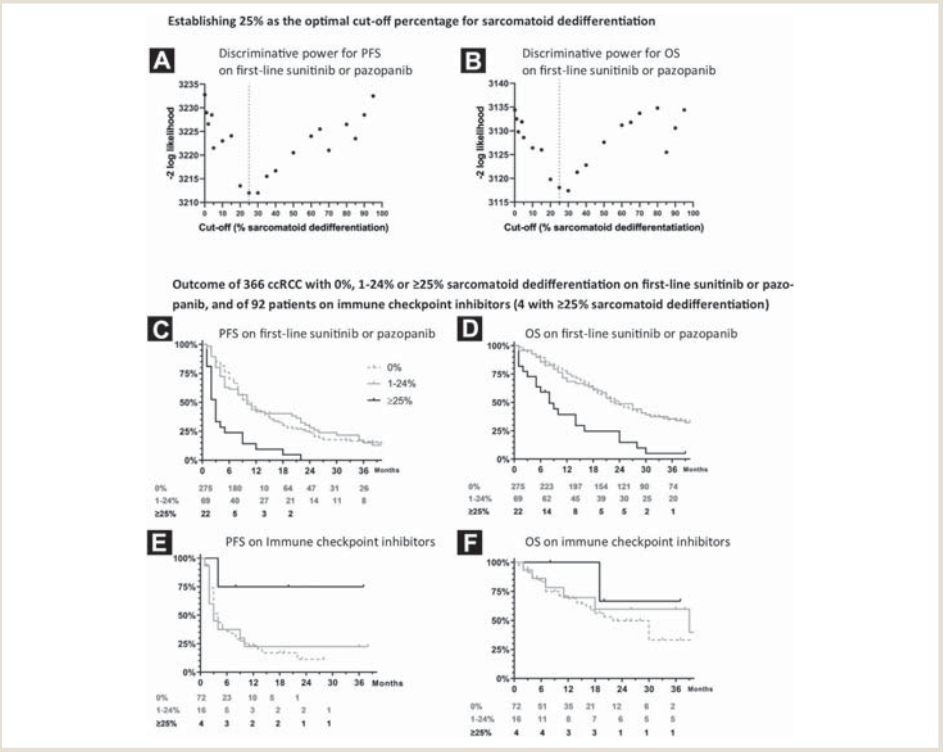
In patients from CIT-rein from whom fresh frozen primary tumor is available, expression of 98 genes is routinely determined with a custom panel (Fluidigm). This panel contains, among others, genes involved in angiogenesis and immune response. Molecular subtype is determined with an established 35-gene classifier algorithm as described previously.[12]

Angiogenic gene expression was quantified using 9 available genes involved in vascular endothelial growth factor (VEGF)-dependent angiogenesis. Fold changes were summarized as one "Angio score," using principal component analysis. Samples were also labeled as "Angio high" or "Angio low," depending on whether their Angio score was above or equal/below the median. Similarly, an "Immune score" was calculated using 13 available genes involved in immune response. As all genes are part of a routine panel, these scores were not trained as predictors for treatment outcome, nor is the Immune score an optimal signature of T_{eff}-cells.

Statistical Analysis

The optimal cutoff value for sarcomatoid dedifferentiation in order to predict progression-free survival (PFS) and overall survival (OS) was determined after considering all possible cutoffs in a Cox proportional hazard model, and selecting the dichomatization leading to the highest likelihood.

Figure 2 Sarcomatoid ccRCC. A, B, A Percentage of 25% Sarcomatoid Dedifferentiation Had the Optimal Discriminative Power to Predict Outcome On First-line Sunitinib or Pazopanib. C-F, ccRCC With ≥ 25% Sarcomatoid Dedifferentiation Had Very Poor Outcomes on First-line Sunitinib or Pazopanib, Compared With 0% to 24%. In a Small Case Series, Outcomes on ICPIs Were Promising. Survival Curves of Patients With 0% or 1% to 24% Sarcomatoid Dedifferentiation Were Overlapping. G-J, ccRCC With ≥ 25% Sarcomatoid Dedifferentiation Were Molecularly Different From ccRCC With 0% to 24% Sarcomatoid Dedifferentiation. They Were Mostly IMDC Poor Risk, ccrcc4 Molecular Subtype, and Angio Low. The Median Angio and Immune Scores are Reported in Graphs I and J



Abbreviations: ccRCC = clear-cell renal cell carcinoma; ICPIs = immune checkpoint Inhibitors; IMDC = International RCC Database Consortium; OS = overall survival; PFS = progression-free survival.

To assess the predictive impact of different biomarkers, we focused on their impact on response rate (RR) and PFS, as OS is heavily influenced by prognostic factors and subsequent therapies. The association of predictors with RR was analyzed using logistic regression. Their association with PFS was analyzed with Cox proportional hazards regression.

The Kruskal-Wallis and Mann-Whitney *U* tests were used to compare expression of individual genes between IMDC groups, molecular subtypes, and sarcomatoid yes/no. The associations between IMDC risk groups, molecular subtype, Angio high/low, Immune high/low, and sarcomatoid yes/no were tested with the Fisher exact test.

Analyses were done using SAS 9.4, GraphPad Prism 7.2, and XLStat 2018.7.

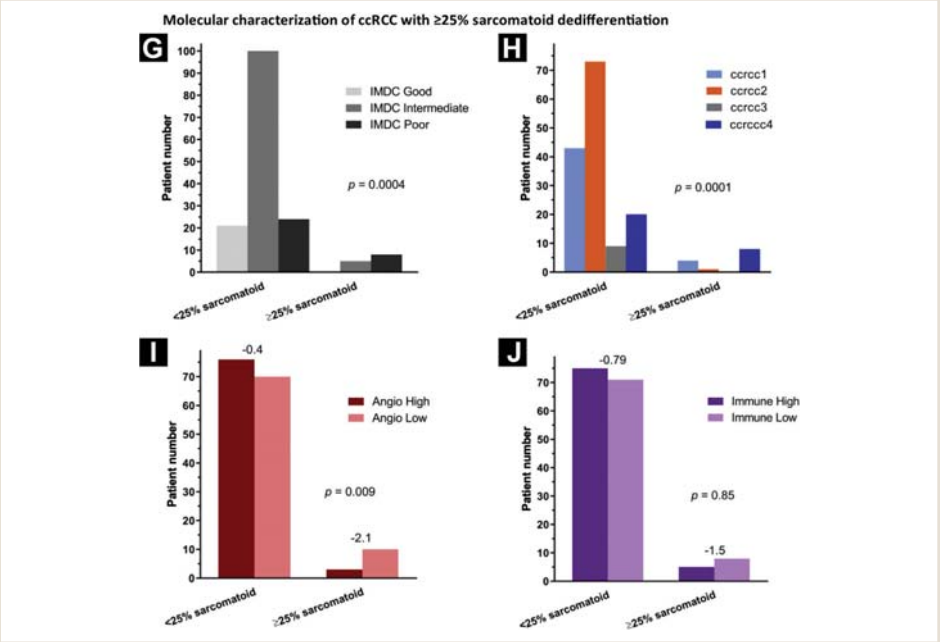
Results

Molecular Differences Between IMDC Risk Groups

Molecular data and IMDC risk group were available for 163 patients, 145 of whom were treated with first-line sunitinib or pazopanib (see Supplemental Table 1 in the online version).

A more favorable IMDC risk group, a ccrcc2 or ccrcc3 molecular subtype, and higher Angio score were all positive predictors for longer PFS and higher RR on first-line sunitinib or pazopanib (Table 1; Figure 1). The discriminative value of these predictors,

Figure 2 continued



quantified by their C-index, was comparable. The combination of markers did not result in a meaningful increase in discriminative value.

IMDC risk group, molecular subtype, and Angio score were strongly associated with each other (Figure 1). The pro-angiogenic ccrcc2 molecular subtype could identify patients with higher Angio scores across IMDC risk groups. The IMDC good-risk group was enriched for the ccrcc2 subtype and Angio-high tumors (both 77%; $P < .001$). The IMDC poor-risk group was enriched for the unfavorable ccrcc4 subtype (41%) and Angio-low tumors (74%). The IMDC intermediate-risk group was heterogeneous, with a normal distribution of the molecular subtypes (46% ccrcc2) and 50% Angio-high and -low tumors. The expression of specific targets of sunitinib and pazopanib (VEGFR1, -2, -3) was highest in IMDC good-risk patients and lowest in IMDC poor-risk patients (see Supplemental Table 2 in the online version). Within the intermediate-risk group, the ccrcc2 subtype could identify tumors with higher angiogenic gene expression. Indeed, upon bivariable analysis, the differences in Angio score between IMDC risk groups became non-significant after correction for molecular subtype. Within the IMDC intermediate-risk group, both molecular subtype and Angio score were predictive for PFS.

The expression of genes related to immune response, summarized by the Immune score, did not differ between IMDC risk groups ($P = .7$). Contrarily, the differences in the Immune score between molecular subtypes were highly significant ($P = .0007$). ccrcc4 tumors, and a subgroup of ccrcc2 tumors, were marked by high Immune scores (Figure 1; Supplemental Table 3 in the online version). Thus, molecular subtypes could identify tumors with high immune gene expression across IMDC risk groups.

Sarcomatoid ccRCC

The impact of sarcomatoid dedifferentiation was tested in 366 patients treated with first-line sunitinib or pazopanib (see Supplemental Table 4 in the online version). The optimal cutoff associated with PFS and OS was ≥ 25% sarcomatoid dedifferentiation. PFS and OS curves for patients with 0% or 1% to 24% sarcomatoid dedifferentiation were overlapping (Figure 2). On multivariable analysis, sarcomatoid dedifferentiation ≥ 25% was independently associated with PFS (hazard ratio, 2.9; 95% confidence interval, 1.8-4.5) and OS (hazard ratio, 2.3; 95% confidence interval, 1.4-3.7) (see Supplemental Tables 5 and 6 in the online version). For tumors with 0% to 24% versus ≥ 25% sarcomatoid dedifferentiation, RR was 46% versus 10% ($P = .001$), and PFS was 11 months versus 3 months ($P < .0001$) (Table 1).

Molecular differences and IMDC risk group were tested in 163 patients. Compared with tumors with 0% to 24% sarcomatoid dedifferentiation, tumors with ≥ 25% dedifferentiation were

typically IMDC poor risk (62%; $P = .0004$) and ccrcc4 subtype (62%; $P = .0001$). They had lower Angio scores ($P = .009$) but no difference in Immune scores ($P = .35$). Tumors with 0% versus 1% to 24% sarcomatoid dedifferentiation had similar IMDC risk groups and Angio scores (see Supplemental Tables 7 and 8 in the online version).

In an exploratory analysis, we assessed outcomes of 92 patients treated with ICPI, mostly in the second- or a consecutive line. The 4 tumors with $\geq$ 25% sarcomatoid dedifferentiation all responded, whereas RR in 88 others was only 16%. PFS curves for 0% and 1% to 24% sarcomatoid dedifferentiation were overlapping and inferior to those for $\geq$ 25%.

Pooling of IMDC Intermediate and Poor Risk Groups

As phase III trials have typically reported outcomes of pooled IMDC intermediate-/poor- versus good-risk patients, we explicitly assessed differences between the intermediate- and poor-risk groups.[3,7] Outcomes on first-line sunitinib or pazopanib, distribution of molecular subtypes, Angio score, and proportion of $\geq$ 25% sarcomatoid tumors were all significantly different between the intermediate- and poor-risk groups. Moreover, when we repeated all analyses while comparing IMDC good risk with pooled intermediate/poor risk, the results were not as solid as when considering the 3 groups separately (see Supplemental Tables 9 and 10 in the online version).

Discussion

There is still no optimal way of selecting patients with accRCC for treatment with angiogenesis inhibitors, ICPIs, or the upcoming combinations. Recent trials have suggested IMDC risk group, angiogenic or T_{eff} gene expression, and sarcomatoid dedifferentiation as possible biomarkers, but they all have important limitations.[4,7,11] In this study, we assessed the correlations between these markers and investigated whether the information they provide could be summarized by a single biomarker, the molecular ccrcc1 to ccrcc4 subtypes. A secondary aim was to characterize the large but heterogeneous IMDC intermediate-risk group on a molecular level.

IMDC good-risk patients had higher angiogenic gene expression, associated with response to sunitinib. This was explained by the overrepresentation of the pro-angiogenic ccrcc2 subtype in the IMDC good-risk group. The predictive value for outcome on sunitinib or pazopanib was similar for molecular subtypes, IMDC risk groups, and angiogenic signature. But more importantly, the ccrcc2 subtype could identify tumors with an angiogenic signature within the IMDC intermediate-risk group and was predictive for outcome on sunitinib and pazopanib in this population. As the majority of patients with accRCC are IMDC intermediate risk, molecular subtypes could contribute to personalized therapy decisions in this population.

When looking at immune gene expression, we found no differences between IMDC risk groups. This is in accordance with responses to ICPIs that were observed across all risk groups in the Checkmate214 trial and with similar T_{eff}-signatures observed in the IMmotion151 trial.[4,7] However, molecular subtypes showed marked differences in immune gene expression, with ccrcc4 and a subgroup of ccrcc2 tumors displaying immune activation. Therefore, the lack of difference in Immune score between IMDC risk groups hides more pronounced differences in immune phenotype. IMDC good-risk patients mainly have ccrcc2 tumors, a subgroup of which have an activated immune response. IMDC poor-risk patients mainly have either immune-exhausted ccrcc4 tumors or immune-cold ccrcc1 tumors.[12]

We also found a marked difference between IMDC intermediate- and poor-risk patients on a molecular level. Any comparison of these risk groups should therefore be performed preferentially without pooling IMDC intermediate- and poor-risk patients.

Based on these data, ccrcc1 to ccrcc4 molecular subtypes incorporate in a single marker the information provided by IMDC risk group and expression of angiogenesis and immune response genes. The subtypes were not developed as a marker for a specific therapy and might therefore retain their value in different therapeutic settings and for the development of novel therapeutic options. For example, in the setting of metastasectomy, ccrcc1 and ccrcc4 tumors are at risk for early relapse.[13] Upon treatment with sunitinib or pazopanib, ccrcc2 and ccrcc3 have favorable outcomes. ccrcc4 tumors can be expected to be the best candidates for ICPIs. As earlier research showed that ccrcc4 tumors exhibit an immune suppressive microenvironment with high levels of myeloid inflammation, they will probably benefit especially from a combination of ICPIs and angiogenesis inhibitor, where the latter suppresses myeloid inflammation.[12] For the immune-cold ccrcc1 tumors, immune sensitizing therapies should be investigated.

Sarcomatoid dedifferentiation has been associated with poor prognosis and outcome on sunitinib or pazopanib.[19,20] We showed on a large patient series that a cutoff percentage of 25% sarcomatoid dedifferentiation separated tumors with distinct therapeutic sensitivity and molecular characteristics. Tumors with $\geq$ 25% sarcomatoid dedifferentiation had poor outcomes on first-line sunitinib or pazopanib, low angiogenic gene expression, and were typically IMDC poor risk and ccrcc4 subtype. Contrarily, outcomes and molecular characteristics were similar for tumors with 0% versus 1% to 24% sarcomatoid dedifferentiation. Interestingly, 4 sarcomatoid tumors treated with ICPIs at our institution all responded, whereas outcomes were poorer in 88 patients with 0% to 24% sarcomatoid dedifferentiation.

This study has its limitations. Most importantly, the CIT database only contains a limited number of patients treated with ICPIs, to assess the hypothesized predictive value of molecular subtypes in this context. Two ongoing prospective trials are evaluating this question (Nivoren NCT03013335; Bionikk NCT02960906). Secondly, molecular subtype is currently determined on fresh frozen tissue. To facilitate the classification procedure, a major future aim is to validate the classifier on paraffin-embedded tissue.

In conclusion, molecular subtypes in accRCC can explain differences in IMDC risk group, expression of angiogenic and immune response genes, and sarcomatoid dedifferentation. Favorable outcomes on first-line sunitinib or pazopanib are predicted by IMDC good risk group, ccrcc2 molecular subtype, and angiogenic gene expression. Molecular subtypes could select patients within the IMDC intermediate risk group for angiogenesis inhibitors, but validation on paraffin-embedded tissue is needed before potential clinical implementation. As molecular subtypes also identify tumors with different immune profiles, where IMDC risk groups do not, they have a potential use in the setting of ICPIs and combination therapies. For sarcomatoid dedifferentiaton, a cutoff value of 25% separates tumors with distinct prognosis and molecular characteristics. Tumors with $\geq$ 25% sarcomatoid dedifferentiation have low angiogenic gene expression and are

typically IMDC poor risk and ccrcc4 subtype. They have poor outcomes on sunitinib or pazopanib, but ICPIs seem a promising option.

Clinical Practice Points

- Metastatic RCC can be treated with angiogenesis inhibitors, ICPIs, or combination therapy. There is currently no reliable way of patient selection. IMDC good risk is associated with better outcome on sunitinib, whereas ICPIs are effective across IMDC risk groups. The majority of patients are intermediate risk, with very heterogeneous outcomes on all therapies.
- ccRCC can be divided into 4 molecular subtypes, ccrcc1 to ccrcc4. Here, we show that the molecular ccrcc2 subtype defines highly angiogenic tumors, explaining the different therapeutic sensitivity of IMDC risk groups. It is enriched in IMDC good-risk patients and can identify patients that are likely to respond to sunitinib or pazopanib within the intermediate-risk group. Immune signature is different between the 4 molecular subtypes, but not between IMDC risk groups.
- Sarcomatoid RCC have poor prognosis and poor outcome on sunitinib and pazopanib. Here, we show that ccRCC with $\geq$ 25% sarcomatoid component are molecularly different from those with 0% to 24%, whereas there were no differences beween those with 0% and 1% to 24%. Sarcomatoid ccRCC ($\geq$25%) were enriched for the ccrcc4 subtype and had low angiogenic signature and poor outcome on sunitinib or pazopanib. In a small series treated with ICPIs, results were promising.
- Molecular subtypes therefore provide a maximum of information as a single generic biomarker. They are associated with angiogenic and immune signature, IMDC risk group, sarcomatoid dedifferentiation, and outcome on suntinib and pazopanib. They may be used for patient selection in the future, especially within the IMDC intermediate-risk group.

Acknowledgments

A. Verbiest, B. Beuselinck, and E. Lerut have received grants from FWO (Research Foundation-Flanders). Annelies Verbiest has received a grant from "Kom op tegen Kanker" (Stand up to Cancer), the Flemish cancer society.

Disclosure

B. Beuselinck reports consultancy fees from Amgen, Ipsen, Pfizer, and Novartis and research grants from Bristol-Myers Squibb and Ipsen. The remaining authors have stated that they have no conflicts of interest.

Supplemental Data

Supplemental tables accompanying this article can be found in the online version at https://doi.org/10.1016/j.clgc.2019.05.009.

References

1. Hsieh JJ, Purdue MP, Signoretti S, et al. Renal cell carcinoma. *Nat Rev Dis Primers* 2017; 3:17009.
2. Powles T, Albiges L, Staehler M, et al. Updated European Association of Urology Guidelines recommendations for the treatment of first-line metastatic clear cell renal cancer. *Eur Urol* 2018; 73:5.
3. Motzer RJ, Penkov K, Haanen J, et al. Avelumab plus axitinib versus sunitinib for advanced renal-cell carcinoma. *N Engl J Med* 2019; 380:1103-15.
4. Rini B, Huseni M, Atkins M, et al. Molecular correlates differentiate response to atezolizumab (atezo)+ bevacizumab (bev) vs sunitinib (sun): Results from a phase III study (IMmotion151) in untreated metastatic renal cell carcinoma (mRCC). *ESMO Annu Congress* 2018:LBA31.
5. Rini BI, Plimack ER, Stus V, et al, KEYNOTE-426 Investigators. Pembrolizumab plus axitinib versus sunitinib for advanced renal-cell carcinoma. *N Engl J Med* 2019; 380:1116-27.
6. National Institutes of Health; US National Library of Medicine. Lenvatinib/everolimus or lenvatinib/pembrolizumab versus sunitinib alone as treatment of advanced renal cell carcinoma (CLEAR), Available at: https://clinicaltrialsgov/ct2/show/NCT02811861. Accessed: December 18, 2018.
7. Motzer RJ, Tannir NM, McDermott DF, et al, CheckMate 214 Investigators. Nivolumab plus ipilimumab versus sunitinib in advanced renal-cell carcinoma. *N Engl J Med* 2018; 378:1277-90.
8. Heng DY, Xie W, Regan MM, et al. Prognostic factors for overall survival in patients with metastatic renal cell carcinoma treated with vascular endothelial growth factor-targeted agents: results from a large, multicenter study. *J Clin Oncol* 2009; 27:5794-9.
9. Rini BI, Hutson TE, Figlin RA, et al. Sunitinib in patients with metastatic renal cell carcinoma: clinical outcome according to International Metastatic Renal Cell Carcinoma Database Consortium Risk Group. *Clin Genitourin Cancer* 2018; 16:298-304.
10. Beuselinck B, Vano YA, Oudard S, et al. Prognostic impact of baseline serum C-reactive protein in patients with metastatic renal cell carcinoma (RCC) treated with sunitinib. *Clin Genitourin Cancer* 2014; 114:81-9.
11. McDermott DF, Huseni MA, Atkins MB, et al. Clinical activity and molecular correlates of response to atezolizumab alone or in combination with bevacizumab versus sunitinib in renal cell carcinoma. *Nat Med* 2018; 24:749-57.
12. Beuselinck B, Job S, Becht E, et al. Molecular subtypes of clear cell renal cell carcinoma are associated with sunitinib response in the metastatic setting. *Clin Cancer Res* 2015; 21:1329-39.
13. Verbiest A, Couchy G, Job S, et al. Molecular subtypes of clear-cell renal cell carcinoma are prognostic for outcome after complete metastasectomy. *Eur Urol* 2018; 74:474-80.
14. The Cancer Genome Atlas Network. Comprehensive molecular characterization of clear cell renal cell carcinoma. *Nature* 2013; 499:43-9.
15. Brooks SA, Brannon AR, Parker JS, et al. ClearCode34: a prognostic risk predictor for localized clear cell renal cell carcinoma. *Eur Urol* 2014; 66:77-84.
16. Verbiest A, Couchy G, Job S, et al. Molecular subtypes of clear cell renal cell carcinoma are associated with outcome during pazopanib therapy in the metastatic setting. *Clin Genitourin Cancer* 2018; 16:e605-12.
17. Beuselinck B, Verbiest A, Couchy G, et al. Pro-angiogenic gene expression is associated with better outcome on sunitinib in metastatic clear-cell renal cell carcinoma. *Act Oncol* 2018; 57:498-508.
18. Harrell FE, Califf RM, Pryor DB, Lee KL, Rosati RA. Evaluating the yield of medical tests. *JAMA* 1982; 247:2543-6.
19. Beuselinck B, Lerut E, Wolter P, et al. Sarcomatoid dedifferentiation in metastatic clear cell renal cell carcinoma and outcome on treatment with anti–vascular endothelial growth factor receptor tyrosine kinase inhibitors: a retrospective analysis. *Clin Genitourin Cancer* 2014; 12:e205-14.
20. Golshayan AR, George S, Heng DY, et al. Metastatic sarcomatoid renal cell carcinoma treated with vascular endothelial growth factor–targeted therapy. *J Clin Oncol* 2009; 27:235-41.

Supplemental Table 1	Characteristics of 163 Patients of Whom IMDC Risk Group and Molecular Data Were Available
Characteristic	**N**
Total	163
Male	113
First-line suntinib or pazopanib	
Sunitinib	110
Pazopanib	35
Median age at start of first-line, y	63
IMDC risk group	
Good	22
Intermediate	107
Poor	34
ccrcc1 to -4 subtype	
ccrcc1	49
ccrcc2	70
ccrcc3	10
ccrcc4	34
Angio score	
High	80
Low	83
T stage	
T1	28
T2	19
T3	109
T4	4
Synchronous metastases	86
Fuhrman grade	
1	0
2	6
3	53
4	104
Sarcomatoid dedifferentiation	
<25%	146
≥25%	13

Abbreviation: IMDC = International Metastatic RCC Database Consortium.

Supplemental Tables 2 and 3. Differences in Angiogenic and Immune Gene Expression Between IMDC Risk Groups and ccrcc1 to ccrcc4 Molecular Subtypes.

Supplemental Table 2 Angiogenic Gene Expression is Higher for IMDC Good > Intermediate > Poor Risk and ccrcc2 > ccrcc3 > ccrcc1 > ccrcc4 Subtypes

	IMDC Risk Group				ccrcc1 to -4 Molecular Subtype				
Gene	Good	Intermediate	Poor	*P* Value	ccrcc1	ccrcc2	ccrcc3	ccrcc4	*P* Value
VEGFA	7.8	5.0	2.8	<.001	3.2	8.5	2.0	2.5	<.0001
VEGFC	0.86	0.92	0.55	.3	0.83	0.92	1.2	0.52	.04
VEGFR1	3.3	1.8	0.10	<.001	1.0	3.3	0.95	0.52	<.0001
VEGFR2	1.6	0.87	0.38	<.001	0.38	1.6	0.61	0.29	<.0001
VEGFR3	1.1	0.83	0.40	<.001	0.64	1.2	0.68	0.36	<.0001
HIF1A	0.81	0.63	0.64	.3	0.81	0.64	2.2	0.50	.008
HIF2A	1.2	0.98	0.49	<.001	0.67	1.3	0.95	0.39	<.0001
PDGFRB	1.5	1.4	1.1	.07	1.4	1.6	1.6	0.89	.008
ANGPTL4	88	70	35	.05	72	103	2.6	27	<.0001

The largest difference is in expression of VEGFR2, the most important target of sunitinib and pazopanib (+321% for IMDC Good vs. Poor and +440% for ccrcc2 vs. ccrcc4).
Abbreviation: IMDC = International Metastatic RCC Database Consortium.

Supplemental Table 3 Immune Gene Expression is Not Different Between IMDC Risk Groups, but Significantly Higher for ccrcc4 > ccrccc2 > ccrcc1 and ccrcc3

	IMDC Risk Group				ccrcc1-4 Molecular Subtype				
Gene	Good	Intermediate	Poor	*P* Value	ccrcc1	ccrcc2	ccrcc3	ccrcc4	*P* Value
PD-L1	1.0	1.0	1.2	.9	0.51	1.2	1.5	1.2	.008
IFN-G	2.4	2.8	2.0	.5	1.4	2.9	1.2	6.3	.0007
PD1	3.4	6.1	5.3	.5	3.8	5.7	3.4	11.1	.001
PD-L2	1.3	1.6	1.3	.9	0.78	1.8	1.4	2.2	<.0001
LAG3	3.6	5.1	5.5	.5	3.6	5.0	3.2	11	<.0001
CD3G	3.2	3.3	2.2	.3	2.0	3.7	1.9	4.0	.0004
CXCL13	4.3	8.4	9.5	.3	4.9	8.2	2.5	34	<.0001
CXCL9	2.7	3.3	2.6	.9	1.9	4.3	1.8	10	<.0001
CSF1R	3.4	3.3	4.1	.8	2.6	3.8	2.9	3.7	.5
IL10	2.7	2.4	2.3	1	2.4	2.5	2.7	2.4	.9
IL12a	0.55	0.53	0.36	.3	0.35	0.51	1.0	0.57	.05
TNF	0.88	0.86	0.94	1	0.58	0.87	1.4	1.4	.0006
TIM3	1.3	1.1	0.94	.3	0.97	1.3	1.5	0.83	.02
CD8a	4.8	6.3	4.3	.5	3.6	7.4	2.1	10	<.0001

Median fold change is reported and compared with the Kruskal Wallis test.
Abbreviation: IMDC = International Metastatic RCC Database Consortium.

Supplemental Tables 5 and 6. Multivariable Cox regression for impact of sarcomatoid dedifferentiation on progression-free and overall survival on first-line sunitinib or pazopanib (n = 366). The following predictors were included in the models: sarcomatoid dedifferentiation (0%-24% vs. ≥ 25%), IMDC (Good vs. Intermediate vs. Poor), time of metastases (synchronous vs. metachronous), Fuhrman grade (< 4 vs. 4), T-stage (1-2 vs. 3-4), and age at start of first-line therapy. Molecular subtype and Angio score were not included, as these were only known for a minority of patients. Sarcomatoid dedifferentiation and IMDC risk group remained independent predictors of progression-free and overall survival.

Supplemental Table 4 Characteristics of 366 Patients Treated With First-line Sunitinib or Pazopanib, for Whom Percentage of Sarcomatoid Dedifferentiation was Available

	Non-sarcomatoid (< 25%), n = 344 (%)	Sarcomatoid (≥ 25%), n = 22 (%)
Median age at start, y	64	61
Male	228 (66)	16 (73)
First-line therapy		
Sunitinib	248 (72)	18 (82)
Pazopanib	96 (28)	4 (18)
IMDC risk group		
Good	45 (14)	0
Intermediate	195 (60)	9 (43)
Poor	85 (26)	12 (57)
ccrcc1 to -4 subtype		
ccrcc1	44 (30)	3 (23)
ccrcc2	67 (45)	2 (15)
ccrcc3	9 (6)	0
ccrcc4	26 (18)	8 (62)
Angio score		
High	76 (52)	3 (23)
Low	70 (48)	10 (77)
T stage		
T1	63 (19)	3 (14)
T2	64 (20)	3 (14)
T3	186 (57)	13 (62)
T4	13 (4)	2 (10)
Synchronous metastases	163 (47)	15 (68)
Fuhrman grade		
1	5 (1)	0
2	37 (11)	0
3	143 (42)	0
4	157 (46)	22 (100)

Abbreviation: IMDC = International Metastatic RCC Database Consortium.

Supplemental Table 5 Progression-free Survival

Predictor	Hazard Ratio	95% Confidence Interval	*P* Value
≥ 25% sarcomatoid dedifferentiation	2.9	1.8-4.5	<.0001
IMDC intermediate risk (vs. good)	1.4	0.9-2.2	.1
IMDC poor risk (vs. good)	2.3	1.5-3.7	.0003

Abbreviation: IMDC = International Metastatic RCC Database Consortium.

Supplemental Table 6 Overall Survival

Predictor	Hazard Ratio	95% Confidence Interval	*P* Value
≥ 25% sarcomatoid dedifferentiation	2.3	1.4-3.7	.001
IMDC intermediate risk (vs. good)	1.9	1.2-3.1	.009
IMDC poor risk (vs. good)	3.2	1.9-5.4	<.0001

Abbreviation: IMDC = International Metastatic RCC Database Consortium.

Supplemental Tables 7 and 8. Comparison of ccRCC with 0% and 1% to 24% Sarcomatoid Dedifferentiation.

Supplemental Table 7 IMDC Risk Group and Angio Score do Not Differ Between Tumors With 0% Versus 1% to 24% Sarcomatoid Dedifferentiation

	0%, n (%)	1%-24%, n (%)	*P* Value
IMDC risk group			.4
Good	18 (17)	3 (8)	
Intermediate	72 (67)	28 (74)	
Poor	17 (16)	7 (18)	
Median Angio score	−0.4	−0.4	.99
ccrcc1 to -4 molecular subtype			
ccrcc1	28 (26)	19 (37)	Numbers too small for Fisher exact test
ccrcc2	55 (55)	14 (27)	
ccrcc3	6 (6)	3 (6)	
ccrcc4	18 (17)	16 (31)	

P values calculated with the Fisher exact test (IMDC) and logistic regression (Angio score).
Abbreviation: IMDC = International Metastatic RCC Database Consortium.

Supplemental Table 8 Expression of Angiogenesis Genes is Similar Between ccRCC With 0% and 1% to 24% Sarcomatoid Dedifferentiation, and Higher Compared With ccRCC With ≥25% Sarcomatoid Dedifferentiation

	Sarcomatoid Component		
Gene	0%	1%-24%	≥ 25%
VEGFA	5.7	4.7	2.7
VEGFC	0.82	0.89	0.58
VEGFR1	1.8	1.9	0.46
VEGFR2	0.89	0.87	0.17
VEGFR3	0.83	0.85	0.38
HIF1A	0.71	0.83	0.47
HIF2A	1.0	0.81	0.29
PDGFRB	1.4	1.5	0.99
ANGPTL4	63	72	32

Median fold change is reported.
Abbreviation: ccRCC = Clear-cell renal cell carcinoma.

Supplemental Tables 9 and 10. The 3 IMDC Risk Groups are Distinctly Different; Therefore, Pooling of Intermediate and Poor Risk Should be Avoided When Possible.

Supplemental Table 9 Comparison of IMDC Good-risk Patients With Pooled Intermediate/Poor Risk

	IMDC Risk Group		*P* Value
	Good	Intermediate/Poor	
Outcome on first-line suntinib or pazopanib			
RR, %	63	41	.08
PFS, mos	25	11	.02
Angio score			
High	77% (17)	45% (63)	.02
Low	23% (5)	55% (78)	
Individual angiogenesis genes, median fold change			
VEGFA	7.8	4.3	.01
VEGFC	0.86	.80	.5
VEGFR1	3.3	1.59	.004
VEGFR2	1.6	.66	.003
VEGFR3	1.1	.72	.02
HIF1A	0.81	.63	.2
HIF2A	1.2	.79	.01
PDGFRB	1.5	1.4	.2
ANGPTL4	88	63	.2
ccrcc1 to -4 molecular subtype			
ccrcc1	14% (3)	33% (46)	.002
ccrcc2	77% (17)	38% (53)	
ccrcc3	9% (2)	6% (8)	
ccrcc4	0	24% (34)	
Sarcomatoid dedifferentiation			
<25%	100% (21)	91% (124)	.14
≥25%	0	9% (13)	

The differences are less pronounced and *P* values are higher than upon comparison of the 3 IMDC risk groups separately.
Abbreviations: IMDC = International Metastatic RCC Database Consortium; PFS = progression-free survival; RR = response rate.

Supplemental Table 10 **Clinical and Molecular Characteristics are Significantly Different Between IMDC Intermediate- and Poor-risk Patients**

	IMDC Risk Group, % (n)		*P* Value
	Intermediate	Poor	
Outcome on first-line sunitinib or pazopanib			
RR, %	46	19	.03
PFS, mos	13	5	.0009
Angio score			
High	50 (54)	26 (9)	.01
Low	50 (53)	74 (25)	
Individual angiogenesis genes, median fold change			
VEGFA	5.0	2.8	.003
VEGFC	0.92	0.55	.2
VEGFR1	1.8	0.10	.02
VEGFR2	0.87	0.38	.004
VEGFR3	0.83	0.40	.0007
HIF1A	0.63	0.64	.7
HIF2A	0.98	0.49	.004
PDGFRB	1.4	1.1	.06
ANGPTL4	70	35	.03
ccrcc1 to -4 molecular subtype			
ccrcc1	33 (35)	32 (11)	.03
ccrcc2	43 (46)	21 (7)	
ccrcc3	6 (6)	6 (2)	
ccrcc4	19 (20)	24 (14)	
Sarcomatoid dedifferentiation			
<25%	95 (100)	75 (24)	.002
≥25%	5 (5)	25 (8)	

P values were calculated with the Fisher exact test (Angio score, ccrcc1 to ccrcc4, sarcomatoid), logistic regression (RR), Kaplan-Meier method (PFS), and Mann Whitney *U* test (fold change).
Abbreviations: IMDC = International Metastatic RCC Database Consortium; PFS = progression-free survival; RR = response rate.

Chapter 9

MicroRNA expression profiles in molecular subtypes of clear-cell renal cell carcinoma are associated with clinical outcome and repression of specific mRNA targets

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PLOS ONE 2020. In press.

Chapter 9: Contents

Abstract 117

Introduction 118

Results 119

- Exploratory unsupervised clustering reveals two miRNA clusters that overlap with molecular subtypes 119
- Molecular subtype-specific miRNA expression is involved in pathways that regulate tumor biology 120
- MiRNA expression is correlated with both overall survival and molecular subtype 127
- MiRNA expression is not predictive for response to sunitinib or pazopanib 128

Discussion 128

Conclusion 131

Methods 132

- Small RNAseq preprocessing 132
- Unsupervised clustering 133
- Differential expression 133
- Functional characterization 133
- Correlation with clinical outcome 134

References 134

Supplemental tables and figures 138

Abstract

Clear-cell renal cell carcinomas (ccRCC) can be divided into four transcriptomic subtypes, two of which have a favorable and two an unfavorable prognosis. To assess mechanisms driving these subtypes, we investigated their miRNA expression patterns. miRNAs are master regulators of mRNAs, that are widely deregulated in cancer. Unsupervised clustering in our dataset (n=128) and The Cancer Genome Atlas (TCGA) validation set identified two distinct miRNA clusters that overlapped with the transcriptomic subtypes, underscoring the validity of these subtypes on a multi-omics level and suggesting a driving role for miRNAs. Discriminatory miRNAs for the favorable subtypes repressed epithelial-to-mesenchymal transition, based on gene set enrichment analysis and target-mRNA expression levels. Strikingly, throughout the entire dataset, miRNAs associated with favorable subtypes were also associated with longer overall survival after diagnosis, and miRNAs associated with unfavorable subtypes with shorter overall survival (Pearson $r=-0.54$, $p<0.0001$). These findings indicate a general shift in miRNA expression between more and less aggressive tumors. This adds to current literature, which usually suggests only a small subset of miRNAs as markers of aggressive disease.

In conclusion, this study reveals distinct mRNA expression patterns underlying transcriptomic ccRCC-subtypes, whereby miRNAs associated with favorable subtypes counteract epithelial-to-mesenchymal transition. There is a general shift in miRNA expression in ccRCC, between more and less aggressive tumors.

Introduction

miRNAs are small non-coding RNAs that repress gene expression. They bind to their complementary sequence on mRNAs, thus inducing the mRNA's degradation or preventing translation. miRNAs regulate key cellular processes in an intricate way, with each miRNA having up to hundreds of mRNA targets, and each mRNA being targeted by several miRNAs. (1) In general, regulation by miRNAs aids to maintain cells in a differentiated state. Conversely, cancer cells are hallmarked by a global downregulation of miRNA expression, which allows them to develop a more undifferentiated phenotype. (2,3) Apart from this widespread loss of miRNA expression in cancer, individual miRNAs may be upregulated and can play either an oncogenic or a tumor suppressor role. To add another layer of complexity, this role can vary depending on the tumor type and specific circumstances. In clear-cell renal cell carcinoma (ccRCC) as well as in other cancer types, several miRNAs have been associated with prognosis, but often with inconsequent or contradictory results in different series. (4-9)

On the mRNA level, we have previously described four molecular subtypes in advanced ccRCC, which are based on unsupervised clustering of whole transcriptome data. (10) Two of these subtypes have a favorable prognosis: ccrcc2 (45%, highly angiogenic) and ccrcc3 (5%, resembling normal kidney). The two others are associated with an aggressive stem cell-like phenotype and poor outcomes, but have different immune phenotypes: ccrcc1 (30%, immune-cold) and ccrcc4 (20%, inflamed). Similar mRNA-based molecular subtypes have been described by other groups. (4,11,12) Although these subtypes also display different mutation profiles, they are not fully determined by specific genomic alterations. Therefore, they are also driven by epigenetic regulatory processes that remain incompletely understood. Methylation status plays a role, with the poor prognostic ccrcc1 and ccrcc4 tumors being globally hypomethylated. On the other hand, as miRNAs are master regulators of gene expression, they are also excellent candidates to partially explain the different gene expression profiles between the subtypes. (10) Indeed, previous clustering of miRNA data in The Cancer Genome Atlas (TCGA) cohort has revealed four miRNA clusters, two of which overlapped with transcriptomic mRNA clusters that were also described by TCGA. (4) Among key discriminatory miRNAs, miR-21 was strongly correlated with poor prognosis and repressed targets in the Von Hippel Lindau - Hypoxia Inducible Factor 1-alpha (VHL – HIF1A) axis, which is critically deregulated in ccRCC. In other tumor types as well, miRNA expression has

been shown to differ between molecular subtypes. For instance, in colorectal cancer, the poor prognostic 'mesenchymal' molecular subtype is associated with downregulation of miRs 194, 200b, 203 and 429. (13) In bladder cancer, the less aggressive 'luminal' molecular subtype is associated with downregulation of miR-99a, miR-100, miR-145 and miR-125b. (14)

In this study, we performed a miRNome analysis of primary tumors from a large series of metastatic ccRCC patients. We aimed to identify miRNAs driving mRNA molecular subtypes, to assess their repression of mRNA-targets and to determine the association between subtype-specific miRNA expression and clinical outcome.

Results

We included 128 patients for miRNome and clinical analysis, and determined the molecular subtype in 95 cases where fresh-frozen tissue was available. 45 tumors had a favorable ccrcc2_3 subtype (39 ccrcc2, 6 ccrcc3), 50 had an unfavorable ccrcc1_4 subtype (24 ccrcc1, 26 ccrcc4). Of the 16 paired normal and tumor samples, we determined molecular subtype for 11 cases. Patient characteristics are summarized in Table 9.1. Raw and normalized expression data will be deposited in ArrayExpress upon publication.

In the TCGA validation set, combined miRNA and mRNA expression data with ccrcc1 to -4 molecular subtype were available for 107 samples.

Exploratory unsupervised clustering reveals two miRNA clusters that overlap with molecular subtypes

We evaluated miRNA expression patterns across all samples in an unbiased way, using principal component analysis and unsupervised clustering. These analyses revealed a marked differentiation of tumoral *vs* normal kidney samples (Figure 9.1 A-C). As shown in the heatmap, the tumor cluster further split into two clusters that largely overlapped with the molecular subtypes. Unsupervised clustering using only the tumor samples, separated the samples into one cluster that for 81% consisted of the unfavorable ccrcc1_4 tumors, and a second cluster that for 62% consisted of the favorable ccrcc2_3 tumors ($p = 0.0005$). These findings were validated on the TCGA dataset, where we identified two miRNA clusters with 66% and 91% overlap with favorable and unfavorable molecular subtypes respectively ($p = 4.7e\text{-}9$). The heterogeneity of miRNA expression was higher in the unfavorable ccrcc1_4 subtypes. We also compared 11 tumor

samples with their matched normal kidney (7 ccrcc2_3, 4 ccrcc1_4). The miRNAs that were differentially expressed with normal kidney, largely overlapped between ccrcc2_3 and ccrcc1_4 tumors (Figure 9.1D). However, the extent to which they were differentially expressed between normal kidney and tumors, differed between ccrcc2_3 and ccrcc1_4.

Table 9.1. *Patient characteristics.*

All patients	**128**	
Male	86	67%
Median age at diagnosis	62 yrs	
Synchronous metastases	62	48%
Median DFI if metachronous	27 mo	
Median OS after diagnosis	49 mo	
Median OS after stage IV	34 mo	
1st line targeted therapy[A]		
Sunitinib	68	53%
Pazopanib	31	24%
Sorafenib	11	9%
Temsirolimus	9	7%
Nivolumab-Ipilimumab	6	5%
Other	3	2%
IMDC risk group		
Favorable	14	11%
Intermediate	81	63%
Poor	33	26%
ccrcc1 to -4 molecular subtype		
ccrcc1	24	25%
ccrcc2	39	41%
ccrcc3	6	6%
ccrcc4	26	27%
Unknown	33	

[A] All patients were treated for metastatic disease. No patients received adjuvant therapy. DFI = disease-free interval (months); OS = overall survival (months); IMDC = International Metastatic ccRCC Database Consortium.

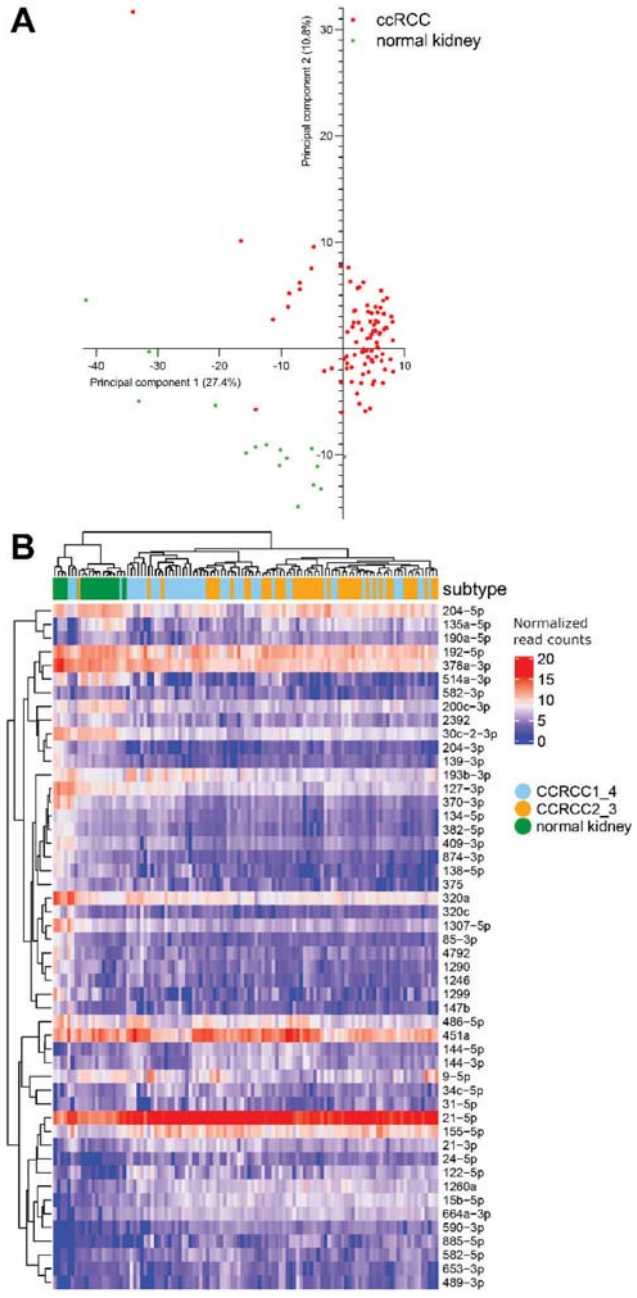

Figure 9.1. *Exploratory analyses of all samples (tumor and normal kidney combined, Leuven dataset). These showed a marked separation of normal vs tumoral kidney samples.* **(A)** *Principal component analysis (n = 111).* **(B)** *Unsupervised cluster analysis of the 50 most variable miRNA (n = 111). (continued on next page)*

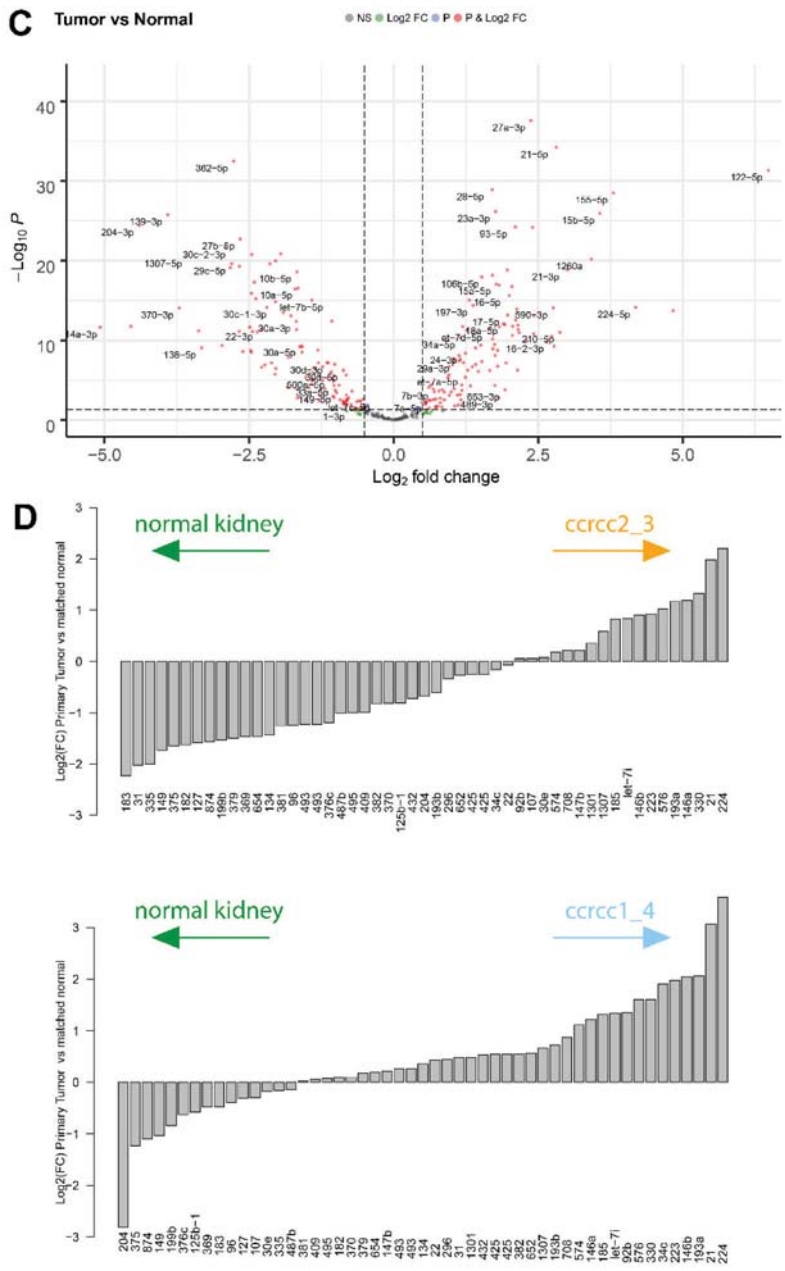

Figure 9.1. continued. **(C)** *Volcano plot (n = 95).* **(D)** *miRNAs with significant differential expression between matched normal and tumoral samples (n = 16). In panels C and D, higher positive fold changes indicate higher expression in tumor samples and lower negative fold changes indicate higher expression in normal kidney.*

Molecular subtype-specific miRNA expression is involved in pathways that regulate tumor biology

We identified the miRNAs that displayed significantly different expression levels between the ccrcc2_3 and ccrcc1_4 subtypes (Figure 9.2A, Table 9.2, exhaustive list in Table 9.S1). The favorable and unfavorable subtypes had markedly different expression of several miRNAs, many of which have been widely associated with relevant pathways in cancer biology (Table 9.3). These findings were validated on the TCGA data: the subtype-specific expression of miRNAs, as quantified by fold change, was strongly correlated between our dataset and the TCGA with a Pearson coefficient of r = 0.59 (p < 2.2e-16) (Figure 9.2B).

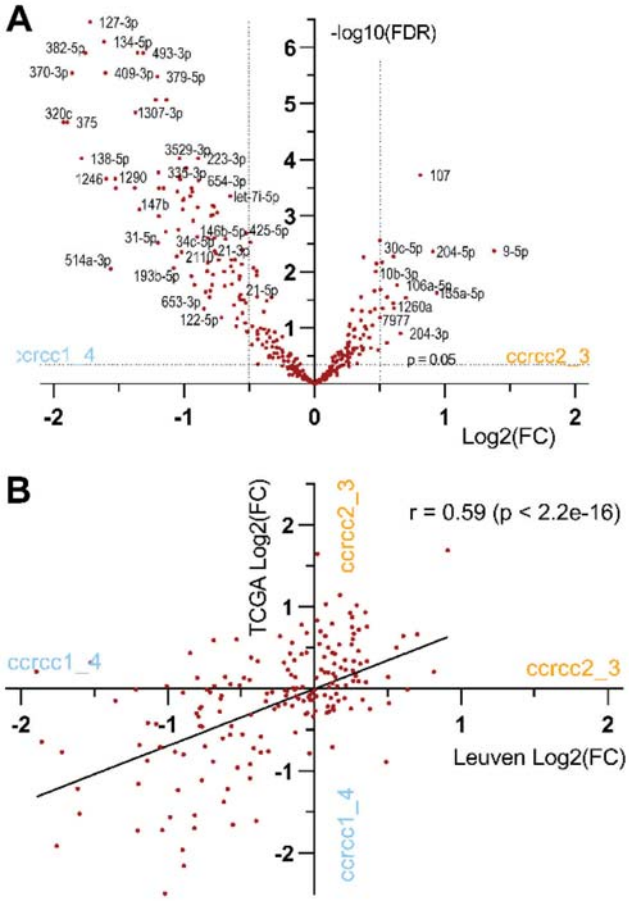

***Figure 9.2**. Illustration of subtype-specific miRNA expression. Ccrcc2_3 and ccrcc1_4 tumors showed distinct miRNA expression patterns.* **(A)** *Volcano plot of miRNAs with subtype-specific expression (Leuven dataset, n = 95).*

(B) *Subtype-specific miRNA expression in the Leuven dataset was validated on TCGA by Pearson correlation (r = 0.59) (n = 95 Leuven and n = 107 TCGA). The correlation was performed on 189 miRNAs that were shared between the Leuven and TCGA dataset.*

FC = fold change; FDR = false discovery rate.

Table 9.2. *List of miRNAs that exhibited subtype-specific expression (FDR < 0.05) and that were either significantly associated with OS (Leuven dataset) or suppressed an mRNA target that was downregulated with a fold change of < 0.5 compared to the other molecular subtype (TCGA dataset). miRNAs with higher expression in ccrcc1_4 subtypes were systematically associated with shorter OS, and those with higher expression in ccrcc2_3 with longer OS. Downregulated mRNA targets are listed below the miRNA. Only experimentally validated mRNA targets were considered (sources: TarBase, TargetScan Human, miRecords, Inguinity Expert Findings). mRNA subtype-specific fold changes are illustrated in Figure 9.3B.*

miRNA upregulated in ccrcc2_3 tumors				
	log2(FC)	**FDR**	**HR(OS)**	**FDR**
101-3p	0.43	0.051	0.91	0.54
PTGS2				
23c	0.46	0.046	0.89	0.23
PLAU				
7977	0.52	0.045	0.69	0.00009
1260a	0.61	0.044	0.70	0.0002
30c-5p	0.61	0.0053	0.80	0.074
F2, MLLT11, CDCP1, SYT4, RUNX2, SLC7A1				
106a-5p	0.63	0.017	0.91	0.46
MMP3. CXCL8. RUNX1				
204-5p	0.91	0.004	0.73	0.0009
MMP3, MMP9, FBN2, ITGB, AURKB, SPDEF				
135a-5p	0.94	0.024	0.84	0.014
RUNX2				
9-5p	1.38	0.004	1.11	0.12
JAK3, FOXG1, ONECUT2				

FC = fold change; FDR = false discovery rate; HR = hazard ratio: OS = overall survival since diagnosis.

miRNA upregulated in ccrcc1_4 tumors				
	log2(FC)	**FDR**	**HR(OS)**	**FDR**
370-3p	-1.86	2.9e-6	1.17	0.025
134-5p	-1.61	7.9e-7	1.27	0.025
409-3p	-1.60	2.9e-6	1.22	0.0495
1307-3p	-1.37	0.00001	1.31	0.0295
182-5p	-1.22	8.6e-6	1.25	0.025
335-3p	-1.20	0.0001	1.26	0.025
224-5p	-1.19	0.001	1.24	0.0498
KLK1				
193b-3p	-1.19	0.0003	1.28	0.007
574-5p	-1.14	8.6e-6	1.44	0.016
193b-5p	-1.08	0.009	1.27	0.012
146a-5p	-1.05	0.0004	1.17	0.2
ATOH8, C8A, IL12RB2				
3529-3p	-1.03	0.00009	1.39	0.013
34c-5p	-1.02	0.004	1.32	0.0004
96-5p	-1.01	0.0005	1.17	0.12
AQP5, MYRIP				
199b-5p	-0.93	0.001	1.29	0.014
369-3p	-0.90	0.002	1.24	0.0498
146b-5p	-0.82	0.002	1.46	0.0004
652-3p	-0.78	0.0007	1.37	0.039
149-5p	-0.78	0.001	1.38	0.025
1301-3p	-0.77	0.0007	1.42	0.032
21-3p	-0.75	0.005	1.49	0.0004
629-5p	-0.73	0.006	1.36	0.025
185-5p	-0.68	0.003	1.41	0.016
let-7i-5p	-0.64	0.0004	1.70	0.002
TRIM71				
320a	-0.63	0.017	1.28	0.019
212-5p	-0.62	0.01	1.36	0.022
155-5p	-0.62	0.039	1.18	0.16
AGTR1				
222-3p	-0.61	0.006	1.34	0.039
425-5p	-0.52	0.002	1.61	0.014
21-5p	-0.44	0.028	1.69	0.003

Table 9.3. *Brief literature review of selected miRNAs with the highest discriminatory power for molecular subtype and OS. OncomiR = miRNA that is associated with cancer. AntagomiR = synthetic RNA that is perfectly complementary to a specific miRNA target.*

miRNAs that were relatively upregulated in ccrcc1 and -4 tumors and associated with poor OS	
let-7i-5p	The let-7 family is expressed higher in RCC compared to normal kidney. In RCC cell lines, a let-7 inhibitor inhibits proliferation and induces apoptosis. (27) Let-7a is correlated with intermediate prognosis in ccRCC. (4) In other tumor types, let-7 family members are described as tumor suppressors targeting oncogenes like MYC and RAS-family. (27,36)
21	Well-known oncomiR. (3) It is associated with poor outcomes in RCC. Inhibition results in cell cycle arrest, apoptosis and reduced invasive capacity. (4,22,23,24,37)
425-5p	OncomiR. (39,40) Associated with poor survival in ccRCC treated with sunitinib and in chromophobe RCC. (23,40) Upregulation in RCC cell lines promotes invasiveness and inhibited apoptosis. (28)
146b-5p	Highly expressed by regulatory T-cells (which are mostly found in ccrcc4 tumors). Antagomirs suppress T_{regs} in vitro and in vivo. (41) The related miR-146a is hypoxia-regulated and promotes ccRCC proliferation and invasion. (42)
34c-5p	The miR-34 tumor suppressor family are direct targets of p53, they repress cell proliferation and induced apoptosis. In RCC however, higher expression of miR-34a is reported in non-responders to sunitinib and in early progressors. In colon carcinoma higher expression of miR-34-b/c is reported in aggressive disease. (7,26,43,44)
1307-3p	Serum levels are higher in patients with breast cancer, compared to those without. (45)
134-5p	Tumor suppressing role in various cancer types. (46,47)
193b-5p	Tumor suppressing role in various cancer types. In RCC, higher expression is associated with higher stage and early relapsing disease. (26)
miRNAs that were relatively upregulated in ccrcc2 and -3 tumors and associated with long OS	
204-5p	miR-204 suppresses mRNA targets that otherwise promote tumor invasiveness and metastases. 204-5p expression is lower in the TCGA poor-prognostic miRNA cluster. miR-204 suppresses ccRCC tumor growth by controlling oncogenic autophagy. Expression is higher in non-metastatic compared to metastatic ccRCC. (4,5,18)
135a-5p	miR-135a is a tumor suppressor in RCC, that induces tumor cell apoptosis and represses metastasis. (48-50)
30c-5p	The miR-30 family acts as tumor suppressor in various cancer types. miR-30c is upregulated in non-metastatic compared to metastatic ccRCC and inhibits epithelial-to-mesenchymal transition.(5,20)

In order to identify functional miRNA-mRNA interactions of subtype-specific miRNAs, we correlated these miRNAs with their predicted mRNA targets using the TCGA dataset (full transcriptome data were available for TCGA, but not for our own dataset). Among several repressed mRNAs, 29 targets of 12 miRNAs displayed significantly different expression levels between the molecular subtypes, with log2(FC) ≥ 0.5 or ≤ -0.5 in ccrcc2_3 *vs* ccrcc1_4 (Table 9.2, Figure 9.3B). These specific mRNAs play an important role in the epithelial-to-mesenchymal transition observed in tumor cells: many of the targets that were repressed in the favorable ccrcc2_3 subtypes are mRNAs that promote

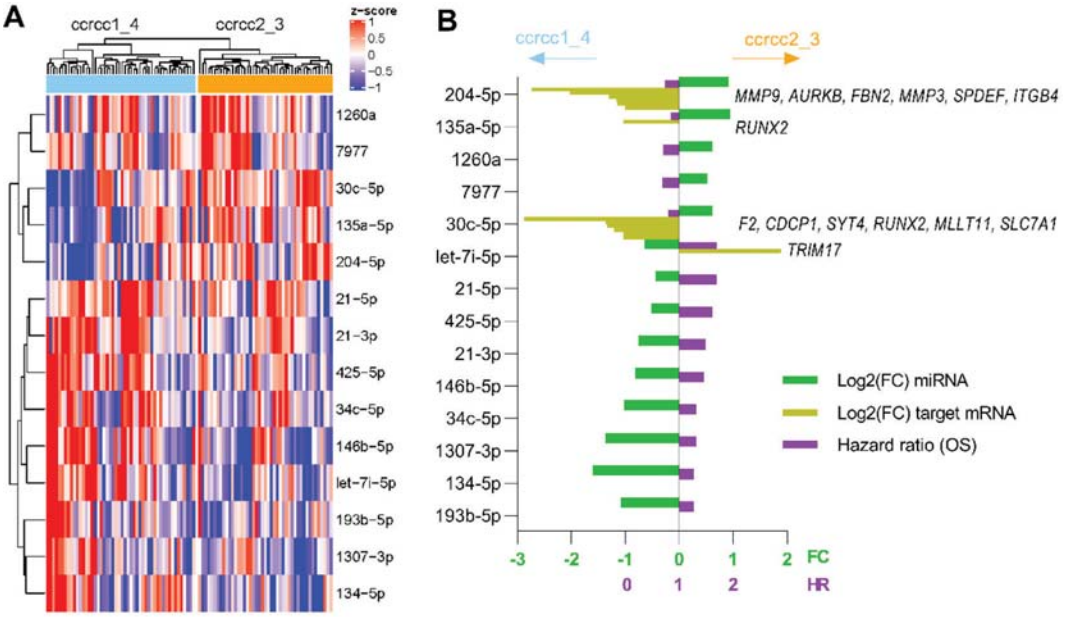

Figure 9.3. *Summary of selected miRNAs with the highest discriminatory power for both molecular subtype and OS (Leuven dataset, n = 95). All miRNAs that were associated with poor prognostic ccrcc1_4 subtypes were systematically associated with poor OS, and vice versa.* **(A)** *Heatmap* **(B)** *Bar plots quantifying subtype-specific miRNA expression, hazard ratio for OS and subtype-specific mRNA expression of predicted mRNA targets that were downregulated with a fold change ≤ 0.5 compared to the other subtype. All results are significant after correction for multiple testing (FDR < 0.05).*

FC = fold change; OS = overall survival.

tumor invasiveness and metastasis (*MMP3, MMP9, AURKB, FBN2, ITGB4, SPDEF, F2, RUNX2, CDCP1*, repressed by miRs 204-5p, 30c-5p, 135a-5p and 106a-5p). Contrarily, miRNA 96-5p, which was upregulated in the unfavorable ccrcc1_4 subtypes, repressed Myosin VIIa- and Rab-Interacting Protein (*MYRIP*), the latter being involved in anchoring and scaffolding processes to retain cell structure. (15)

We then performed KEGG gene set enrichment analysis to identify cellular pathways affected by subtype-specific miRNA expression. Although these results do not fully capture the complex multi-level regulation by miRNAs and should be interpreted with caution, they did show a relative upregulation of several pathways that promote cell structure and adhesion in the favorable ccrcc2_3 subtypes (supplemental Figure 9.S1). The names of these KEGG pathways are: "cell adhesion molecules", "gap junction", "regulation of actin cytoskeleton", "tight junction", "focal adhesion" and "adherens junction".

MiRNA expression is correlated with both overall survival and molecular subtype

To assess the translational relevance of our findings we tested the correlation of miRNA expression with OS using Cox regression. Our hypothesis was that the limited subset of miRNAs with marked subtype-specific expression would also show a prognostic value. However, strikingly, OS analysis revealed a much more remarkable association: throughout the entire dataset, miRNAs with relative upregulation in ccrcc1_4 were associated with worse OS, and miRNAs with relative upregulation in ccrcc2_3 correlated with better OS. Pearson correlation of subtype-specific expression and OS, which were quantified as log2(fold change) and hazard ratio respectively, showed a very robust correlation with $r = -0.54$ (95% CI (-0.61,-0.46) and $p < 0.0001$) (Figure 9.4). This indicates a general shift of miRNA expression between less and more aggressive tumors, rather than only a limited subset of miRNAs being associated with aggressive disease. An overview of miRNAs that were significantly correlated with both molecular subtype and OS is provided in Table 9.2. Results were similar when we repeated outcome analysis using time to metastasis and OS since metastasis, as well as after randomly dividing the cohort in two groups, which underscores the validity of these results (supplemental Figure 9.S2 and Table 9.S1).

To test whether individual miRNAs have independent prognostic value, we performed multivariate Cox regressions with the well-established IMDC risk groups and with molecular subtype. Of the 14 miRNAs with the highest discriminatory power for both molecular subtype and OS (Figure 9.3 and Table 9.3), all but miR 30c-5p remained independent predictors for OS after diagnosis (supplemental Table 9.S2). Molecular subtype remained an independent predictor as well, except when tested against miRs 21-3p (upregulated in unfavorable ccrcc1_4) and 204-5p (upregulated in favorable ccrcc2_3). This is in line with the TCGA miRNA clusters, where the poor prognosis miRNA cluster could be distinguished based on upregulation of 21-3p together with downregulation of 204-5p. (4)

MiRNA expression is not predictive for response to sunitinib or pazopanib

The majority of patients (77%) were treated in 1st line with the anti-angiogenic agents sunitinib or pazopanib. These agents were standard 1st line therapies for advanced ccRCC before the introduction of immune checkpoint inhibitors, and are currently routinely used from 2nd line on. (16) To assess whether specific

miRNAs could be predictive for response to sunitinib or pazopanib, we tested their association with response rate and progression-free survival. No miRNAs were significantly associated with response rate after correction for multiple testing, and only three were significantly associated with progression-free survival (miRs 185-5p, 193b-5p and 193b-3p). As the latter were also strongly associated with OS since diagnosis, we deemed them to be prognostic rather than predictive.

Discussion

In this study, we analyzed miRNomes of ccRCC to assess the link between miRNA expression and transcriptomic molecular subtypes, mRNA expression and clinical outcomes.

The ccrcc1 to -4 molecular subtypes showed clearly different miRNA expression patterns, both in our dataset and the TCGA validation set. First, unsupervised clustering of miRNAs revealed two clusters that overlapped with the favorable (ccrcc2_3) and unfavorable (ccrcc1_4) subtypes. This is in line with

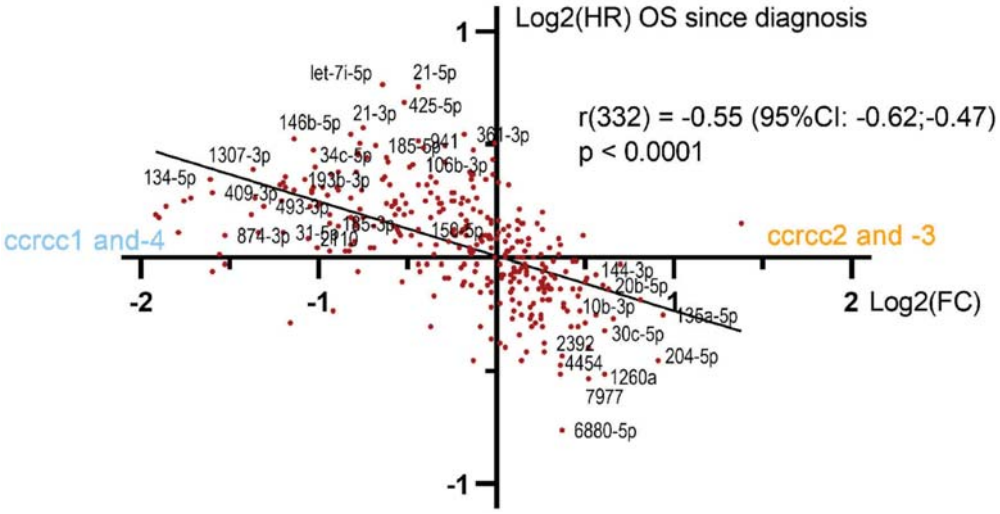

Figure 9.4. *Scatterplot illustrating the correlation between subtype-specific miRNA expression (Log2(FC)) and HR for OS (Leuven dataset, n = 95)). There was a strong correlation throughout the dataset, with a Pearson coefficient of -0.54 (332 degrees of freedom). Hazard ratio for OS is plotted on a log2-scale for graphical reasons; the regression line was fitted on the original data. HR = hazard ratio; OS = overall survival; FC = fold change.*

earlier findings from TCGA, where four miRNA clusters were found, of which only two overlapped with molecular subtypes. (4) Secondly, a supervised analysis revealed several miRNAs that were discriminatory between the subtypes. Moreover, miRNAs that were expressed higher in unfavorable subtypes were systematically associated with poor OS, and miRNAs that were expressed higher in favorable subtypes with short OS. This held true in the entire dataset, and not only for those miRNAs with significant subtype-specific expression (Figure 9.4, supplemental Table 9.S1). These findings underscore the robustness of these subtypes.

A striking finding, which was not expected at study set-up, was the strong correlation between subtype-specific miRNA expression and OS, throughout the entire dataset. Otherwise put, there was a shift in global miRNA expression between less *vs* more aggressive tumors. This is in contrast to published literature, which typically describes small subsets of miRNAs that are significantly associated with prognosis in some studies but not in others. Our study reveals that aggressive tumors are not set apart by only a few distinctive miRNAs. Rather, these tumors display an entirely different miRNome expression, regardless of single miRNAs reaching the significance threshold for OS. Importanly, this is the case in both advanced and earlier stage disease, as findings were similar in our dataset (stage IV ccRCC) and the TCGA (mostly stage I-III ccRCC).

Many of the most discriminatory miRNAs for both molecular subtype and OS are well described oncomiRs or tumor suppressors in ccRCC or other tumor types. Previously, two miRNA signatures have been published that distinguished localized from metastatic ccRCC. (5,9) According to these signatures, miRs 10b, 139-5p, 30a-3p, 30c-5p, 139-5p and 144-5p were discriminative for localized disease, and miRs 130b and 199b-5p for metastatic tumors. Our series validated the prognostic value of all these miRs, with the former all being associated with ccrcc2_3 and long OS, and the latter with ccrcc1_4 and short OS. As we only used biological material from metastatic tumors we could not validate the capability of these signatures to predict the risk of relapse.

When correlating miRNA expression levels with their predicted mRNA targets, several subtype-specific miRNAs repressed targets that are involved in tumor epithelial-to-mesenchymal transition. Tumors with a mesenchymal phenotype have an aggressive behavior and poor prognosis. In RCC, they are best represented by the notoriously unfavorable sarcomatoid RCC, which are typically of the unfavorable ccrcc4 subtype. (10, 17) In the favorable ccrcc2_3 subtypes, miR-204-5p was strongly upregulated, predicted long OS, and repressed several targets that are involved in tumor invasiveness and metastasis (*MMP3, MMP9, AURKB, FBN2, ITGB4, SPDEF*). miR-204 is a well-studied tumor suppressor

that inhibits epithelial-to-mesenchymal transition, as well as the immune suppressive interleukin 11. (8,18) In ccRCC, miR-204 has been shown to suppress tumor growth by controlling oncogenic autophagy, and it is expressed at higher levels in non-metastatic ccRCC compared to metastatic disease. (5,19) Indeed, in the TCGA cohort, the poorest prognosis miRNA cluster showed downregulation of miR-204. (4) Expression of miR-30 family members, and especially 30c-5p, was also markedly favorable. The members of this family have a well-established role as tumor suppressors inhibiting epithelial-to-mesenchymal transition in various cancer types. (20) In our series, it repressed targets involved in invasiveness and metastasis (*F2, RUNX2, CDCP1*). Earlier studies in ccRCC showed that miR-30c inhibits epithelial-to-mesenchymal transition and reduces HIF2A activity, which blocked xenograft tumor growth. (21,22) It is expressed at higher levels in non-metastatic compared to metastatic ccRCC. (5) Another miRNA that was expressed higher in ccrcc2_3 tumors and showed a trend towards good prognosis is miR-10b. miR-10b was discriminatory for the good-prognosis miRNA cluster in the TCGA cohort, is part of two prognostic ccRCC signatures and is expressed higher in non-metastatic ccRCC. (4,5,9) On the other hand, when looking at unfavorable miRNAs, miR-21 was strongly correlated with ccrcc1_4 subtypes and poor OS. MiR-21 is indeed one of the best described oncomiRs and may contribute to the general downregulation of miRNAs in cancer. (3) This was also described by TCGA, where miR-21 was discriminative for the miRNA cluster with poorest prognosis and repressed targets in the VHL – HIF1A axis. (4) Zaman et al. showed that miR-21 was increased in high stage tumors and that its inhibition in ccRCC cell lines led to cell cycle arrest, apoptosis and reduced invasive and migratory capacities; other groups have reported similar findings. (23-26)

Counterintuitively, some of the miRNAs that are most discriminatory for the unfavorable ccrcc1_4 subtypes and poor OS, are well-known tumor suppressors: let-7i-5p, 34c-5p, 134-5p and 193-5p. miRNA regulation is indeed so complex, that contradictory results are often reported for even the best established miRNA pathways. (2) One specific hypothesis on the current results is that downregulation or loss of these tumor suppressive miRs may act as a key oncogenic mechanism in the ccrcc2_3 subtypes, whereas ccrcc1_4 subtypes depend on other oncogenic pathways. This is supported by the finding that the expression of miR-34c-5p, 134-5p and 193-5p is lower in ccrcc2_3 tumors compared to normal kidney, suggesting active loss (Figure 9.1D). Moreover, miR-34c and 193b were also associated with early progressive disease in ccRCC in other series. (7) Let-7i, although part of the well-established let-7 tumor suppressor family, was strongly associated with ccrcc1_4 tumors and poor OS. This is in line with a previous study reporting that a let-7 inhibitor caused

apoptosis in ccRCC cell lines, and with the TCGA where let-7a was correlated with intermediate prognosis. (4,27)

We did not identify any miRNAs that were predictive for response to the anti-angiogenic agents sunitinib or pazopanib in 99 patients that received 1st line treatment with these therapies. These findings are consistent with other studies, that only described a correlation of miRNAs with progression-free survival or OS, but not response rates. (6,7,28)

While our miRNome-wide approach was indispensable to reveal the general shifts in miRNA expression between less and more aggressive tumors, it also constitutes a limitation of this study. We did not assess single miRNAs that may be fundamentally involved in the regulation of angiogenic or immune pathways, which constitute therapeutic targets in ccRCC, and can therefore not exclude that some predictive miRNAs may exist which did not survive correction for multiple testing. We did not seek to validate the effects of discriminative miRNAs with functional studies either, but such studies have been done previously for most of the most discriminative miRNAs.

Conclusion

The ccrcc1 to -4 transcriptomic molecular subtypes in ccRCC have fundamentally different miRNA expression profiles that underlie their mRNA expression, which underscores the robustness of these subtypes on a multi-omics scale. There was a very strong association between subtype-specific miRNA expression (favorable *vs* unfavorable) and outcome (long *vs* short OS), throughout the entire dataset. This reflects a general shift in miRNA expression from less to more aggressive tumors. Many of the discriminative miRNAs repressed mRNA targets that are involved in tumor epithelial-to-mesenchymal transition.

Methods

Patients and samples

We selected primary ccRCC samples from the Leuven University Hospitals ccRCC tumor bank, which prospectively collects tissue and detailed clinical data from metastatic ccRCC patients after informed consent. Patients were eligible if archived tissue of the primary tumor was available, provided they had not

received systemic therapy prior to tissue retrieval. Clear-cell histology was confirmed by an expert uro-pathologist. As a reference, we included paired normal kidney samples from 16 patients as well (healthy tissue block from the nephrectomy specimen removed during ccRCC surgery). The protocol for miRNome analysis has been described previously. (6) Briefly, total RNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue using the Recover All Total Nucleic Acid Isolation kit for FFPE (Ambion). Libraries were constructed with the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs E7300) and sequenced for 50 bases in a single-read format (Genome Analyzer IIx, Illumina). In cases where fresh-frozen primary tumor tissue was available, this was used to determine ccrcc1 to -4 transcriptomic molecular subtype, using the established 35-gene classifier algorithm as described previously. (10) The TCGA ccRCC cohort was used as a validation set. In TCGA samples for which combined miRNome and transcriptome data derived from fresh-frozen tissue were available, we determined molecular subtype using the classification algorithm.

Small RNAseq preprocessing

Raw reads were trimmed using Trim Galore! (Babraham Bioinformatics) and aligned against the miRbase mature miRNA sequences using Bowtie1 (Johns Hopkins University), resulting in raw read counts for 2589 miRNAs. (29) For the clustering and differential expression results, raw count data was analyzed according to the DESeq2 workflow (Bioconductor) after filtering out miRNA with a normalized count smaller than five in more than 50 samples. (30) For the correlation with overall survival (OS) since diagnosis of ccRCC, counts were normalized using the upperquartile method as implemented in the R package edgeR after removing lowly expressed miRNAs (zero counts per million reads in more than 100 samples). (31)

Unsupervised clustering

To assess the presence of miRNA clusters and their overlap with previously determined mRNA molecular subtypes, we performed unsupervised clustering. For this, a principal component analysis was run on variance stabilized counts obtained from DESeq2 (version 1.25.16). In addition, the samples were clustered hierarchically using the 50 most variable miRNAs using correlation as a distance metric as implemented in the R package ComplexHeatmap (version 2.1.1, Bioconductor). (32) To further investigate the miRNA based clustering, variance

stabilized counts of all expressed miRNAs were normalized and tumor samples were partitioned into two clusters according to the 'partitioning around medoids' algorithm as implemented in the R package cluster (version 2.1.0). A Chi-Square test was performed to assess the association of the favorable and unfavorable prognosis mRNA subtypes with the two miRNA clusters.

Differential expression

Differential miRNA expression between the favorable (ccrcc2_3) *versus* (*vs*) the unfavorable prognostic subtypes (ccrcc1_4) and tumor *vs* normal samples was assessed using DESeq2 (version 1.25.16). As a validation of the differential expression results, miRNA data from the TCGA ccRCC cohort was analyzed in a similar fashion using DESeq2.

Functional characterization

For miRNAs with subtype-specific expression (defined by log2 (fold change) ≥ 0.5 or ≤ -0.5 and false discovery rate (FDR) < 0.05), Ingenuity Pathway Analysis (IPA, Qiagen) was used to collect the predicted downstream mRNA targets. Moreover, this data was integrated with the subtype-specific expression of mRNA targets in the TCGA dataset to detect putative miRNA-mRNA functional interactions (microRNA Target Filter module, IPA).

Kyoto Encyclopedia of Genes and Genomes (KEGG) gene set enrichment analysis on the differentially expressed miRNAs was performed using the R package RBiomirGS (version 0.2.12). (33) Briefly, the miRNA differential expression list is used to search multiple databases for miRNA-mRNA interactions, resulting in a target mRNA list. For each miRNA, the fold change and p-value from the miRNA differential expression list are used to calculate an expression score. From this, a miRNA impact score for the target mRNAs is generated. With the mRNA score and a gene set database, gene set enrichment is calculated using logistic regression.

Correlation with clinical outcome

We performed univariate Cox regression to correlate individual miRNAs with OS since diagnosis and progression-free survival on 1st line sunitinib or pazopanib. We used a Mann-Whitney U test to analyze expression differences between patients with partial response *versus* progressive disease at first

evaluation. P-values were corrected for multiple testing using the Benjamini and Hochberg method, and are reported as FDR. (34) In another effort to confirm the validity of these results, we also performed Cox regression for other outcome parameters (OS since metastasis and time to metastasis). Lastly, as internal validation, we repeated all analyses after randomly dividing our patient population into two cohorts.

In a selection of miRNAs with the strongest association with both molecular subtype and OS, we performed multivariate Cox regressions with International Metastatic ccRCC Database Consortium (IMDC) risk groups and molecular subtype. (35)

Molecular analyses were done with R version 3.5.1, clinical outcome analyses with SAS version 9.4. All statistical tests were two-sided.

References

1. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136(2):215–33.
2. Rupaimoole R, Wu SY, Pradeep S, Ivan C, Pecot C V, Gharpure KM, et al. Hypoxia-mediated down-regulation of miRNA biogenesis promotes tumor progression. Nat Commun. 2014;5:5202.
3. Lin S, Gregory RI. MicroRNA biogenesis pathways in cancer. Nat Rev Cancer. 2015;15(6):321–33.
4. Creighton CJ, Morgan M, Gunaratne PH, Wheeler DA, Gibbs RA, Robertson G, et al. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature. 2013;499(7456):43–9.
5. Heinzelmann J, Arndt M, Pleyers R, Fehlmann T, Hoelters S, Zeuschner P, et al. 4-miRNA score predicts the individual metastatic risk of renal cell carcinoma patients. Ann Surg Oncol. 2019;26:3765–73.
6. García-Donas J, Beuselinck B, Inglada-Pérez L, Graña O, Schöffski P, Wozniak A, et al. Deep sequencing reveals microRNAs predictive of antiangiogenic drug response. JCI Insight. 2016;1(10):e86051.
7. Gámez-Pozo A, Antón-Aparicio LM, Bayona C, Borrega P, Gallegos Sancho MI, García-Domínguez R, et al. MicroRNA expression profiling of peripheral blood samples predicts resistance to first line sunitinib in advanced renal cell carcinoma patients. Neoplasia. 2012;14(12):1144–52.
8. Li T, Pan H, Li R. The dual regulatory role of miR-204 in cancer. Tumor Biol. 2016;37(9):11667–77.
9. Wu X, Weng L, Li X, Guo C, Pal SK, Jin JM, et al. Identification of a 4-microRNA signature for clear cell renal cell carcinoma metastasis and prognosis. PLoS One. 2012;7(5):e35661.
10. Beuselinck B, Job S, Becht E, Karadimou A, Verkarre V, Couchy G, et al. Molecular subtypes of clear cell renal cell carcinoma are associated with sunitinib response in the metastatic setting. Clin Cancer Res. 2015;21(6):1329–39.

11. Hakimi AA, Voss MH, Kuo F, Sanchez A, Liu M, Nixon BG, et al. Transcriptomic profiling of the tumor microenvironment reveals distinct subgroups of clear cell renal cell cancer: data from a randomized phase III trial. Cancer Discov. 2019;9(4):510–25.

12. Brooks SA, Brannon AR, Parker JS, Fisher JC, Sen O, Kattan MW, et al. ClearCode34: A prognostic risk predictor for localized clear cell renal cell carcinoma. Eur Urol. 2014;66(1):77–84.

13. Cantini L, Isella C, Petti C, Picco G, Chiola S, Ficarra E, et al. MicroRNA-mRNA interactions underlying colorectal cancer molecular subtypes. Nat Commun. 2015;6:8878.

14. Weinstein JN, Akbani R, Broom BM, Wang W, Verhaak RGW, McConkey D, et al. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature. 2014;507(7492):315–22.

15. El-Amraoui A, Schonn J, Küssel-Andermann P, Blanchard S, Desnos C, Henry J, et al. MyRIP, a novel Rab effector, enables myosin VIIa recruitment to retinal melanosomes. EMBO Rep. 2002;3(5):463–70.

16. Albiges L, Powles T, Staehler M, Bensalah K, Giles RH, Hora M, et al. Updated European Association of Urology Guidelines on Renal Cell Carcinoma: Immune Checkpoint Inhibition Is the New Backbone in first line Treatment of Metastatic Clear-cell Renal Cell Carcinoma. Eur Urol. 2019;76(2):151-6.

17. Conant J, Peng Z, Evans M, Naud S, Cooper K. Sarcomatoid renal cell carcinoma is an example of epithelial-mesenchymal transition. J Clin Pathol. 2011;64(12):1088-92.

18. Putoczki TL, Ernst M. IL-11 signaling as a therapeutic target for cancer. Immunotherapy. 2015;7(4):441–53.

19. Mikhaylova O, Stratton Y, Hall D, Kellner E, Ehmer B, Drew AF, et al. VHL-Regulated miR-204 suppresses tumor growth through inhibition of LC3B-mediated autophagy in renal clear cell carcinoma. Cancer Cell. 2012;21(4):532–46.

20. Yang S-J, Yang S-Y, Wang D-D, Chen X, Shen H-Y, Zhang X-H, et al. The miR-30 family: versatile players in breast cancer. Tumor Biol. 2017;39(3).

21. Huang J, Yao X, Zhang J, Dong B, Chen Q, Xue W, et al. Hypoxia-induced downregulation of mi R-30c promotes epithelial-mesenchymal transition in human renal cell carcinoma. Cancer Sci. 2013;104(12):1609–17.

22. Mathew LK, Lee SS, Skuli N, Rao S, Keith B, Nathanson KL, et al. Restricted Expression of miR-30c-2-3p and miR-30a-3p in Clear Cell Renal Cell Carcinomas Enhances HIF2α Activity. Cancer Discov. 2014;4(1):53–60.

23. Zaman MS, Shahryari V, Deng G, Thamminana S, Saini S, Majid S, et al. Up-regulation of microRNA-21 correlates with lower kidney cancer survival. PLoS One. 2012;7(2):e31060.

24. Ge Y-Z, Wu R, Xin H, Zhu M, Lu T-Z, Liu H, et al. A tumor-specific microRNA signature predicts survival in clear cell renal cell carcinoma. J Cancer Res Clin Oncol. 2015;141(7):1291–99.

25. Vergho D, Kneitz S, Rosenwald A, Scherer C, Spahn M, Burger M, et al. Combination of expression levels of miR-21 and miR-126 is associated with cancer-specific survival in clear-cell renal cell carcinoma. BMC Cancer. 2014;14(1):25.

26. Faragalla H, Youssef YM, Scorilas A, Khalil B, White NMA, Mejia-Guerrero S, et al. The clinical utility of miR-21 as a diagnostic and prognostic marker for renal cell carcinoma. J Mol Diagnostics. 2012;14(4):385–92.

27. Yu Z, Ni L, Chen D, Zhang Q, Su Z, Wang Y, et al. Identification of miR-7 as an oncogene in renal cell carcinoma. J Mol Histol. 2013;44(6):669–77.

28. Khella HWZ, Butz H, Ding Q, Rotondo F, Evans KR, Kupchak P, et al. miR-221/222 are involved in response to sunitinib treatment in metastatic renal cell carcinoma. Mol Ther. 2015;23(11):1748–58.

29. Langmead B, Trapnell C, Pop M, Salzberg S. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009;10(3): R25.

30. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550-71.

31. Robinson MD, McCarthy DJ, Smyth GK. EdgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26(1):139-40.

32. Gu Z, Eils R, Schlesner M. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. Bioinformatics. 2016;32(18):2847-49.

33. Zhang J, Storey KB. RBiomirGS: an all-in-one miRNA gene set analysis solution featuring target mRNA mapping and expression profile integration. PeerJ. 2018;6:e4262.

34. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J R Stat Soc Ser B. 1995;57(1):289–300.

35. Heng DY, Xie W, Regan MM, Warren MA, Golshayan AR, Sahi C, et al. Prognostic factors for overall survival in patients with metastatic renal cell carcinoma treated with vascular endothelial growth factor–targeted agents: results from a large, multicenter study. J Clin Oncol. 2009;27(34):5794-9.

36. Johnson CD, Esquela-Kerscher A, Stefani G, Byrom M, Kelnar K, Ovcharenko D, et al. The let-7 microRNA represses cell proliferation pathways in human cells. Cancer Res. 2007;67(16):7713–22.

37. Mayr C, Hemann MT, Bartel DP. Disrupting the Pairing Between let-7 and Hmga2 enhances oncogenic transformation. Science. 2007;315(5818):1576–9.

38. Faragalla H, Youssef YM, Scorilas A, Khalil B, White NMA, Mejia-Guerrero S, et al. The clinical utility of miR-21 as a diagnostic and prognostic marker for renal cell carcinoma. J Mol Diagnostics. 2012;14(4):385–92.

39. Zhang Z, , Li Y, Fan L, Zhao Q, Tan B, Li Z, et al. microRNA-425-5p is upregulated in human gastric cancer and contributes to invasion and metastasis in vitro and in vivo. Exp Ther Med. 2015;9(5):1617–22.

40. Wang J, Li Z, Ge Q, Wu W, Zhu Q, Luo J, et al. Characterization of microRNA transcriptome in tumor, adjacent, and normal tissues of lung squamous cell carcinoma. J Thorac Cardiovasc Surg. 2015;149(5):1404–14.

41. Quan J, Li Y, Pan X, Lai Y, He T, Lin C, et al. Oncogenic miR-425-5p is associated with cellular migration, proliferation and apoptosis in renal cell carcinoma. Oncol Lett. 2018;16(2):2175–84.

42. Lu Y, Hippen KL, Lemire AL, Gu J, Wang W, Ni X, et al. miR-146b antagomir–treated human Tregs acquire increased GVHD inhibitory potency. Blood. 2016;128(10):1424–35.

43. Yang L, Zhao G, Wang F, Li C, Wang X. Hypoxia-regulated miR-146a targets cell adhesion molecule 2 to promote proliferation, migration, and invasion of clear cell renal cell carcinoma. Cell Physiol Biochem. 2018;49(3):920–31.

44. Raver-Shapira N, Marciano E, Meiri E, Spector Y, Rosenfeld N, Moskovits N, et al. Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. Mol Cell. 2007;26(5):731–43.

45. Hiyoshi Y, Schetter AJ, Okayama H, Inamura K, Anami K, Nguyen GH, et al. Increased microRNA-34b and-34c predominantly expressed in stromal tissues is associated with poor prognosis in human colon cancer. PLoS One. 2015;10(4):e0124899.

46. Shimomura A, Shiino S, Kawauchi J, Takizawa S, Sakamoto H, Matsuzaki J, et al. Novel combination of serum microRNA for detecting breast cancer in the early stage. Cancer Sci. 2016;107(3):326–34.

47. Li J, Wang Y, Luo J, Fu Z, Ying J, Yu Y, et al. miR-134 inhibits epithelial to mesenchymal transition by targeting FOXM1 in non-small cell lung cancer cells. FEBS Lett. 2012;586(20):3761–5.

48. Gao Y, Liu T, Huang Y. MicroRNA-134 suppresses endometrial cancer stem cells by targeting PO-GLUT1 and Notch pathway proteins. FEBS Lett. 2015;589(2):207–14.

49. Yamada Y, Hidaka H, Seki N, Yoshino H, Yamasaki T, Itesako T, et al. Tumor-suppressive micro RNA-135a inhibits cancer cell proliferation by targeting the c-MYC oncogene in renal cell carcinoma. Cancer Sci. 2013;104(3):304–12.

50. Zhou W, Bi X, Gao G, Sun L. miRNA-133b and miRNA-135a induce apoptosis via the JAK2/STAT3 signaling pathway in human renal carcinoma cells. Biomed Pharmacother. 2016;84:72.

Supplemental tables and figures

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Table 9.S2. *Multivariate Cox regressions for overall survival after diagnosis, testing selected miRNAs against International Metastatic ccRCC Database Consortium (IMDC) risk groups and molecular subtype. All miRs except for miR 30c-5p remained independent predictors for OS. Molecular subtype remained an independent predictor as well, except when tested against miRs 21-3p (up in unfavorable ccrcc1_4) and 204-5p (up in favorable ccrcc2_3). IMDC risk groups: good (G), intermediate (I) and poor (P).*

miRNA and MDC			**miRNA and moecular subtype**		
	p-value	**hazard ratio**		**p-value**	**hazard ratio**
let-7i-5p	0.0001	1.7	let-7i-5p	0.003	1.6
IMDC-I vs G	0.082	1.9	ccrcc2_3 vs 1_4	0.033	0.6
IMDC-P vs G	0.001	3.7			
21-3p	0.0001	1.4	21-3p	0.006	1.4
IMDC-I vs G	0.068	2.0	ccrcc2_3 vs 1_4	0.057	0.6
IMDC-P vs G	0.005	3.2			
21-3p	< 0.0001	1.7	21-3p	0.003	1.6
IMDC-I vs G	0.034	2.2	ccrcc2_3 vs 1_4	0.010	0.5
IMDC-P vs G	0.0001	4.1			
30c-5p	0.095	0.9	30c-5p	0.124	0.8
IMDC-I vs G	0.058	2.0	ccrcc2_3 vs 1_4	0.019	0.5
IMDC-P vs G	0.002	3.6			
34c-5p	< 0.0001	1.3	34c-5p	0.004	1.2
IMDC-I vs G	0.036	2.2	ccrcc2_3 vs 1_4	0.020	0.6
IMDC-P vs G	0.002	3.5			
134-5p	0.007	1.2	134-5p	0.034	1.2
IMDC-I vs G	0.049	2.1	ccrcc2_3 vs 1_4	0.021	0.6
IMDC-P vs G	0.001	3.6			
135a-5p	0.001	0.8	135a-5p	0.038	0.9
IMDC-I vs G	0.024	2.3	ccrcc2_3 vs 1_4	0.013	0.5
IMDC-P vs G	0.001	4.0			
146b-5p	< 0.0001	1.4	146b-5p	0.001	1.4
IMDC-I vs G	0.036	2.2	ccrcc2_3 vs 1_4	0.008	0.5
IMDC-P vs G	0.002	3.5			
193b-5p	0.002	1.2	193b-5p	0.036	1.2
IMDC-I vs G	0.031	2.3	ccrcc2_3 vs 1_4	0.008	0.5
IMDC-P vs G	0.001	3.7			
204-5p	< 0.0001	0.7	204-5p	< 0.0001	0.7
IMDC-I vs G	0.045	2.1	ccrcc2_3 vs 1_4	0.109	0.7
IMDC-P vs G	0.003	3.3			
425-5p	0.003	1.6	425-5p	0.032	1.5
IMDC-I vs G	0.016	2.5	ccrcc2_3 vs 1_4	0.011	0.5
IMDC-P vs G	0.001	3.7			
1260a	< 0.0001	0.7	1260a	0.003	0.8
IMDC-I vs G	0.037	2.2	ccrcc2_3 vs 1_4	0.011	0.5
IMDC-P vs G	0.001	3.7			
1307-3p	0.010	1.3	1307-3p	0.017	1.3
IMDC-I vs G	0.062	2.0	ccrcc2_3 vs 1_4	0.004	0.5
IMDC-P vs G	0.001	3.7			
7977	< 0.0001	0.7	7977	< 0.0001	0.7
IMDC-I vs G	0.048	2.1	ccrcc2_3 vs 1_4	0.008	0.5
IMDC-P vs G	0.001	3.9			

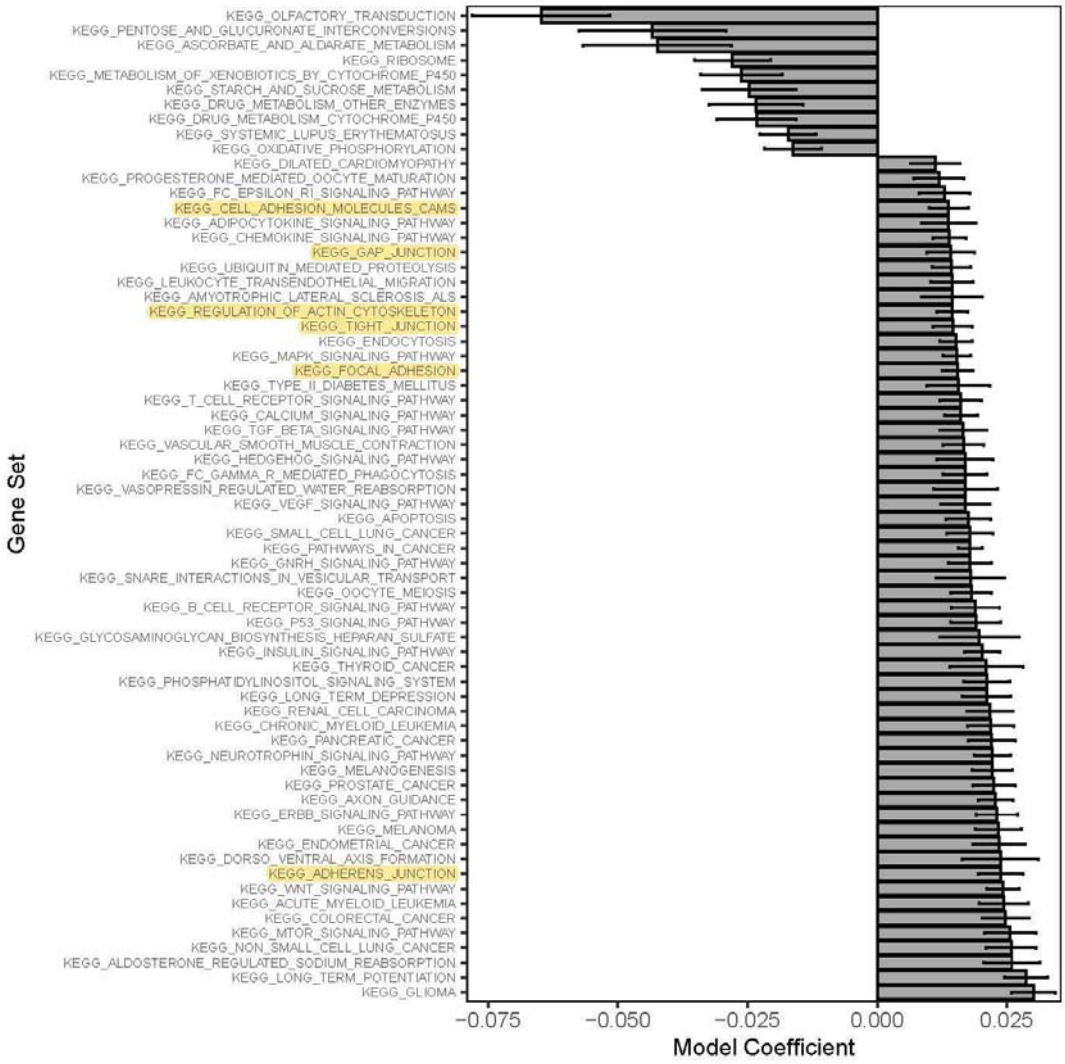

Figure 9.S1. *KEGG gene set enrichment analysis on subtype-specific miRNAs showed less suppression of pathways involved in retaining an epithelial phenotype in ccrcc2_3 tumors, suggesting less possibility for epithelial-to-mesenchymal transition in these tumors***.**

The biological interpretation of the model coefficient can be stated as follows (in the context of two-group comparison, i.e., ccrcc2_3 vs ccrcc1_4): if the coefficient is positive, miRNA inhibition on target mRNAs might be lifted, thereby leading to less suppression on the gene set of interest in the experimental group.

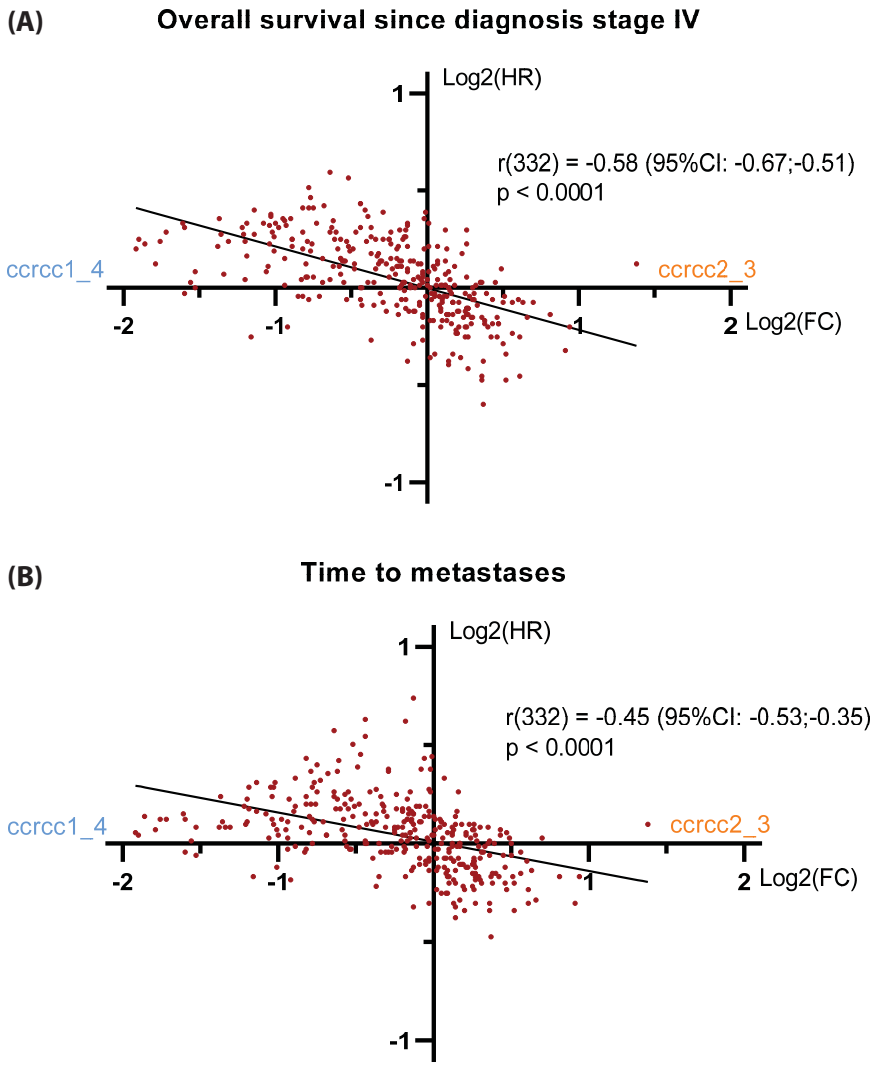

Figure 9.S2. *Differences in miRNA expression were similar across outcome parameters: OS since diagnosis (main text), OS since stage IV* ***(A)*** *and time to metastases* ***(B)****. HR = hazard ratio; FC = fold change.*

Chapter 10

Identification of molecular subtypes in clear-cell renal cell carcinoma on paraffin-embedded tissue and molecular characterization of metastatic lesions

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Project ongoing

The authors listed have contributed significantly to the data presented below. As this chapter describes a work in progress, they have not been given the opportunity to critically review all preliminary results. The PhD candidate therefore takes full responsibility should there be any flaws or inconsistencies.

Chapter 10: Contents

Summary 145
Disclaimer 146
Introduction 146
Preliminary results and discussion 147
The ccrcc4 subtype is highly inflamed 150
Ccrcc1 to -4 subtypes correlate well with the predictive IMmotion150 signatures 150
Ccrcc1 to -4 molecular subtypes outperform Angio score for outcome on anti-angiogenic therapy 152
The molecular subtypes and myeloid cell infiltration are prognostic for overall survival 154
Ccrcc1 tumors appear driven by chr14q loss 154
Ccrcc3 tumors overexpress mitochondrial pathways 154
Metastases often share the same subtype within patients 156
Angiogenic signature is highly conserved within patients 156
Sarcomatoid dedifferentiation is conserved within patients and less frequent in metastases 156
Conclusion and ongoing work 158
Methods 159
Method details 159
Quantification and statistical analysis 160
Analysis of clinical outcomes 162
References 163
Supplemental tables and figures 165

Summary

Clear-cell renal cell carcinoma (ccRCC) can be divided into four mRNA expression-based molecular subtypes, ccrcc1 to -4, with different prognosis and response to targeted therapy. The major drawback of the current classification protocol is the necessity to use fresh-frozen tissue. Here, we validate the ccrcc1-4 subtypes on 132 formalin-fixed paraffin-embedded (FFPE) stage IV primary ccRCC, through unsupervised clustering of RNAseq data. The obtained clusters are robust and correlate well with their frozen-based counterparts. The ccrcc2 subtype is highly angiogenic, overlapping almost perfectly with the "Angio High" predictive signature from the IMmotion150 trial, and has a 60% response rate to 1st line anti-angiogenic therapy. The dismall ccrcc4 subtype shows high expression of MYC-targets and immunological signatures, including interferon gamma, cytotoxic T-cell and M2 macrophage signatures. The ccrcc1 subtype's prognosis and gene expression is intermediate between ccrcc2 and ccrcc4, with gene set enrichment analysis suggesting frequent arm level loss of chr14q. The rare ccrcc3 subtype is atypical, with overexpression of mitochondrial pathways. It may resemble Birt-Hogg-Dubé related ccRCC and require tailored treatment decisions. The ccrcc1 to -4 subtypes outperform the IMmotion150 signatures in predicting outcome on 1st line anti-angiogenic therapy, and survive multivariate analysis together with Myeloid score to predict overall survival at diagnosis. RNAseq of 174 ccRCC metastases reveals that metastases tend to share the same subtype within patients, and Angio score and sarcomatoid features are highly conserved within patients, whereas this is the case to a lesser extent for immune cell signatures. There is no clear signal for an influence of metastatic location on Angio or immune signatures, but a more in-depth analysis including more samples is ongoing.

In conclusion, we have developed a robust and user-friendly pipeline for the molecular classification of FFPE ccRCC samples. The novel FFPE-based subtypes outperform the IMmotion150 signatures for response prediction to anti-angiogenic therapy and shed new light on the pathogenesis of ccrcc1 and ccrcc3 tumors. In ccRCC metastases, angiogenic gene expression and sarcomatoid features are highly conserved.

Disclaimer

Upon revision of the bioinformatics pipeline, we have unfortunately discovered an error in the preprocessing of the raw RNAseq read counts. We therefore consider our current results not fully reliable and are repeating the analyses. Nevertheless we present our preliminary results here, as their correlation with clinical and biological data is so solid that they seem unlikely to undergo major changes upon proper analysis.

Introduction

Ever since we discovered the ccrcc1 to -4 molecular subtypes on fresh-frozen clear-cell renal cell carcinomas (ccRCC) in 2015, our long-term goal has been to develop a predictor based on formalin-fixed paraffin-embedded (FFPE) tissue. (1) The reasons are that fresh-frozen samples are not routinely collected and that they are delicate and tedious to work with, which thwarts the implementation of the classification protocol in other labs. Our initial approach was to develop an FFPE-based classifier by clustering a limited gene set (n=192) using a customized NanoString nCounter® panel on 150 tumors. Unfortunately, only 60% of samples yielded reliable results, mainly due to poor RNA quality of FFPE tissue, and these samples did not group into robust clusters, possibly because the gene set was too limited. These problems are reminiscent of those encountered with the consensus subtypes in colon cancer. (2) We therefore decided to switch to RNA sequencing (RNAseq), which has recently become both affordable and reliable for low-quality RNA. Even more importantly, RNAseq yields whole transcriptome data, thus providing much richer information beyond merely a basis to establish molecular subtypes. With this advantage in mind, we embarked on a more ambitious goal: the molecular characterization of ccRCC metastases. In ccRCC, as in most other tumor types, treatment decisions for metastatic disease are based on the features of the primary tumor, even though the extent to which metastases resemble their primary tumor or are influenced by their metastatic niche remains largely unknown. We have taken advantage of the extensive tissue bank at University Hospitals Leuven to include a large set of ccRCC metastases for RNAseq.

Preliminary results and discussion

Validation of the ccrcc1 to -4 molecular subtypes on FFPE tissue

We were able to obtain good-quality transcriptomic data for 87% of all samples that were put through RNA extraction and sequencing (319/368, supplemental Figure 10.S1), including core needle biopsies. The robustness of this protocol is reassuring, as the quality of FFPE-derived RNA is typically low and core needle biopsies are becoming more frequent with dwindling rates of cytoreductive nephrectomy. (3) We performed unsupervised consensus clustering on the 25% most variable genes of the bulk RNAseq data of 132 primary FFPE ccRCC and identified four biologically different clusters (Figure 10.1). The concordance between the new FFPE-based clusters and the ccrcc1 to -4 subtypes that were previously identified on frozen tissue from the same tumors, was high for ccrcc2, -3 and -4 tumors: 60%, 75% and 60% respectively. The discordant samples reflect not only reclassification arising from a new clustering, but also intratumor heterogeneity: the corresponding FFPE and frozen samples were derived from different parts of the tumor, and there is known heterogeneity of molecular subtypes within the same lesion (49% on our own frozen samples and 80% for the Brannon ccA/ccB subtypes). (4) In contrast, the concordance between the fourth FFPE cluster and previously identified ccrcc1 tumors, was only 39%. This suggests that the ccrcc1 subtype is an intermediate subtype without distinctive expression signatures. This hypothesis was corroborated by principal component analysis, that showed a clear separation of the ccrcc2, -3 and -4 subtypes, with ccrcc1 tumors interspersed between ccrcc2 and -4 (Figure 10.1B). It is also supported by previous miRNA expression analyses on the frozen-tissue derived clusters, where ccrcc1 tumors showed intermediate miRNA expression patterns (unpublished data).

The baseline clinical and histological characteristics of the FFPE-derived ccrcc1 to -4 subtypes, corresponded well with those described previously on fresh-frozen tissue (Table 10.1). The ccrcc4 subtype showed the most aggressive phenotype, with enrichment for sarcomatoid tumors, higher Fuhrman grades, synchronous metastases and International Metastatic ccRCC Database Consortium (IMDC) Poor risk patients. The rare ccrcc3 subtype was characterized by the most favorable features, followed by the ccrcc2 subtype. Ccrcc1 tumors had intermediate characteristics between ccrcc4 and ccrcc2. We were able to validate the predictive value of the FFPE-based molecular subtypes for 1st line treatment with sunitinib or pazopanib. Ccrcc2 tumors had very high response rates (60%), whereas most ccrcc4 were progressive at first follow-up (63%).

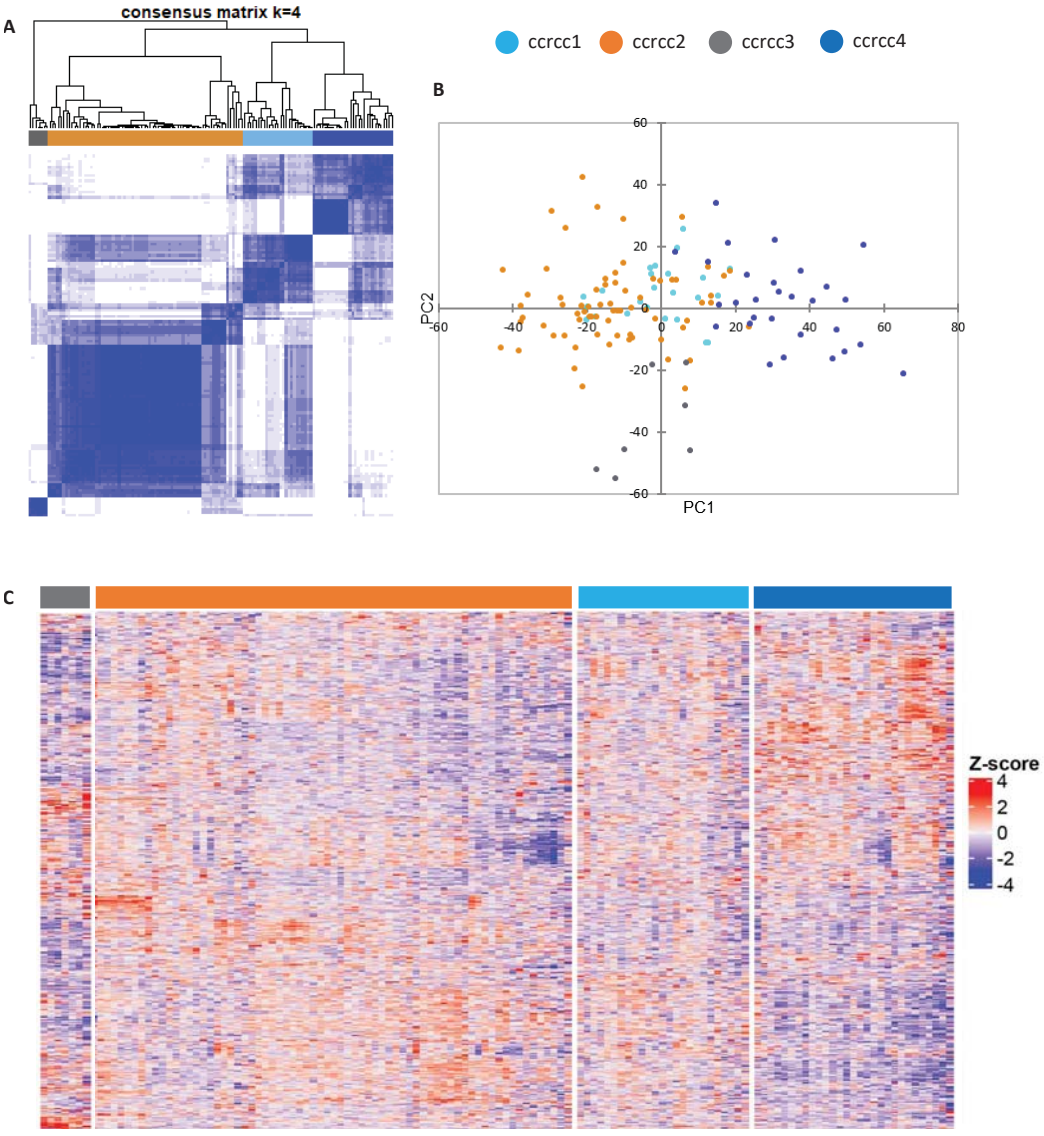


Figure 10.1. *Unsupervised clustering of RNAseq data of 132 primary ccRCC in the metastatic setting reveals four molecular subtypes. (**A**) Consensus cluster matrix. (**B**) Subtype ccrcc2, -3 and –4 separate spontaneously on a principal component plot, with subtype ccrcc1 interspersed between ccrcc2 and –4. (**C**) Heatmap of the most variable genes.*
PC = principal component.

Table 10.1. Patient characteristics.

	ccrcc1		ccrcc2		ccrcc3		ccrcc4		p-value
Number	25	*19%*	71	*54%*	7	*5%*	29	*22%*	
Male	16	*64%*	54	*76%*	3	*43%*	18	*62%*	
OS since diagnosis (mo)	53		74		55		18		p<0.0001
Characteristics at diagnosis (n=132)									
Age (median)	62		64		62		63		
Synchonous metastases	17	*68%*	34	*48%*	1	*14%*	17	*59%*	
T-stage									
1	4	*16%*	17	*25%*	1	*14%*	0	*0%*	
2	3	*12%*	12	*18%*	0	*0%*	2	*7%*	
3	17	*68%*	35	*51%*	6	*86%*	23	*82%*	
4	1	*4%*	4	*6%*	0	*0%*	3	*11%*	
Unknown			3				1		
Nodal status									
Negative	15	*83%*	40	*82%*	4	*80%*	14	*52%*	
Positive	3	*17%*	9	*18%*	1	*20%*	13	*48%*	
Unknown	7		22		2		2		
Fuhrman grade									
1	0	*0%*	0	*0%*	0	*0%*	0	*0%*	
2	2	*8%*	3	*4%*	0	*0%*	0	*0%*	
3	5	*20%*	27	*39%*	4	*57%*	1	*3%*	
4	18	*72%*	40	*57%*	3	*43%*	28	*97%*	
Unknown			1						
Sarcomatoid features									
0%	19	*79%*	53	*83%*	7	*100%*	10	37%	
1-24%	5	*21%*	11	*17%*	0	*0%*	10	*37%*	
≥25%	0	*0%*	0	*0%*	0	*0%*	7	26%	
Unknown	1		7				2		
IMDC at start of systemic therapy (n=117)									
Good	3	*13%*	6	*10%*	2	*29%*	0	*0%*	
Intermediate	14	*61%*	42	*71%*	4	*57%*	16	*62%*	
Poor	6	*26%*	11	*19%*	1	*14%*	10	*38%*	
Unknown	1		1						
Outcome on 1st line sunitinib/pazopanib (n=93)									
PFS (mo)	16		14		8		2		p<0.0001
Best response									
Partial/complete	10	*50%*	25	*60%*	1	*20%*	3	*18%*	
Stable disease	9	*45%*	12	*29%*	3	*60%*	3	*18%*	p<0.0001
Progressive disease	1	*5%*	5	*12%*	1	*20%*	11	*65%*	
OS (mo)	22.5		25		37		6		p<0.0001

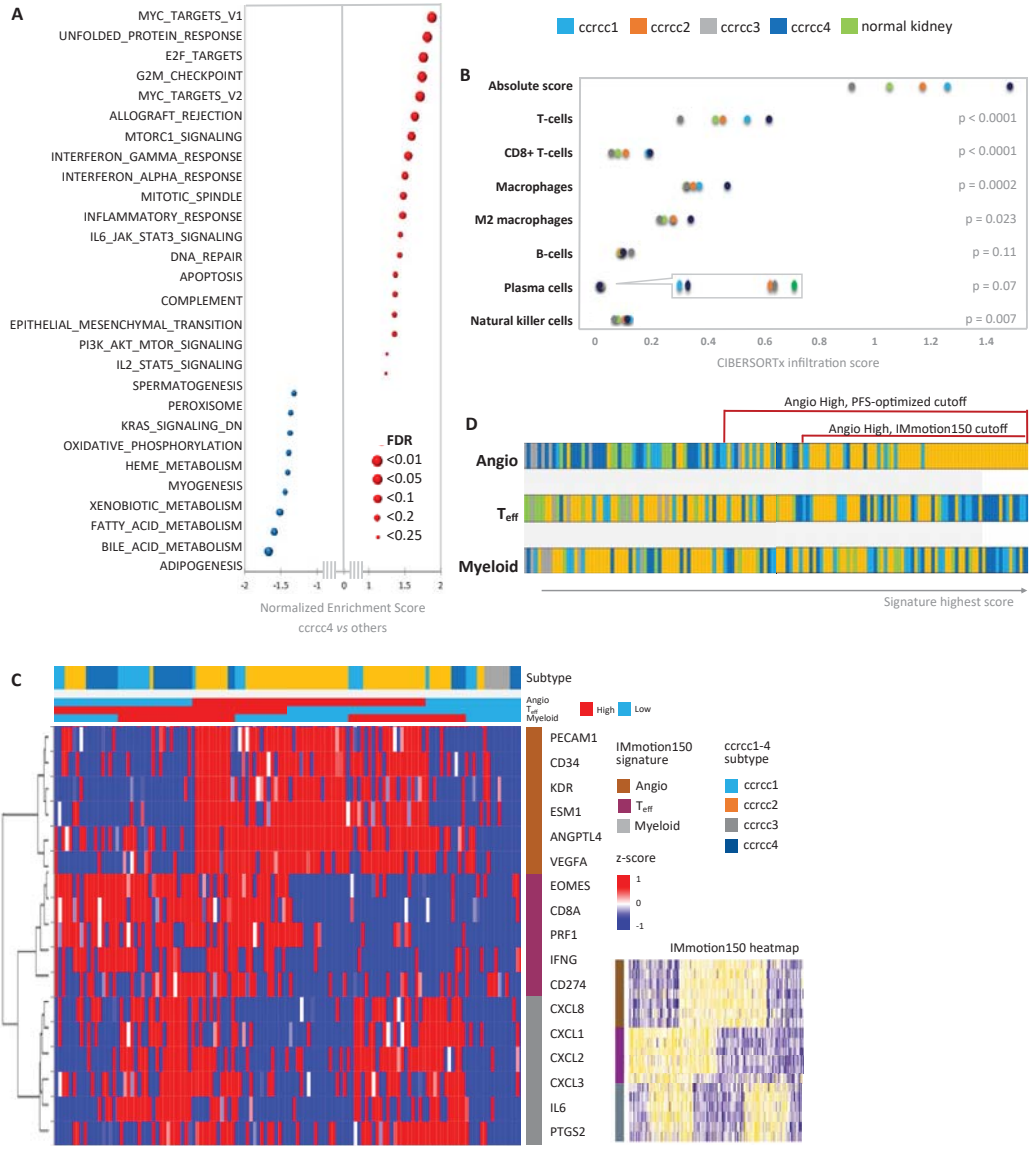
OS = overall survival; IMDC = International Metastatic RCC Database Consortium; PFS = progression-free survival.

The ccrcc4 subtype is highly inflamed

Gene set enrichment analysis (GSEA) of Hallmark and Immunologic gene sets showed an aggressive phenotype of ccrcc4 tumors with enrichment of MYC-targets, proliferation and apoptosis pathways, whereas metabolic signatures were downregulated compared to other tumors. Most strikingly, there was a global upregulation of immune pathways in ccrcc4 tumors (3198 of 4872 Immunologic gene sets upregulated, but none significantly downregulated with FDR<0.05). GSEA results for ccrcc2 tumors were the opposite of the results for ccrcc4, again implying that these two are the most characteristic ccRCC subtypes, whereas ccrcc1 tumors are intermediate and ccrcc3 too rare to have a major impact (Figure 10.2A). In concordance with these findings, the Cytolytic score, a measure for cytotoxic T-cell activity, was highest for ccrcc4 tumors ($p<0.0001$), as well as the estimate of total immune cell infiltration by CIBERSORTx ($p<0.0001$) (Figure 10.2B). Interestingly, immune deconvolution by CIBERSORTx suggested that despite their high immune infiltrate, including high levels of CD8+ T-cells and M2 macrophages, the numbers of plasma cells were lower in ccrcc4 tumors compared to ccrcc2. Plasma cells are necessary for maturation of tumor-specific cytotoxic T-cells in tertiary lymphoid structures, which have previously been shown to be lacking in many highly infiltrated ccRCC. (5)

Ccrcc1 to -4 subtypes correlate well with the predictive IMmotion150 signatures

The most important predictive molecular biomarkers tested in a randomized controlled trial setting thus far, are the Angio, Teff and Myeloid signatures of the IMmotion150 trial. (6) In this phase 2 trial, high Angio scores were predictive for response to sunitinib, whereas a combined T_{eff} high/Myeloid high score was predictive for response to atezolizumab + bevacizumab. In T_{eff} high/Myeloid low tumors, the addition of bevacizumab to atezolizumab did not seem to improve efficacy. To our surprise, upon calculation of these signatures in our cohort, we found almost identical frequencies of Angio, T_{eff} and Myeloid high/low tumors, providing a robust cross-platform validation of these signatures (Figure 10.2C). Importantly, there was a strong correlation between the signatures and the molecular subtypes (Figure 10.2C-E). Ccrcc2 tumors were a highly selective marker for Angio high tumors (sensitivity 85%, specificity 77%). Ccrcc4 tumors



E

	Angio High	Teff High	Myeloid High
ccrcc1	32%	64%	64%
ccrcc2	79%	39%	47%
ccrcc3	0%	0%	0%
ccrcc4	7%	76%	59%
P-value	<0.0001	0.0002	0.017

Figure 10.2. *(**A**) Gene set enrichment analysis (Hallmark gene set) of ccrcc4 vs other subtypes shows upregulation of MYC-targets and inflammatory pathways, and downregulation of metabolic pathways. (**B**) Immune deconvolution through CIBERSORTx shows higher levels of T-cell, macrophage and natural killer cell infiltration, but not B-cell infiltration, in ccrcc4 > ccrcc1 > ccrcc2 > ccrcc3 tumors. (C-E) The predictive IMmotion150 Angio, Teff and Myeloid signatures differ significantly between the ccrcc1 to –4 subtypes.*
FDR = false discovery rate; PFS = progression free survival.

were mostly T_{eff} high (76%) and Angio low (93%), whereas ccrcc1 tumors had intermediate Angio and T_{eff} scores. Myeloid scores were only slightly higher in ccrcc4. Ccrcc3 tumors had very low Angio, T_{eff} and Myeloid scores, dipping even below those of normal kidney tissue. This suggests a very different tumor biology from that of classical ccRCC, with implications for personalized treatment decisions.

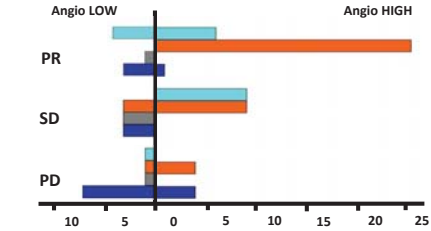
Ccrcc1 to -4 molecular subtypes outperform Angio score for outcome on anti-angiogenic therapy

The main distinguishing characteristic of ccrcc2 tumors is their highly angiogenic profile. Yet, although response rates to 1st line sunitinib/pazopanib were indeed highest among ccrcc2 tumors, the ccrcc1 subtype had comparable PFS and OS (Figure 10.3A-C). This is in concordance with Hakimi et al, who reported that three non-inflamed ccRCC subtypes (≈ ccrcc1,-2,-3) had comparable outcomes on 1st line sunitinib/pazopanib, whereas outcomes of inflamed tumors (≈ ccrcc4) were much worse. (7) This led us to hypothesize that IMmotion150 cutoff for Angio high tumors, which is based on the median and corresponds well with ccrcc2 tumors, may be too restrictive. Indeed, the optimal Angio score cutoff for predicting PFS in our series was well below the median and classified many additional ccrcc1 tumors as Angio High. The new cutoff outperformed the median-based cutoff in identifying tumors with partial response, and performed equally for tumors with progressive disease (Figure 10.2D, 3A). When tested in a multivariate model, only the molecular subtypes and IMDC risk groups remained predictive for PFS, and only the molecular subtypes for objective response. Angio score lost its significance in a multivariate analysis, whereas other signatures (T_{eff}, Myeloid, Cytolytic score and infiltration scores by CIBERSORTx) where not significantly associated with PFS or response on univariate Cox or logistic regression (Figure 10.3D).

A recent analysis of microarray data from the COMPARZ trial has suggested different biomarkers for outcome on 1st line sunitinib *vs* pazopanib. (7) An angiogenic signature was correlated with improved response, PFS and OS on sunitinib but not pazopanib. In contrast, an M2 macrophage signature was correlated with worse response, PFS ad OS on pazopanib, but not sunitinib. In our cohort, there was no differential effect of these markers depending on type of TKI. Moreover, although the angiogenic signature was indeed correlated with response, PFS and OS for both sunitinib and pazopanib, this was not the case for M2 macrophage infiltration with either TKI (supplemental Table 10.S1). Our findings should be interpreted with caution though, as the patient numbers in

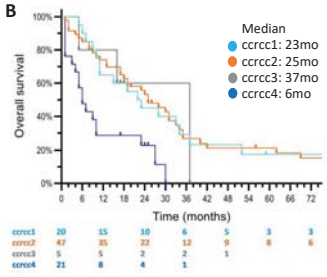
Outcomes on first-line sunitinib/pazopanib

A Best response by PFS-optimized cutoff for Angio score



	PR	SD	PD
ccrcc1	50%	54%	5%
ccrcc2	60%	29%	12%
ccrcc3	20%	60%	20%
ccrcc4	18%	18%	65%
Angio High	55%	31%	14%
Angio Low	30%	33%	37%

B



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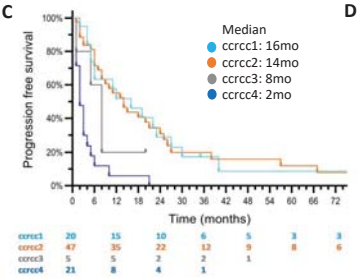
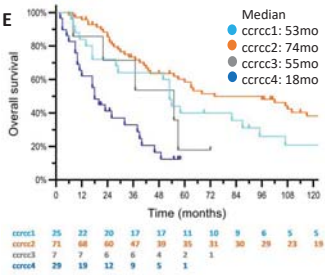


D Multivariate Cox regression model for PFS

	HR (95% CI)	p-value
ccrcc3 vs –2	3.0 (1.0-9.3)	0.052
ccrcc1 vs –2	1.1 (0.63-2.0)	0.69
ccrcc4 vs –2	4.2 (2.2-8.0)	<.0001
IMDC I vs G	2.2 (0.94-5.2)	0.068
IMDC P vs G	4.0 (2.2-8.0)	0.004

Overall survival since diagnosis

E



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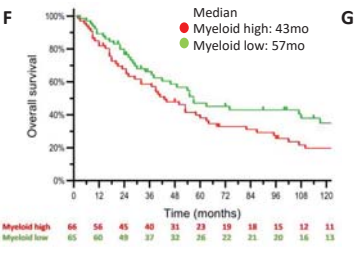

G Multivariate Cox regression model for OS

	HR (95% CI)	p-value
ccrcc3 vs –2	3.5 (1.3-9.3)	0.013
ccrcc1 vs –2	1.4 (0.84-2.4)	0.20
ccrcc4 vs –2	4.0 (2.3-6.9)	<.0001
Myeloid score	1.8 (1.3-2.6)	0.001

Figure 10.3. *Panels showing the outcomes on first-line sunitinib/pazopanib and OS since diagnosis. (A) The cutoff used to define "Angio High" tumors is based on optimal predictive power for PFS, not the median-based cutoff from IMmotion150 (cf Fig2D). (D, G) The multivariable model used all variables with p<0.1 on univariate Cox regression. For PFS: molecular subtype, Angio score, Myeloid score, IMDC. For OS: age at diagnosis, ccrcc1 to –4 subtype, Angio score, Teff score, Myeloid score, plasma cell infiltration, T-cell infiltration, macrophage infiltraten, absolute immune cell infiltration. Angio, Teff and Myeloid scores were included as continuous variable. Immune cell infiltration was estimated through CIBERSORTx.*

PFS = progression free survival; PR = partial remission; SD = stable disease; PD = progressive disease, OS = overall survival; IMDC = International Metastatic ccRCC Database Consortium.

our cohort were much smaller compared to the COMPARZ cohort.

The molecular subtypes and myeloid cell infiltration are prognostic for overall survival

We tested the impact on OS from diagnosis of molecular subtype, the IMmotion 150 signatures, Cytolytic score, CIBERSORTx immune infiltration scores, age and gender, using Cox regression models. The final multivariate model consisted of both molecular subtype and Myeloid signature. For OS from start of systemic therapy, the model also included IMDC (Figure 10.3E-G). The association of Myeloid score with OS was surprisingly robust (HR 1.8, p=0.001 on multivariate). Although the immune suppressing capacity of myeloid cells is well described, they have not yet been proposed as a prognostic marker in ccRCC to the best of our knowledge. Myeloid signature was not associated with PFS or response rate on 1st line sunitinib/pazopanib, in contrast with the molecular subtypes.

Ccrcc1 tumors appear driven by chr14q loss

There were no discriminative signatures for ccrcc1 tumors upon GSEA of Hallmark and Immunologic genes. However, upon analyzing all other signatures in the Molecular Signatures Database, we found significant downregulation of seven loci on the long arm of chromosome 14 (chr14q) (Figure 10.4A). As arm level loss of chr14q is a frequent event in ccRCC, occurring in 45% of cases, this led us to hypothesize that samples were allocated to the ccrcc1 cluster if they showed intermediate gene expression and loss of chr14q. (8) Indeed, loss of chr14q can explain the more unfavorable characteristics of ccrcc1 tumors compared to ccrcc2, as *HIF1A* is located on chr14q and its loss results in compensatory upregulation of *HIF2A*, which has more oncogenic effects. (9,10) Copy number analyses to test this hypothesis are ongoing.

Ccrcc3 tumors overexpress mitochondrial pathways

The rare ccrcc3 subtype had very atypical features. They had favorable prognostic features and an indolent disease course, despite low response rates to VEGFR-TKI (Table 10.1, Figure 10.3). The patients were more often young (29% < 45 yrs) and female (57%). Their Angio, T_{eff} and myeloid scores were particularly low, dipping even below those of normal kidney tissue (Figure 10.2C-E). This suggests a very different tumor biology from that of classical ccRCC, with implications for tailored treatment decisions. Upon GSEA, the ccrcc3 tumors

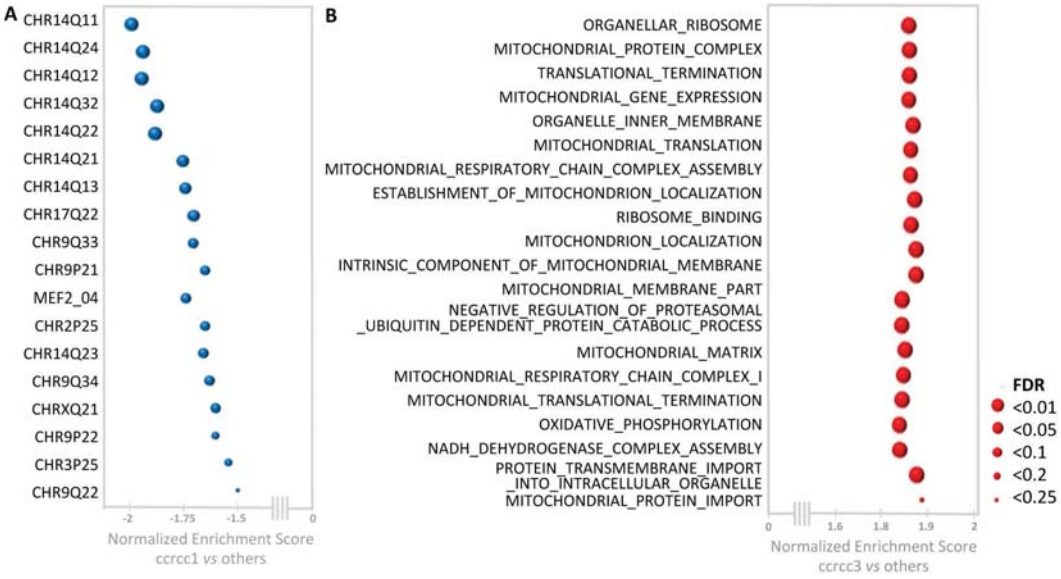

Figure 10.4. *Gene set enrichment analysis of all gene sets in the Molecular Signatures Database of ccrcc1 (A) and ccrcc3 (B) tumors vs other subtypes. Results of the 20 gene sets with the highest absolute normalized enrichment score and false discovery rate (FDR) < 0.25 are shown.*

showed a striking upregulation of mitochondrial pathways as sole distinguishing feature (Figure 10.4B). This led us to hypothesize that there may be a link between ccrcc3 tumors and Birt-Hogg-Dubé related RCC, which are hallmarked by the accumulation of mitochondria. Birt-Hogg-Dubé syndrome is caused by a germline mutation in the *FLCN* gene, resulting in a dysfunctional folliculin protein. This gives rise to RCC that accumulate mitochondria and upregulate the mTOR pathway. Indeed, the ccrcc3 tumors also showed upregulation of mTOR pathways and of FLCN, which may be compensatory mechanism for folliculin dysfunction. Upon histological review of ccrcc3 tumors, they all showed a typical eosinophilic cytoplasm, which is seen in some types of RCC, among which Birt-Hogg-Dubé RCC. To test the hypothesis that ccrcc3 tumors may be driven by a somatic loss of FLCN, we are now performing mutation analysis on ccrcc3 tumors, and RNAseq of confirmed Birt-Hogg-Dubé RCC for molecular classification. If Birt-Hogg-Dubé RCC are indeed similar to ccrcc3 tumors, this implies that they have a disease biology that is fundamentally different from classical RCC. These findings will therefore help to personalize the therapeutic approach for these patients.

Metastases often share the same subtype within patients

We performed RNAseq of 174 metastases from 85 patients and classified these using the K-nearest neighbor method (Figure 10.5A). Within patients, molecular subtype was conserved in 78% of metastasis pairs (177/226), showing a remarkable homogeneity (Figure 10.5B). However, among pairs of primary and metastatic lesions, the subtype was conserved in only 59% of cases (55/94). This implies that the primary tumor may not be sufficiently representative for characterization of its metastases, as suggested before by Serie et al. (11) Intriguingly, it also suggests more homogeneity among metastases compared to the primary tumors. Among primary-metastatic couples with a different subtype, the metastatic lesion had a more aggressive subtype in 75% of cases. It can therefore be expected that ccrcc1 and ccrcc4 subtypes are more frequent in metastatic lesions, compared to primary tumors. This was not the case in our series, but this is probably an effect of sampling bias, as ccrcc2 tumors are more likely to undergo metastasectomy.

Angiogenic signature is highly conserved within patients

To assess to what extent the angiogenic and immune microenvironment is conserved among different lesions within patients, we used paired t-tests to compare the IMmotion150 signatures and CIBERSORTx immune infiltration scores between primary tumors and the average scores of their paired metastases. These showed no significant differences between primary tumors and their metastases (Table 10.2). Moreover, R-squared models that considered a random patient effect, showed that angiogenic gene expression was highly conserved: 68% of Angio score variability is explained by patient effect. A substantial portion of the variability of immune scores was explained by patient effect as well, e.g. 37% of T_{eff} score and 35% of Myeloid score (Table 10.3). For M2 macrophage and total immune cell infiltration, as estimated by CIBERSORTx, molecular subtype could explain a substantial part of the variability in addition to patient effect. The location of metastatic lesions did not influence angiogenic and immune scores according to R-squared models. However, the low number of measurements per location and crude signatures used could mask more subtle differences.

Sarcomatoid dedifferentiation is conserved within patients and less frequent in metastases

The ccrcc4 subgroup was highly enriched for sarcomatoid RCC, both in

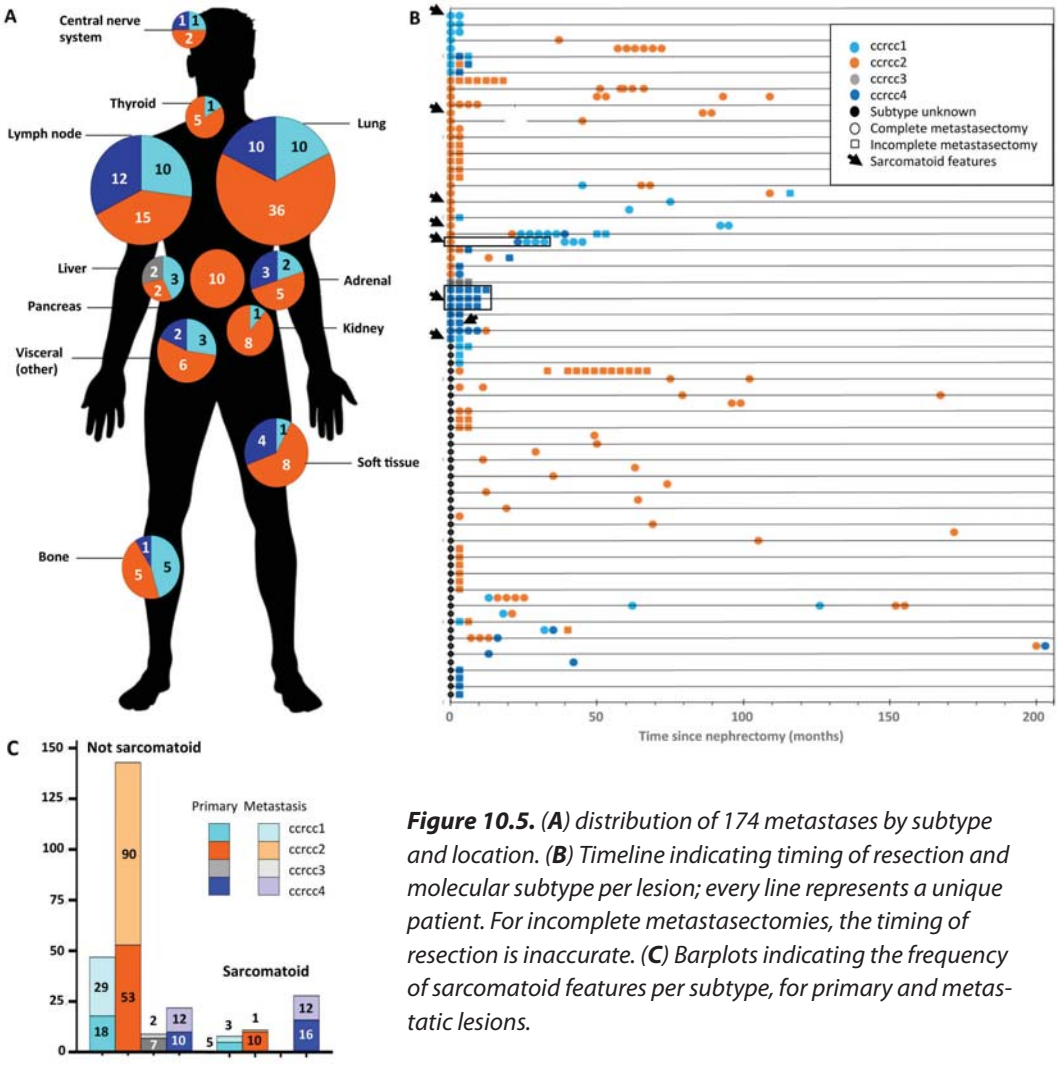

Figure 10.5. *(**A**) distribution of 174 metastases by subtype and location. (**B**) Timeline indicating timing of resection and molecular subtype per lesion; every line represents a unique patient. For incomplete metastasectomies, the timing of resection is inaccurate. (**C**) Barplots indicating the frequency of sarcomatoid features per subtype, for primary and metastatic lesions.*

Table 10.2. *Paired t-tests reveal no substantial differences in angiogenic and immune signatures between primary tumors and the average scores of their paired metastases.*

	Mean difference	p-value
IMmotion150 signatures		
Angio score	-0.18	0.18
T_{eff} score	0.09	0.40
Myeloid score	0.01	0.41
CIBERSORTx estimate of immune cell infiltration		
Absolute score	0.12	0.043
CD8+ T-cells	0.02	0.17
M2 macrophages	0.02	0.42
Plasma cells	0.0004	0.84

Table 10.3. *R-square models estimating the impact of patient effect, molecular subtype and metastatic location on angiogenic and immune signatures.*

Variable	Patient	Subtype*	Location*	Patient + subtype	Patient + location	Subtype + location	Patient + subtype + location
IMmotion150 signatures							
Angio score	0.68	0.44	0.02	0.67	0.70	0.48	0.68
T_{eff} score	0.37	0.17	0.06	0.39	0.43	0.22	0.46
Myeloid score	0.35	0.09	0.04	0.32	0.34	0.12	0.32
CIBERSORTx estimate of immune cell infiltration							
Absolute score	0.21	0.25	0.08	0.31	0.25	0.30	0.36
CD8+ T-cells	0.30	0.15	0.06	0.34	0.36	0.21	0.41
M2 macrophages	0.22	0.10	0.05	0.33	0.28	0.14	0.38
Plasma cells	0.53	0.02	0.03	0.54	0.52	0.06	0.54

Patient is considered as a random effect, subtype and location as fixed effects.
**The marginal R-squared (which considers only fixed factors) is used to estimate the proportion of the variance explained by subtype and location. The conditional R-squared is used in the other models.*

primary and metastatic lesions (Figure 10.5C). The frequency of sarcomatoid features was lower in metastases (11% *vs* 26% in primary RCC, p=0.001 after correction for molecular subtype), which is surprising as one would expect more aggressive histological features in metastatic lesions. This difference could not be explained by sampling bias (assuming that primary sarcomatoid RCC are less likely to have metastases resected): on the contrary, sarcomatoid features were present in a high number of primary RCC with resected metastases (29%). The presence of sarcomatoid features was highly conserved in primary and metastatic lesions within patients: sarcomatoid metastases were found in 4 of 9 patients with a sarcomatoid primary tumor, but only in 1 of 25 patients without a sarcomatoid primary tumor (Figure 10.5B).

Conclusion and ongoing work

We have developed a robust and user-friendly pipeline for the molecular classification of FFPE ccRCC samples. The novel FFPE-based subtypes outperform the IMmotion150 signatures for prediction of response to anti-angiogenic therapy and shed new light on the pathogenesis of ccrcc1 and ccrcc3 tumors. In ccRCC metastases, angiogenic gene expression and sarcomatoid features are highly conserved.

After correction of our bioinformatics pipeline, we will repeat the current clustering and develop a classifier algorithm, that will be tested on a large

validation cohort that is available from an independent lab. This classifier algorithm will allow for the implementation of the ccrcc1-4 subtypes as a biomarker across different labs. To test our hypotheses on chr14q loss in ccrcc1 and somatic *FLCN* mutation in ccrc3 tumors, we will perform targeted copy number and mutations analyses. We have also sequenced a new batch of primary tumors to test the prognostic impact of the molecular subtypes on disease-free survival after complete metastasectomy and response to immune checkpoint inhibitors. Most importantly, a new batch of metastases is being sequenced for more in-depth analysis of inter- and intrapatient heterogeneity. We are complementing RNAseq with immunohistochemistry to assess the spatial distribution of immune cell populations and especially their organization into tertiary lymphoid structures.

Methods

Subject details

This study was approved by the ethical committee of University Hospitals Leuven (study number s53479). We included patients with ccRCC who were treated at University Hospitals Leuven. At our center, we prospectively collect detailed clinical data in two databases: (1) ccRCC patients treated with systemic therapy since 2005 (n=338) and (2) ccRCC patients undergoing metastasectomy since 1990 (n=191). Clinical data were frozen for analysis on February 11th, 2020. Inclusion criteria for primary tumors were: availability of archived FFPE tissue from the treatment-naive primary tumor and either 1st line systemic treatment with sunitinib, pazopanib or ICI, or availability of paired metastatic tissue, or previous inclusion of paired fresh-frozen tissue to establish the original ccrcc1 to -4 classifier. For metastases, we used the availability of treatment-naive FFPE tissue as sole inclusion criterion. We took only one sample of every lesion (primary or metastatic). We also included normal kidney tissue samples as a reference.

Method details

Sample selection and preparation

We requested archived H&E slides for all samples that were selected from the clinical databases. The slides were reviewed by an expert uro-pathologist (Evelyne Lerut, Tania Roskams or Marcella Baldewijns) to confirm clear-cell

histology and estimate Fuhrman/ISUP grade and percentage of sarcomatoid dedifferentiation. After that, the tissue block with the highest tumoral content was selected for further processing. After trimming, we cut seven slices of 5µm from each block. The first and last slide were H&E stained and the tumoral section was delignated after microscopic review.

RNA extraction

We macrodissected blanco slides to include only tumor tissue, using a total surface area of 50-1800mm^2 with 5µm tickness. As both primary and metastatic ccRCC tend to grow as enlarging, often encapsulated lesions that push aside surrounding tissue instead of infiltrating it, contamination with surrounding normal tissue was no concern. We extracted RNA with the Maxwell® RSC RNA FFPE kit (Promega) according to the manufacturer's instructions. For 113 samples, which had previously been histologically reviewed and sent to the INSERM 1162 lab as part of a collaboration, we received RNA that had been extracted following the same protocol.

RNA sequencing

We prepared cDNA libraries using the Forward QuantSeq 3' mRNA-Seq Library Prep Kit for Illumina (Lexogen) according to the manufacturer's instructions for FFPE tissue. Of note, we used 5µl of RNA as input, incubated the samples for 60 minutes during the "first strand cDNA synthesis" step and performed 16 PCR cycles. cDNA concentrations and fragment length were measured with the QubitTM dsDNA HS assay (Thermofisher) and Bioanalyzer HS DNA electrophoresis (Agilent). We used Illumina cBOT for clonal cluster generation and performed RNAseq using the Illumina HiSeq 4000 kit according to the manufacturer's instructions.

Quantification and statistical analysis

Pre-processing and normalization of transcriptomic data

The reads were trimmed to remove adaptor sequences and low-quality regions following Lexogen's recommendations. Next, the reads were mapped to the human genome (hg19) using Hlsat2 (v2.1.0) and quantified using featureCounts (v1.6.4). Raw reads were processed using DESeq2 (v 1.26.0) and normalized using the VarianceStabilizingTransformation (vst function). A small batch effect corresponding to the site of processing (RNA extraction in Leuven or INSERM) was removed using ComBat as implemented in the R package sva (v 3.34.0). Analyses were performed with R version 3.5.1.

Cluster analysis

We used 132 treatment-naive primary ccRCC for unsupervised clustering. The 25% genes with the highest variance across primary tumors were retained and used to cluster the samples using the partitioning around medoids (PAM) algorithm, as implemented in the R package ConsensusClusterPlus (v 1.50.0). We selected a four-cluster solution, as the existence of four molecular subtypes with similar characteristics has previously been described not only by our group, but also by the TCGA research program and Hakimi et al. (7,8)

In order to classify metastatic lesions, the 112 primary tumor samples with an itemConsensus larger than 0.6 were retained as a "core" set of samples to develop a classifier using K-nearest neighbors. The caret package (v 6.0.84) was used to determine the K nearest neighbor number with the highest accuracy following repeated cross validation using 10 folds and 10 repeats and then used to classify the metastatic samples. Analyses were performed with R version 3.5.1.

Analysis of specific gene signatures

GSEA - We performed gene set enrichment analysis (GSEA) of every cluster against the other three clusters, using all the signatures contained in the Molecular Signatures database (MSig DB), including KEGG and Biocarta pathway, GO gene sets and specific oncogenic and immunologic signatures. (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp)

IMmotion150 signatures - In the biomarker analysis of the IMmotion150 trial, which compared atezolizumab + bevacizumab *vs* atezolizumab *vs* sunitinib, McDermott et al found three gene expression signatures to be correlated with outcome. (6) The gene signatures were defined as follows: Angio (VEGFA, KDR, ESM1, PECAM1, ANGPTL4 CD34), T_{eff} (CD8A, EOMES, PRF1, IFNG, CD274) and Myeloid inflammation (IL-6, CXCL1, CXCL2, CXCL3, CXCL8, PTGS2). Following their approach, we calculated the average expression of all genes in a given signature to attribute to every sample an Angio score, T_{eff} score and Myeloid score. In analogy with IMmotion150, a sample's signature was considered high if its score was above the median gene signature score of all tumors. We decided to use the median signature score of the primary tumors as cutoff to define High/Low, both for primary tumors and metastases. This approach can capture differences in signature scores between primary tumors and metastases that would otherwise not be apparent. To calculate the cutoff for Angio score with optimal power to discriminate for PFS (as opposed to the median-based IMmotion150 cutoff), we calculated the -2log(likelihood) of Cox regression models with different cutoffs for Angio High/Low, and selected the cutoff that generated the lowest -2log(likelihood). Differences between High/Low tumors

were analyzed using the chi-squared test (two-sided, significance level $p < 0.05$).

CIBERSORTx – Absolute immune cell infiltration was estimated using the LM22 immune cell signature, which was published along with CIBERSORT and has been validated on RNAseq data. (12,13) Differences between subtypes were analyzed with the Kruskal-Wallis test.

Cytolytic score – Cytolytic score was calculated as described by Rooney et al. as the geometric mean (log-average) of GZMA and PRF1. (14) Differences between subtypes were analyzed with the Kruskal-Wallis test.

Characterization of metastatic lesions

We used paired t-tests to compare the IMmotion150 signatures and CIBERSORTx immune infiltration scores between primary tumors and the average scores of their paired metastases. We calculated R-squared models to estimate the amount of the variance in IMmotion150 signatures and CIBERSORTx immune infiltration scores that is explained by patient, molecular subtype, metastatic location or combinations of thereof. Patient was considered a random effect and the conditional R-squared was calculated for models including patient effect. Subtype and location were considered fixed effects and marginal R-squared was calculated for models considering only these effects. (15) The impact of primary *vs* metastatic lesion on the presence of sarcomatoid features was tested in a multivariable logistic regression model with molecular subtype as covariate.

Analysis of clinical outcomes

OS was defined as time since diagnosis of primary ccRCC or since start of systemic therapy (specified in the text) to death from any cause or censoring. PFS was defined as time since start of systemic therapy and progression, death or censoring. OS and PFS were estimated using univariate Cox regression for the following variables: age at diagnosis, gender, IMDC risk group, molecular subtype, IMmotion150 signatures (Angio, T*eff*, Myeloid, treated as continuous variables), Cytolytic score and immune infiltration scores as estimated through CIBERSORTx (B-cells, plasmacells, T-cells, CT8+ T-cells, natural killer cells, macrophages, M2 macrophages, absolute infiltration). Variables with $p<0.1$ were included for multivariate Cox regression. IMDC was not tested for OS since diagnosis, as the risk factor "time to systemic therapy < 1 year" is unknown at diagnosis. Responses were measured by RECIST and defined as response, stable disease or progressive disease. Differences in response rate were analyzed with logistic regression models of response *vs* progressive disease, for the same variables as listed for OS and PFS. Patient numbers for every analysis are listed

in Table 10.1. All tests are two-sided, with a significance level of $p<0.05$.

References

1. Beuselinck B, Job S, Becht E, Karadimou A, Verkarre V, Couchy G, et al. Molecular subtypes of clear cell renal cell carcinoma are associated with sunitinib response in the metastatic setting. Clin Cancer Res. 2015;21(6):1329–39.
2. Fontana E, Eason K, Cervantes A, Salazar R, Sadanandam A. Context matters—consensus molecular subtypes of colorectal cancer as biomarkers for clinical trials. Ann Oncol. 2019;30(4):520–7.
3. Méjean A, Ravaud A, Thezenas S, Colas S, Beauval J-B, Bensalah K, et al. Sunitinib alone or after nephrectomy in metastatic renal-cell carcinoma. N Engl J Med. 2018;379(5):417–27.
4. Gulati S, Martinez P, Joshi T, Birkbak NJ, Santos CR, Rowan AJ, et al. Systematic evaluation of the prognostic impact and intratumor heterogeneity of clear cell renal cell carcinoma biomarkers. Eur Urol. 2014;66(5):936–48.
5. Giraldo NA, Becht E, Pagès F, Skliris G, Verkarre V, Vano Y, et al. Orchestration and prognostic significance of immune checkpoints in the microenvironment of primary and metastatic renal cell cancer. Clin Cancer Res. 2015;21(13):3031–40.
6. McDermott DF, Huseni MA, Atkins MB, Motzer RJ, Rini BI, Escudier B, et al. Clinical activity and molecular correlates of response to atezolizumab alone or in combination with bevacizumab versus sunitinib in renal cell carcinoma. Nat Med. 2018;24(6):749–57.
7. Hakimi AA, Voss MH, Kuo F, Sanchez A, Liu M, Nixon BG, et al. Transcriptomic profiling of the tumor microenvironment reveals distinct subgroups of clear cell renal cell cancer: Data from a randomized phase III trial. Cancer Discov. 2019;9(4):510–25.
8. Creighton CJ, Morgan M, Gunaratne PH, Wheeler DA, Gibbs RA, Robertson G, et al. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature. 2013;499:43–9.
9. Gordan JD, Lal P, Dondeti VR, Letrero R, Parekh KN, Oquendo CE, et al. HIF-α effects on c-Myc distinguish two subtypes of sporadic VHL-deficient clear cell renal carcinoma. Cancer Cell. 2008;14(6):435–46.
10. Monzon FA, Alvarez K, Peterson L, Truong L, Amato RJ, Hernandez-McClain J, et al. Chromosome 14q loss defines a molecular subtype of clear-cell renal cell carcinoma associated with poor prognosis. Mod Pathol. 2011;24(11):1470–9.
11. Serie DJ, Joseph RW, Cheville JC, Ho TH, Parasramka M, Hilton T, et al. Clear cell type A and B molecular subtypes in metastatic clear cell renal cell carcinoma: tumor heterogeneity and aggressiveness. Eur Urol. 2017;71(6):979–85.
12. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al. Robust enumeration of cell subsets from tissue expression profiles. Nat Methods. 2015;12(5):453–7.
13. Chen B, Khodadoust MS, Liu CL, Newman AM, Alizadeh AA. Profiling tumor infiltrating immune cells with CIBERSORT. In: Cancer Systems Biology. Springer; 2018. p. 243–59.

14. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. Cell. 2015;160(1–2):48–61.

15. Nakagawa S, Schielzeth H. A general and simple method for obtaining R2 from generalized linear mixed-effects models. Methods Ecol Evol. 2013;4(2):133–42.

Supplemental tables and figures

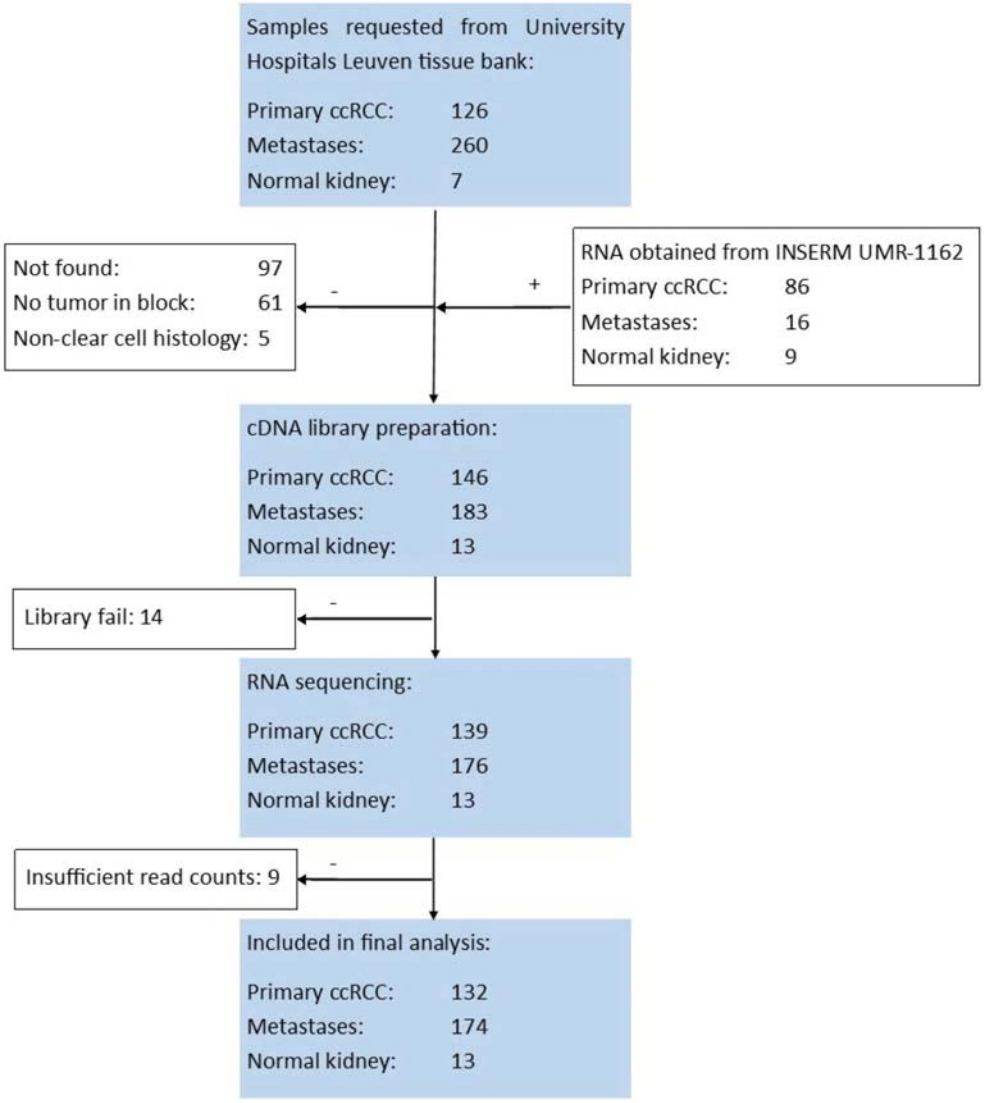

Figure 10.S1. *Work flow for the included samples.*

Table 10.S1. *Angio score, but not M2 macrophage infiltration, was correlated with PFS and response on 1st line sunitinib/pazopanib in our cohort. There was no signal that suggested a differential impact of these markers between patients treated with sunitinib or pazopanib.*

	Overall survival			**Progression-free survival**			**PR *vs* PD**	
	HR	**95% CI**	**p-value**	**HR**	**95% CI**	**p**	**AUC**	**p**
M2 macrophages								
Sunitinib (n=54)	20	0.64-641	0.09	2.6	0.08-91	0.59	0.62	0.15
Pazopanib (n=39)	1.9	0.06-66	0.69	0.74	0.03-20	0.85	0.59	0.44
All	5.6	0.53-60	0.16	1.4	0.13-14	0.80	0.54	0.71
Angio score								
Sunitinib (n=54)	0.77	0.53-1.1	0.19	0.55	0.00-0.81	0.003	0.64	0.10
Pazopanib (n=39)	0.76	0.52-1.1	0.17	0.75	0.50-1.1	0.16	0.78	0.053
All	0.81	0.62-1.1	0.12	0.63	0.00-0.82	0.001	0.68	0.016

HR = hazard ratio; CI = confidence interval of hazard ratio; PR = partial response; PD = progressive disease; AUC = area under the curve of logistic regression model.

Chapter 11

Polymorphisms in the Von Hippel–Lindau gene are associated with overall survival in metastatic clear-cell renal-cell carcinoma patients treated with VEGFR tyrosine kinase inhibitors

Bonus Chapter

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Clinical genitourinary cancer. 2018;16(4):266-273

Original Study

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Polymorphisms in the Von Hippel–Lindau Gene Are Associated With Overall Survival in Metastatic Clear-Cell Renal-Cell Carcinoma Patients Treated With VEGFR Tyrosine Kinase Inhibitors

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Abstract

We assessed whether 3 single nucleotide polymorphisms (SNPs) in the *VHL* gene are associated with outcome in metastatic clear-cell renal-cell carcinoma patients treated with first-line vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitors (TKIs) by genotyping 199 patients. After multivariable analysis, 2 SNPs in close linkage disequilibrium, rs1642742 and rs1642743, remained independent predictors of poor overall survival after initiation of VEGFR-TKI. They are also associated with higher levels of sarcomatoid dedifferentiation.

Background: Clear-cell renal-cell carcinoma (ccRCC) is characterized by loss of a functional Von Hippel–Lindau (VHL) protein. We investigated the potential of 3 single nucleotide polymorphisms (SNPs) in *VHL* as biomarkers in metastatic ccRCC (m-ccRCC) patients treated with vascular endothelial growth factor receptor (VEGFR) tyrosine kinase inhibitors (TKIs). **Patients and Methods:** We genotyped 3 *VHL* SNPs in 199 m-ccRCC patients: rs1642742 T > C, rs1642743 A > G, and rs1678607 C > A. Primary end points were response rate (RR), progression-free survival (PFS), and overall survival (OS) after start of first-line TKI. RR was compared with Fisher's exact test, and PFS and OS with Kaplan-Meier analysis and multivariable Cox regression. Secondary end points were association with *VHL* promotor hypermethylation, *VHL* mutation status, *VHL* loss of heterozygosity, ≥ 25% sarcomatoid dedifferentiation, and expression of genes implicated in angiogenesis and immunoresponse (Fisher's exact test and unpaired *t* tests). **Results:** The minor alleles of rs1642742 and rs1642743, known to be in close linkage disequilibrium, were associated with poor outcome, following a recessive pattern. For the rs1642742 CC versus TT/TC genotype, OS was 11 versus 26 months (hazard ratio = 2.3; 95% confidence interval, 1.2-6.6; $P = .015$). For the rs1642743 GG versus AA/AG genotype, OS was 15 versus 28 months (hazard ratio = 2.6; 95% confidence interval, 1.4-5.0; $P = .004$). After multivariable analysis, both remained linked with poor OS ($P = .018$ and $P = .009$, respectively). There was a trend toward

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Submitted: Dec 11, 2017; Revised: Jan 17, 2018; Accepted: Jan 27, 2018; Epub: Feb 5, 2018

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https://doi.org/10.1016/j.clgc.2018.01.013

shorter PFS and poorer RR. Both SNPs were associated with ≥ 25% sarcomatoid dedifferentiation ($P = .037$ and .006, respectively). No significant results were found for rs1678607. **Conclusion:** rs1642742 and rs1642743 are candidate biomarkers for poor OS in m-ccRCC patients receiving first-line VEGFR-TKI. They are associated with higher levels of sarcomatoid dedifferentiation.

Clinical Genitourinary Cancer, Vol. 16, No. 4, 266-73 © 2018 Elsevier Inc. All rights reserved.
Keywords: Biomarker, rs1642742, rs1678607, Sarcomatoid dedifferentiation, SNP

Introduction

Clear-cell renal-cell carcinoma (ccRCC) is characterized by the ubiquitous loss of a functional Von Hippel–Lindau (VHL) protein, which is caused by mutation, promotor hypermethylation, or loss of heterozygosity.[1,2] This results in an increase in hypoxia-inducible factor (HIF) and, among other effects, subsequent activation of vascular endothelial growth factor (VEGF)-dependent angiogenesis. Targeted therapies directed against the VEGF pathway have become the standard of care as first-line treatment of metastatic ccRCC (m-ccRCC) patients.[3,4] Apart from bevacizumab, which is an anti-VEGF antibody, these therapies are tyrosine kinase inhibitors (TKIs) targeting mainly VEGF receptors (VEGFR). Clinical responses are however highly variable, and even patients whose disease responds well will ultimately develop secondary resistance.[5] On a transcriptomic level, a higher expression of angiogenesis-related genes is associated with better response to VEGFR-TKIs.[6,7] Yet genomic biomarkers for outcome are lacking. Several studies assessing *VHL* status (mutation or promotor hypermethylation) have not found a link with response rate (RR), progression-free survival (PFS), or overall survival (OS) on VEGFR-TKIs.[8]

De Haas et al[9] have reported a potential predictive value of genetic variability in the VEGF pathway in different tumor types treated with bevacizumab. Genetic variability in *VHL* was linked with PFS in a Bayesian genewise association analysis but did not survive correction for multiple testing. Other groups have linked single nucleotide polymorphisms (SNPs) in protein encoding genes more downstream in the VEGF pathway with outcome in m-ccRCC patients treated with sunitinib.[10-13] Because ccRCC is a *VHL*-driven disease, we aimed to assess the impact of SNPs in *VHL* on outcome in m-ccRCC patients treated with first-line VEGFR-TKIs.

Patients and Methods

Three SNPs in *VHL* were selected: 2 described by De Haas et al,[9] rs1678607 and rs1642742; and a neighboring SNP, rs1642743.

Primary end points were association of genotype with RR, PFS, and OS on first-line VEGFR-TKI. Secondary end points were correlation with *VHL* promotor hypermethylation, *VHL* mutation status, *VHL* loss of heterozygosity, sarcomatoid dedifferentiation, and expression of selected genes implicated in angiogenesis and immune pathways.

Patients

Germ-line DNA samples were retrospectively selected from the "CIT-rein" renal-cell carcinoma tumor bank. This French–Belgian multicenter renal-cell carcinoma tumor bank contains over 400 frozen primary kidney tumor samples from patients with metastatic disease. Samples were selected of patients with ccRCC who started first-line VEGFR-TKIs between May 2004 and March 2016 and for whom frozen normal kidney tissue (n = 106) or peripheral blood (n = 93) was available for DNA extraction. Prior treatment with interferon or interleukin 2 was allowed (n = 34; 17%); treatment with another TKI, bevacizumab, or mammalian target of rapamycin inhibitor was not. Drug schedules, dose reduction policy, and timing of radiologic assessments were in accordance with local practice guidelines.

The protocol was approved by the medical ethics review boards of the participating institutions, and signed consent was obtained from all patients. Biologic material of deceased patients was used when approval was given by the institutional review board.

Laboratory Analyses

DNA was isolated from fresh frozen normal kidney tissue sampled during nephrectomy using the QIA Quick Extraction Kit (Qiagen, Germantown, MD) and quantified by fluorometry (Fluoroskan Thermo Labsystems, Cergy-Pontoise, France). From peripheral blood, DNA was isolated with the Qiagen DNA kit, and the final DNA concentration quantified with a NanoDrop spectrophotometer (NanoDrop, Wilmington, DE). High-throughput SNP genotyping was performed with the Sequenom MassArray platform (Sequenom, San Diego, CA).[14] Genotyping analysis was performed by investigators blinded to the clinical data.

Expression data of 95 genes in 103 primary tumor samples were available from previous experiments. Their expression level was determined as described previously.[7] Nineteen genes implicated in angiogenesis or immune response were selected for correlation analysis: *ALDOC, ANGPTL4, CA9, CD274, CD38, EPAS1, EPHB2, FLT1, FLT4, HIF1A, KDR, MYC, PDCD1, PDCD1LG2, PDGFR1, PDGFRB, PLAU, VEGF1*, and *VEGFC*. Expression levels of *VHL* had not been determined, as *VHL* expression is lost in ccRCC.

Also from previous experiments, data were available on *VHL* promotor methylation (65 samples), *VHL* mutational status (79 samples), and *VHL* loss of heterozygosity (73 samples).[7]

Statistical Analyses

The database was closed on October 3, 2016. PFS and OS after TKI were calculated by the Kaplan-Meier method from the first day of TKI treatment until the date of radiologic disease progression or death. Response was defined as objective response (complete or partial remission), stable disease, or progressive disease by the Response Evaluation Criteria in Solid Tumors (RECIST). Two patients who had only bone metastases were excluded from PFS and

response assessment, as these are not target lesions for RECIST. Comparison between genotypes was done by the log-rank test for PFS and OS, and by Fisher's exact test for RR.

The relation of the following baseline prognostic factors with PFS and OS was tested with the Kaplan-Meier method: International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) score, individual factors of the IMDC score, liver metastases, bone metastases, and $\geq$ 25% sarcomatoid dedifferentiation. Variables with $P \leq .2$ on univariate analysis were selected as covariates for multivariate Cox regression analysis. The IMDC score was not used in the multivariate model, as the individual factors of the score were used if significant on univariate analysis.

Fisher's exact test was used to assess the association of genotype with *VHL* promotor hypermethylation, *VHL* mutation, *VHL* loss of heterozygosity, and level of sarcomatoid dedifferentiation. A cutoff of $\geq$ 25% sarcomatoid dedifferentiation was used because its correlation with PFS and OS has been previously demonstrated.[15] The predictive power for OS of the IMDC score in our series was compared to that of the IMDC score and the SNPs using a concordance probability estimate. Unpaired *t* tests were used to correlate SNPs with expression of selected genes. *P* values were calculated by the Mann-Whitney test for nonparametric data. As an internal validation, the analyses were repeated in patient groups treated with sunitinib (n = 148) versus pazopanib or sorafenib (n = 51). Because this study was exploratory in nature, no correction for multiple testing was done.

Statistical analyses were conducted by GraphPad Prism 5 (GraphPad Software, La Jolla, CA), XLSTAT (Addinsoft, Paris, France), and SAS 9.4 for Windows (SAS Institute, Cary, NC, USA).

Results

A total of 199 patients were included. One hundred thirty-four patients (67%) died during follow-up; median follow-up was 19 months. Patient characteristics are listed in Table 1.

Genotyping was successful for rs1642742 in 196 patients (98%), for rs1642743 in 172 (86%), and for rs1678607 in 197 (99%). Allele frequency was similar to reference frequency for a Central European population as published by the 1000 Genomes Project.[16]

Correlation With Outcome on First-Line VEGFR-TKI

rs1642742. PFS and OS curves were overlapping for the CT and TT genotypes. Therefore, all analyses were done using a recessive model comparing the CC with the CT/TT genotype. Median OS was 11 months for the CC genotype versus 26 months for CT/TT (hazard ratio = 2.3; 95% confidence interval, 1.2-6.6; $P = .015$). Genotype remained an independent predictor of OS on multivariable analysis. There was a trend toward shorter PFS for the CC genotype (9 vs. 12 months, $P = .053$). RR was not significantly different (28% vs. 46%, $P = .211$), but there was more progressive disease at first follow-up among CC genotypes (32% vs. 12%, $P = .032$) (Tables 2 and 3, Figure 1).

rs1642743. PFS and OS curves were overlapping for the AA and AG genotypes. Therefore, all analyses were done using a recessive model comparing the GG genotype with AA/AG. Median OS was 15 months for the GG genotype versus 28 months for AA/AG (hazard ratio = 2.6; 95% confidence interval, 1.4-5.0; $P = .004$). Genotype remained an independent predictor of OS on multivariable analysis. Upon bivariable analysis with rs1642742 CC and

Table 1 Patient Characteristics at Diagnosis

Characteristic	Value
Patient Characteristics at Baseline	
Age (years), median (range)	61 (30-82)
Self-reported ethnicity white	100% (168/168)
Male sex	69% (138/199)
rs1642742 C allele[a]	32% (126/392)
rs1642742 CC genotype	10% (20/196)
rs1642743 G allele	37% (128/344)
rs1642743 GG genotype	13% (23/172)
Overlap between CC and GG homozygotes[b]	79% (34/43)
rs1678607 A allele	46/394 (12%)
rs1678607 AA genotype	2/197 (1%)
rs1678607 C allele	348/394 (88%)
RCC at Diagnosis	
Fuhrman grade 1-2	10% (20/195)
Sarcomatoid dedifferentiation $\geq$ 25%	4% (8/178)
Treatment Before Initiation of TKI	
Nephrectomy	94% (187/199)
Interferon or interleukin 2	17% (34/198)
Patient Characteristics at Start of TKI	
Choice of TKI	
Sunitinib	74% (148/199)
Pazopanib	15% (29/199)
Sorafenib	11% (22/199)
Site of Metastasis	
Bone	34% (67/199)
Liver	21% (41/199)
Median no. of metastatic sites	2
Time between diagnosis and start systemic therapy < 12 months	62% (123/199)
Karnofsky performance status $\leq$ 70	15% (28/189)
Serum albumin $\leq$ 3.5 g/dL	16% (23/147)
Neutrophils $\geq$ 7800/mm^3	8% (16/189)
Platelets $\geq$ 450,000/mm^3	13% (25/193)
LDH > 1.5× upper limit of normal	7% (12/184)
Corrected calcium > 10 mg/dL	11% (16/147)
Low hemoglobin (< 13.5 g/dL in men, < 11.5 g/dL in women)	24% (34/143)
IMDC Score	
Good	23% (42/183)
Intermediate	56% (102/183)
Poor	22% (40/183)

Abbreviations: IMDC = International Metastatic Renal Cell Carcinoma Database Consortium; LDH = lactate dehydrogenase; RCC = renal cell carcinoma; TKI = tyrosine kinase inhibitor.
[a]Comparison with 1000 Genomes Project[16] for Central Europe: rs1642742, C allele 32%, CC genotype 8%; rs1642743, G allele 36%, GG genotype 11%.
[b]Linkage disequilibrium according to 1000 Genomes Project[16] ($r^2 = 0.836$).

Table 2 Correlation of SNPs in *VHL* With Outcome and Baseline Characteristics in Metastatic Clear-Cell Renal-Cell Carcinoma Patients Treated With First-Line VEGFR-TKIs

Characteristic	rs1642742 CC (N = 20) Versus TT/CT (N = 176)		rs1642743 GG (N = 23) Versus AA/AG (N = 149)	
	N	Significance	N	Significance
Outcome				
Median OS after initiation of VEGFR-TKI (months)	11 versus 26	$P = .0150$ HR = 2.341 95% CI, 1.179-6.647	15 versus 28	$P = .0041$ HR = 2.587 95% CI, 1.352-4.952
PFS (months)	9 versus 12	Trend $P = .0529$	9 versus 11	Trend $P = .1342$
Best response: PD	32% (6/19) versus 12% (21/174)	$P = .0319$ OR = 3.363 95% CI, 1.123-10.09	30% (7/23) versus 13% (19/146)	Trend $P = .0554$
Best response: PR or CR versus SD or PD	28% (5/18) versus 46% (77/167)	$P = .2110$	36% (8/22) versus 44% (63/143)	$P = .6447$
Baseline Characteristics				
Sarcomatoid dedifferentiation ≥ 25%	17% (3/18) versus 3% (5/157)	$P = .0371$ OR = 6.08 95% CI, 1.468-26.83	20% (4/20) versus 11% (16/147)	$P = .0058$ OR = 10.92 95% CI, 2.656-45.08
VHL promotor hypermethylation	NS		NS	
IMDC score	NS		NS	
Good	26% (5/19) versus 22% (36/161)		23% (5/22) versus 22% (31/138)	
Intermediate	63% (12/19) versus 55% (88/161)		64% (14/22) versus 53% (73/138)	
Poor	11% (2/19) versus 24% (38/161)		14% (3/22) versus 25% (34/138)	
Start TKI < 12 months after nephrectomy	NS 50% (10/20) versus 58% (111/193)		NS 57% (13/23) versus 61% (90/147)	
Time to relapse after nephrectomy (months)	NS 16 versus 27, $P = .3172$		NS 16 versus 17, $P = .2495$	

Abbreviations: CI = confidence interval; CR = complete remission; HR = hazard ratio; IMDC = International Metastatic Renal Cell Carcinoma Database Consortium; NS = not significant; OR = odds ratio; OS = overall survival; PD = progressive disease; PFS = progression-free survival; PR = partial remission; SD = stable disease; TKI = tyrosine kinase inhibitor; VEGFR = vascular endothelial growth factor receptor.

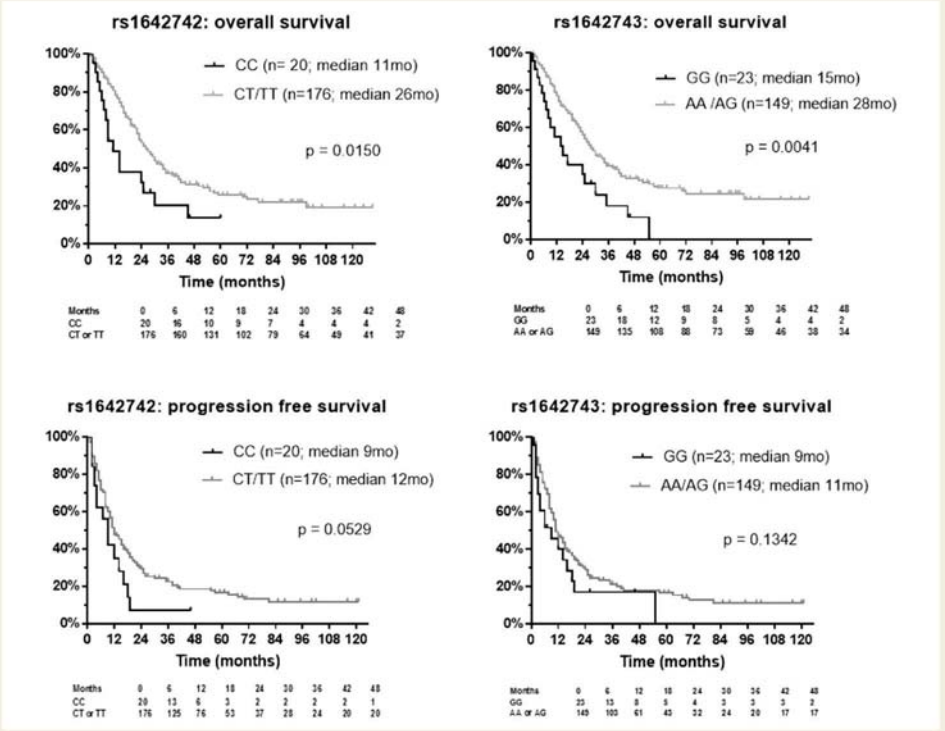
rs1642743 GG, the SNPs were not linked with OS independent of each other. There was a trend toward shorter PFS for the GG genotype (9 vs. 11 months, $P = .134$). There was no difference in RR (36% vs. 44%, $P = .645$) but a trend toward more progressive disease among GG genotypes (30% vs. 13%, $P = .055$) (Tables 2 and 3, Figure 1).

Table 3 Results of Multivariable Analyses for Parameters Correlated With Overall Survival After Initiation of TKI With $P < .2$ in Univariate Analysis

Variable	*P* Value > χ^2	Hazard Ratio	95% Confidence Interval
rs1642742 (N = 155)			
rs1642742 CC	.018	2.065	1.132-3.767
Platelets > 450,000/mm^3	.004	2.245	1.298-3.884
Neutrophils > 7800/mm^3	<.001	3.891	1.910-7.928
KPS ≤ 70	.009	2.051	1.195-3.519
Bone metastases	.019	1.652	1.084-2.518
Start TKI < 12 months after diagnosis of metastases	.017	1.661	1.094-2.523
rs1642743 (N = 140)			
rs1642743 GG	.009	2.123	1.208-3.730
Platelets > 450,000/mm^3	.002	2.411	1.397-4.160
Neutrophils > 7800/mm^3	.000	3.765	1.837-7.714
KPS ≤ 70	.007	2.119	1.223-3.671
Bone metastases	.024	1.646	1.067-2.539
Start TKI < 12 months after diagnosis of metastases	.041	1.573	1.018-2.428

Parameters used in univariate analysis but not withheld because $P > .2$ (results not shown): corrected serum calcium > 10 mg/dL. Parameters with $P < .2$ in univariate analysis but not significant in multivariate analyses (results not shown): serum lactate dehydrogenase > 1.5× upper limit of normal, hemoglobin less than lower limit of normal, presence of liver metastases, sarcomatoid dedifferentiation ≥ 25%.
Abbreviations: KPS = Karnofsky performance status; TKI = tyrosine kinase inhibitor.

Figure 1 Overall and Progression-Free Survival While Receiving First-Line VEGFR-TKI Therapy by Genotype in m-ccRCC Patients



Abbreviations: m-ccRCC = metastatic clear-cell renal-cell carcinoma; TKI = tyrosine kinase inhibitor; VEGFR = vascular endothelial growth factor receptor.

rs1678607. We found no association between rs1678607 C > A and outcome, but only 2 of 197 patients in our series carried the AA genotype (similar to the reference allelic frequency), and survival curves overlapped for the CC and AC genotypes.[16]

Knowledge of genotype added predictive power for OS to the IMDC score, especially in the intermediate prognosis subgroup (Figure 2, Table 4).

As an internal validation, we compared the outcomes of patients treated with sunitinib (n = 148) with those of patients treated with pazopanib or sorafenib (n = 51). These results (RR, PFS, OS) were similar to each other and to the global series (data not shown).

Correlation With Baseline Tumor Characteristics

There were no significant correlations between genotype and expression of 19 genes implicated in angiogenesis and immune response.

Eight patients had ≥ 25% sarcomatoid dedifferentiation, which was more frequent in both unfavorable genotypes: 17% versus 3% for rs1642742 CC versus CT/TT ($P = .037$) and 20% versus 11% for rs1642743 GG versus AA/AG ($P = .006$).

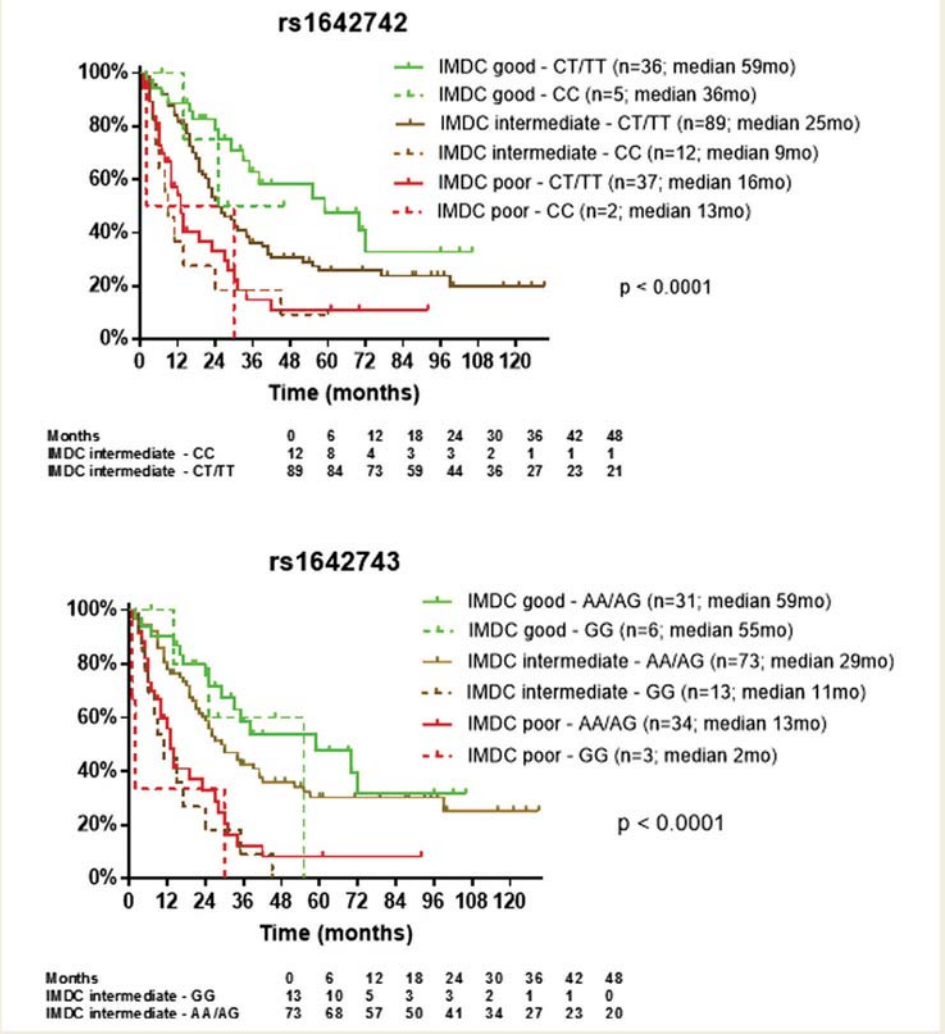
There was no association between genotype and *VHL* promotor hypermethylation, mutation status, or loss of heterozygosity.

In an attempt to distinguish between the prognostic or rather predictive value of these variants, we calculated baseline IMDC scores and interval between nephrectomy and relapse or start of TKI (a longer interval presumed to indicate less aggressive disease behavior). There were no differences between patients with and without an unfavorable genotype.

Discussion

The minor alleles of 2 *VHL* SNPs that are known to be in close linkage disequilibrium, rs1642742 and rs1642743, are associated with poorer outcome in m-ccRCC patients treated with first-line VEGFR-TKI, following a recessive pattern. Their respective CC and GG genotypes are independent predictors of shorter OS on multivariable analysis and show a trend toward shorter PFS, lower RR, and more progressive disease as best response. This is surprising because loss of function of *VHL*, a driving event in oncogenesis of ccRCC, is not associated with survival.[8] The most plausible explanation seems to be that these variants, which are

Figure 2 Overall Survival While Receiving VEGFR-TKI by Genotype in m-ccRCC Patients Stratified by IMDC Score Number At Risk is Shown for IMDC Intermediate Subgroup, Which Contained Most Patients



Abbreviations: IMDC = International Metastatic Renal Cell Carcinoma Database Consortium; m-ccRCC = metastatic clear-cell renal-cell carcinoma; TKI = tyrosine kinase inhibitor; VEGFR = vascular endothelial growth factor receptor.

germ line, are somehow responsible for a change in the microenvironment of ccRCC. Chances are low that rs1642743 itself has any functional effect because it is situated in an intron far from a slice site, not in a microRNA (miRNA) binding site or CpG island, and it is not predicted to affect gene expression or protein function in another way.[17] rs1642742 however is situated in the 3′ untranslated region of *VHL*, both in and close by multiple miRNA binding sites. The C variant is predicted to disrupt the binding of several miRNAs regulating *VHL* expression, among which are members of the tumor-suppressing Let-7 family.[18] In The Cancer Genome Atlas miRNA clusters, an elevated Let-7a discriminated the ccRCC subgroup with the best

Table 4 Predictive Power for Overall Survival Using IMDC Score With and Without SNP

SNP	CPE (95 % CI) for		No. of Patients	No. of Deaths
	IMDC Score	IMDC Score + SNP		
VHL rs1642742	0.605 (0.559, 0.650)	0.615 (0.571, 0.660)	181	121
VHL rs1642743	0.606 (0.557, 0.655)	0.630 (0.583, 0.676)	160	107

Abbreviations: CI = confidence interval; CPE = concordance probability estimate; IMDC = International Metastatic Renal Cell Carcinoma Database Consortium; SNP = single nucleotide polymorphism; VHL = Von Hippel–Lindau.

5-year survival, and the Let-7 family is known to be downregulated in aggressive ccRCC.[2,19]

De Haas et al[9] found that rs1642742 is situated in an expression quantitative locus and that the C allele is associated with higher expression of *VHL*. This is in accordance with our findings because the VHL protein inhibits angiogenesis, whereas an angiogenic gene signature predicts favorable outcome upon treatment with VEGFR-TKIs.[6] These explanations are hypothetical, however, as the correlation with outcome of Let-7a and the VEGF pathway has been described on tumor samples, whereas an effect of the SNP is expected in the microenvironment. When assessing gene expression in tumor samples, we found no correlation between the SNPs and expression of angiogenesis related genes.

There was a marked correlation of the unfavorable SNPs with high level of sarcomatoid dedifferentiation in this series. This finding is of interest because the mechanism for development of sarcomatoid dedifferentiation is not known in ccRCC and yet has a deleterious effect. However, the implications of these results are still of uncertain value.

Whether these SNPs have a prognostic or rather predictive value cannot be derived from the present data. The fact that the IMDC score and time between nephrectomy and relapse or start of TKI were comparable for all groups suggests a predictive value. The fact that VEGFR-TKIs interfere with VHL-HIF-VEGF–dependent angiogenesis also suggests that genetic variability in this pathway could alter treatment response. Yet the number of objective responses, which is the strongest criterion for a predictive biomarker, was not significantly different.

The major limitation of this study is the fact that we did not directly assess the functional impact of the SNPs ourselves. Nor did we correct for multiple testing, as this study was exploratory in nature. However, correction for multiple testing does not seem urgent because the results are concordant: all assessed outcomes are worse for the 2 unfavorable genotypes, and outcomes are similar for rs1642742 and rs1642743, which are in close linkage disequilibrium. As with all SNP studies, these findings still need replication in independent cohorts.

Conclusion

The *VHL* rs1642742 CC genotype and the *VHL* rs1642743 GG genotype are candidate biomarkers for poor OS in m-ccRCC patients treated with first-line VEGFR-TKIs. This correlation remains present after multivariable analysis. Both genotypes are also associated with a higher level of sarcomatoid dedifferentiation. These findings need validation in independent series.

Clinical Practice Points

- VHL-HIF-VEGF–dependent angiogenesis is a driving event in ccRCC.
- VEGFR-TKIs inhibiting this angiogenic pathway are the first-line treatment for metastatic disease.
- The minor alleles of 2 *VHL* SNPs, rs1642742 and rs1642743, are associated with poor OS in patients treated with first-line VEGFR-TKIs.

Acknowledgments

A.V. received a grant from Kom op tegen Kanker (Stand Up to Cancer), the Flemish cancer society. B.B. received a grant from Fondation Martine Midy (Paris, France), a grant from Fonds voor Wetenschappelijk Onderzoek Vlaanderen (Belgium), and a research grant from Pfizer. The funding sources were not implicated in study design, analysis, interpretation, writing, or decision to submit the report.

Disclosure

B.B. received honoraria from Amgen, Pfizer, Janssen, and Bayer and is an investigator of the EudraCT 2011-006085-40/MetaSun trial supported by Pfizer. The other authors have stated that they have no conflict of interest.

References

1. Sato Y, Yoshizato T, Shiraishi Y, et al. Integrated molecular analysis of clear-cell renal cell carcinoma. *Nat Genet* 2013; 45:860-7.
2. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of clear cell renal cell carcinoma. *Nature* 2013; 499:43-9.
3. Motzer RJ, Hutson TE, Tomczak P, et al. Overall survival and updated results for sunitinib compared with interferon alfa in patients with metastatic renal cell carcinoma. *J Clin Oncol* 2009; 27:3584-90.
4. Motzer RJ, Hutson TE, Cella D, et al. Pazopanib versus sunitinib in metastatic renal-cell carcinoma. *N Engl J Med* 2013; 369:722-31.
5. Rini B, Atkins MB. Resistance to targeted therapy in renal-cell carcinoma. *Lancet Oncol* 2009; 10:992-1000.
6. Beuselinck B, Verbiest A, Couchy G. Pro-angiogenic gene expression is associated with better outcome on sunitinib in metastatic clear-cell renal cell carcinoma. *Acta Oncol* 2017, In press.
7. Beuselinck B, Job S, Becht E, et al. Molecular subtypes of clear cell renal cell carcinoma are associated with sunitinib response in the metastatic setting. *Clin Cancer Res* 2015; 21:1329-39.
8. Kim BJ, Kim JH, Kim HS, Zang DY. Prognostic and predictive value of *VHL* gene alteration in renal cell carcinoma: a meta-analysis and review. *Oncotarget* 2017; 8:13979-85.
9. de Haas S, Delmar P, Bansal AT, et al. Genetic variability of VEGF pathway genes in six randomized phase III trials assessing the addition of bevacizumab to standard therapy. *Angiogenesis* 2014; 17:909-20.
10. García-Donas J, Esteban E, Leandro-García LJ, et al. Single nucleotide polymorphism associations with response and toxic effects in patients with advanced renal-cell carcinoma treated with first-line sunitinib: a multicentre, observational, prospective study. *Lancet Oncol* 2011; 12:1143-50.
11. Dornbusch J, Walter M, Gottschalk A, et al. Evaluation of polymorphisms in angiogenesis-related genes as predictive and prognostic markers for sunitinib-

treated metastatic renal cell carcinoma patients. *J Cancer Res Clin Oncol* 2016; 142: 1171-82.
12. Beuselinck B, Jean-Baptiste J, Schoffski P, et al. Validation of VEGFR1 rs9582036 as predictive biomarker in metastatic clear-cell renal cell carcinoma patients treated with sunitinib. *BJU Int* 2016; 118:890-901.
13. Beuselinck B, Karadimou A, Lambrechts D, et al. Single-nucleotide polymorphisms associated with outcome in metastatic renal cell carcinoma treated with sunitinib. *Br J Cancer* 2013; 108:887-900.
14. Reumers J, De Rijk P, Zhao H, et al. Optimized filtering reduces the error rate in detecting genomic variants by short-read sequencing. *Nat Biotechnol* 2011; 30:61-8.
15. Beuselinck B, Lerut E, Wolter P, et al. Sarcomatoid dedifferentiation in metastatic clear cell renal cell carcinoma and outcome on treatment with anti–vascular endothelial growth factor receptor tyrosine kinase inhibitors: a retrospective analysis. *Clin Genitourin Cancer* 2014; 12:e205-14.
16. Genomes Project C, Auton A, Brooks LD, et al. A global reference for human genetic variation. *Nature* 2015; 526:68-74.
17. Dayem Ullah AZ, Lemoine NR, Chelala C. SNPnexus: a web server for functional annotation of novel and publicly known genetic variants (2012 update). *Nucleic Acids Res* 2012; 40(Web Server issue):W65-70.
18. Bhattacharya A, Ziebarth JD, Cui Y. PolymiRTS Database 3.0: linking polymorphisms in microRNAs and their target sites with human diseases and biological pathways. *Nucleic Acids Res* 2014; 42(Database issue):D86-91.
19. Heinzelmann J, Henning B, Sanjmyatav J, et al. Specific miRNA signatures are associated with metastasis and poor prognosis in clear cell renal cell carcinoma. *World J Urol* 2011; 29:367-73.

Chapter 12

Concluding discussion

Chapter 12: Contents

Molecular characterization of ccRCC 181
- Molecular subtype-specific gene expression 181
- microRNA expression patterns 182
- Sarcomatoid RCC 182
- Metastatic lesions 182

Biomarkers in the context of surgical ccRCC treatment 183

Current clinical questions and future work 183
- Cytoreductive nephrectomy 184
- Adjuvant therapy for localized disease 185

Biomarkers in the context of systemic ccRCC treatment 185
- Current clinical questions and future work 186

References (*"Introduction" and "Concluding discussion" sections)* 188

Molecular characterization of ccRCC

The next sections briefly summarize the contribution of the thesis to the molecular characterization of ccRCC.

Molecular subtype-specific gene expression

We have refined the molecular characterization of the ccrcc1 to -4 subtypes, since their first publication in 2015. First and foremost, we have shown that the ccrcc2 subtype, comprising almost half of metastatic ccRCC, is highly angiogenic and therefore susceptible to VEGFR-TKI (chapter 7-8). Indeed, when IMDC Good risk recently submerged as a predictive biomarker for VEGFR-TKI, we could provide a molecular explanation by showing that Good risk patients are highly enriched for angiogenic ccrcc2 tumors. Moreover, the ccrcc2 subtype could identify VEGFR-TKI sensitive tumors among IMDC Intermediate risk patients, which is a large and heterogeneous group with highly variable responses to VEGFR-TKI. In parallel, the lack of predictive value of IMDC for ICI could be explained as we found that T_{eff} signatures are similar between IMDC subgroups. On the contrary, T_{eff} and other immune signatures do differ between molecular subtypes. The subtypes therefore combine information on prognosis, angiogenesis, outcome on TKI and immune phenotype in one single marker.

The RNAseq data we obtained from FFPE tissue have generated new hypotheses on the major differences underlying the molecular subtypes (chapter 10). Ccrcc2 tumors are highly angiogenic and moderately inflamed, whereas ccrcc4 are highly inflamed but with an immune suppressive microenvironment. Ccrcc1 tumors have intermediate expression profiles and prognosis, and are probably distinguished by arm level losses of chr14q. Ccrcc3 tumors on the other hand, have a completely different gene expression pattern from other ccRCC, with angiogenic and immune signatures that are even below those of normal kidney tissue. Their overexpression of mitochondrial and mTOR pathways suggests similarity to *FLCN*-mutation driven ccRCC, as found in Birt-Hogg-Dubé disease: these findings may therefore guide tailored therapy decisions for Birt-Hogg-Dubé patients. We are currently testing these hypotheses on chr14q loss and *FLCN* mutations through copy number and mutation analyses in these tumors.

microRNA expression patterns

We performed miRNome analysis to assess how miRNA, which are master regulators of mRNA, may drive the mRNA-based molecular subtypes (chapter 9). Indeed, we showed that miRNA expression profiles differ greatly between molecular subtypes, with especially miRNA involved in epithelial-to-mesenchymal transmission being upregulated in ccrcc1 and -4 tumors. Strikingly, throughout the entire miRNome, miRNA that were upregulated in ccrcc1 and -4 tumors were consistently associated with poor prognosis, and miRNA upregulated in ccrcc2 and -3 tumors with good prognosis. These findings point towards a global deregulation of miRNA expression in more aggressive tumors. This sheds a new light on the current literature, where studies typically suggest only a few oncogenic miRNAs to be upregulated in aggressive disease.

Sarcomatoid RCC

As sarcomatoid tumors are almost exclusively of the ccrcc4 subtype, we were among the first to show their inflamed phenotype and suggest the benefit of ICI, which by now has been demonstrated extensively. Unfortunately, studies reporting outcomes of sarcomatoid RCC rarely include how sarcomatoid RCC is defined. We have shown that 20% sarcomatoid differentiation is a cut-off with high discriminative power for prognosis and outcome on VEGFR-TKIs. Although such a percentage is intrinsically inaccurate due to intratumoral heterogeneity, it is striking how outcomes of tumors with only a few percent sarcomatoid features overlap perfectly with those of non-sarcomatoid RCC. This cut-off can therefore serve as a baseline for further studies on sarcomatoid RCC.

Metastatic lesions

In medical oncology, we use systemic treatment to prevent or treat metastatic disease, rather than localized tumors. Yet our therapeutic choices are mostly based on the characteristics of the primary tumor. For this reason, the heterogeneity between a primary tumor and its metastases, as well as the effects of the metastatic niches in different host organs, are of major interest. We have performed RNAseq of 174 ccRCC metastases from 85 patients, which is the largest series thus far to our knowledge. A preliminary analysis found, encouragingly, that molecular subtype and angiogenic gene expression are largely conserved (73% and 68%) between different lesions from the same patient. Several immune signatures display substantial intrapatient homogeneity, but here clearly other factors are in play as well.

Biomarkers in the context of surgical ccRCC treatment

Contributions of the thesis

It has long been known that complete metastasectomy in highly selected patients is associated with excellent long-term outcomes. (29)metastasectomy still remains the only potentially curable intervention and plays an important role both in disease control, cancer-specific survival (CSS Based on our data on 113 patients that underwent complete metastasectomy at University Hospitals Leuven, we were able to add the following findings to the literature (chapter 5):

- Repeat metastasectomy is feasible and can result in long-term disease-free survival.
- Patients undergoing metastasectomy do not miss a window of opportunity for VEGFR-TKI.
- It is not possible to infer a causal relationship between metastasectomy and improved overall survival, because patients selected for metastasectomy differ too much from those who are not.

More importantly, we have established a first molecular biomarker to predict the risk of early relapse after metastasectomy: patients with a primary ccrcc1 or ccrcc4 tumor are at much higher risk of early relapse, whereas those with ccrcc2 or ccrcc3 tumors have a high chance of long-term disease-free survival (chapter 6). It is crucial to note that all the patients we included for this analysis had undergone stringent clinical selection and had similar prognostic features. Therefore, the subtypes could be used to prevent the morbidity of extensive surgery in patients with favorable clinical features but a molecular high risk of early relapse.

Current clinical questions and future work

Metastasectomy

As of now, the future place of metastasectomy and other radical local treatments such as stereotactic body radiotherapy is not clear. Do we keep offering them? Do we decrease their use in favor of ever improving systemic therapies? Do we combine them with systemic treatment? Although it was never possible in ccRCC to conclusively prove the benefit of metastasectomy on overall survival or in delaying systemic therapy, its selective use is strongly supported by both its excellent outcomes and the results of a randomized

trial in metastatic non-small cell lung carcinoma, were radical local treatment improved the time to progression at new sites. (67) In ccRCC, the adjuvant use of VEGFR-TKIs after complete metastasectomy had no benefit in several recent trials. (30,31) However, trials that combine (neo)adjuvant immunotherapy with radical metastasectomy are ongoing and hold more promise. After all, whereas VEGFR-TKIs can delay progression but are not curative, ICI may be so for a small subset of susceptible patients with low tumor load. In node positive melanoma for example, the preliminary results of neoadjuvant ipilimumab-nivolumab followed by surgery, show extremely promising rates of pathological responses (80%) and long-term disease-free survival. (68)

Our findings on the spontaneous clinical course after radical metastasectomy without systemic therapy can serve as a baseline as we move towards an age were metastasectomy will be increasingly combined with immunotherapies. The tailoring of therapy can be aided by the molecular subtypes: they predict risk of relapse and provide insight in the microenvironment of both the primary tumor and its metastases. We are currently classifying a new series of metastasectomy patients to validate the predictive value of the FFPE-based subtypes. Furthermore, we are investigating more profoundly the immune contexture of metastases in relation to their primary tumors and host organs. These insights will guide us towards balanced treatment decisions for local and systemic therapy.

Cytoreductive nephrectomy

The place of cytoreductive nephrectomy for advanced ccRCC is again a topic of hot debate. Although the CARMENA trial showed no survival benefit in patients who needed immediate start of systemic therapy, these results date from the sunitinib-era, whereas ICI are now standard 1st line. (27) In the absence of a randomized trial with the current 1st line regimens, there are several strong in favor of cytoreductive nephrectomy. First, the CARMENA trial was only relevant for a small subset of patients, as those who did not need immediate systemic therapy and those with too poor a prognosis to undergo surgery were not included. Second, randomized data from the cytokine-era have demonstrated a survival benefit in the context of (less efficient) immunotherapy. (69,70) Third, nephrectomy specimens from ICI-treated patients who achieved complete responses at distant sites, still showed viable tumor in 82% of the primary sites. (71) Last, many CARMENA patients needed salvage nephrectomy for local complications, whereas the feasibility of such surgery after ICI is unsure. Pilot studies of neo-adjuvant ICI show an acceptable complication rate for less

advanced disease, but a real life series of salvage nephrectomy on ICI reported substantial surgical difficulties and complications. (71,72) Another advantage, which will hopefully become very relevant in the near future, is the collection of tissue for predictive biomarkers. The amount of tissue obtained by biopsy is limited and not fully representative, even though we have been able to determine molecular subtypes on core needle biopsies. As we have shown that the angiogenic profile, molecular subtype and immune contexture of primary tumors are preserved to a substantial extent in their metastases, assessment of these markers on the primary tumor can guide treatment of metastatic lesions.

Adjuvant therapy for localized disease

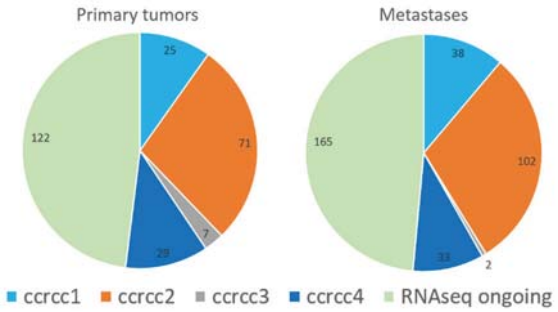
Trials of adjuvant VEGFR-TKIs have consistently failed to show benefit, or resulted in only a small gain of progression-free survival in high-risk patients. (24–26) This comes as no surprise, as TKIs are not curative and tumors at high risk for early relapse, as included in these trials, are less likely to be angiogenic ccrcc2 and therefore sensitive to TKI (unpublished data). Trials testing adjuvant ICI are ongoing. However, these trials also include patients solely based on their risk of relapse, but do not consider the immune microenvironment. Yet it has been shown that among high-risk tumors, the microenvironment of tumors that relapse early is fundamentally different from that of those who do not, and also differs from that of metastatic tumors. For example, high CD8+ T-cell infiltration is associated with good prognosis in early ccRCC, but with poor prognosis in advanced ccRCC. (21,23) Hence, after our efforts in metastatic ccRCC, and our ongoing search for biomarkers for ICI in this context, we will also focus on early ccRCC in the future.

Biomarkers in the context of systemic ccRCC treatment

Contributions of the thesis

One of the main realizations of our group is not just its scientific results, but also the extensive clinical databases and tissue banks that we have established. Detailed clinical data and tissue of metastatic RCC are available for over 600 patients; a summary of the samples being processed for RNAseq is provided below. These data allow us to not only move forward our own lines of research, but also to swiftly assess newly reported biomarkers and to establish flourishing collaborations. For example, we were able to validate the prevalence of the

widely acclaimed predictive signatures from the IMmotion150 trial (chapter 10), and we contribute significantly to the multicenter IMDC database, which is an unrivaled source of real world data on ccRCC.



In terms of predictive biomarkers for VEGFR-TKIs, which were the standard 1st line treatment at the start of this thesis, we were among the first to report the predictive value of angiogenic gene expression, which has by now been confirmed in several randomized trials (chapter 7-8). (16,62) We were also able to establish the predictive value of the molecular subtypes for 1st line treatment with pazopanib (chapter 3), and to provide a molecular explanation for the predictive value of the IMDC risk groups (chapter 8) as discussed above.

Current clinical questions and future work

Although several biomarkers for ICI are emerging, only sarcomatoid dedifferentiation is currently used in the clinic, which can select only a very small subset of patients. The different immune phenotypes of the ccrcc1 to -4 molecular subtypes lead us to expect that they have a predictive value for ICI as well. We have not been able to test this hypothesis during this thesis project, since these treatments have only recently become available, but we are rapidly recruiting patients. Meanwhile, we are currently classifying a large cohort of patients that received 2nd line nivolumab in the real-world NIVOREN trial (NCT03013335), which will allow us to correlate the subtypes with outcome. The ccrcc1 to -4 subtypes are also used for patient allocation in the randomized BIONIKK trial (NCT02960906), which compares different 1st line ICI and VEGFR-TKI regimens and which will complete its first data analysis soon.

Many unresolved questions remain with regard to the current combination regimens of ipilimumab + nivolumab and pembrolizumab + axitinib. We have already discussed in the introduction that it is currently not possible to make patient-tailored recommendations. Moreover, the optimal administration of these regimens is unclear. Pembrolizumab + axitinib entails considerable long-

term toxicity due to the continued use of both drugs. Maybe a run-in time of TKI is sufficient to induce increased T-cell infiltration, generate myeloid cells with immunosupporting features and induce PD-L1 expression, after which monotherapy with ICI is feasible? (73–75) Similarly, the combination ipilimumab + nivolumab induces unpredictable, severe and sometimes irreversible auto-immune toxicities in a substantial fraction of patients. Maybe we can prevent some of these morbidities by administering nivolumab monotherapy, and only boosting with ipilimumab in the absence of response? Or would this strategy decrease the quality of responses? After all, partial response rates to pembrolizumab monotherapy in 1st line do not differ greatly from those to ipilimumab-nivolumab, yet complete responses are rare, whereas depth of response is associated with longer progression-free survival. (76) Furthermore, even though most ICI-related toxicities occur shortly after start, their long-term use causes substantial financial toxicity. Is there a safe point after which we can stop ICI? Indirect data from melanoma do not seem to support the use of ICI beyond two years, but the experience in RCC is anecdotal at best.

One way in which we will further refine our knowledge of the ccRCC immune microenvironment, is by performing 3-plex and later multiplex immunohistochemistry in both treatment-naive, responding and relapsing ccRCC. Immunohistochemistry will reveal the spatial organization of the immune infiltrate in addition to bulk RNA signatures. This will allow us, for instance, to discern tertiary lymphoid structures or related immune niches that contain high endothelial venules. These niches are critical for immune surveillance of tumors, and herald a good prognosis when present at baseline, or after their induction by antiangiogenic immunotherapies. A deeper understanding of their prevalence in relation to response to systemic treatments, will guide us towards more precise tailoring of antiangiogenic and ICI therapy.

References (*"Introduction" and "Concluding discussion" sections)*

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015;65(2):87–108.
2. Fitzmaurice C, Dicker D, Pain A, Hamavid H, Moradi-Lakeh M, MacIntyre MF, et al. The global burden of cancer 2013. JAMA Oncol. 2015;1(4):505–27.
3. Cancer fact sheets, Belgian Cancer Registry, Incidence year 2017, Brussels 2019.
4. Hsieh JJ, Purdue MP, Signoretti S, Swanton C, Albiges L, Schmidinger M, et al. Renal cell carcinoma. Nat Rev Dis Prim. 2017;3(17009):1–19.
5. IARC. WHO classification of tumors of the urinary system and male genital organs. 4th edition 2016.
6. Bottaro DP, Linehan WM. Multifocal renal cancer: genetic basis and its medical relevance. Clin Cancer Res. 2005;11(20):7206–8.
7. Escudier B, Porta C, Schmidinger M, Rioux-Leclercq N, Bex A, Khoo V, et al. Renal cell carcinoma: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2019;30(5):706–20.
8. Creighton CJ, Morgan M, Gunaratne PH, Wheeler DA, Gibbs RA, Robertson G, et al. Comprehensivemolecular characterization of clear cell renal cell carcinoma. Nature. 2013;499(7456):43–9.
9. Varela I, Tarpey P, Raine K, Huang D, Ong CK, Stephens P, et al. Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. Nature. 2011;469(7331):539–42.
10. Dalgliesh GL, Furge K, Greenman C, Chen L, Bignell G, Butler A, et al. Systematic sequencing of renal carcinoma reveals inactivation of histone modifying genes. Nature. 2010;463(7279):360–3.
11. Peña-Llopis S, Vega-Rubín-de-Celis S, Liao A, Leng N, Pavía-Jiménez A, Wang S, et al. BAP1 loss defines a new class of renal cell carcinoma. Nat Genet. 2012;44(7):751.
12. Coppin C, Porzsolt F, Autenrieth M, Kumpf J, Coldman A, Wilt T. Immunotherapy for advanced renal cell cancer. Cochrane database Syst Rev. 2004;(3):CD001425.
13. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N. Molecular and genetic properties of tumors associated with local immune cytolytic activity. Cell. 2015;160(1–2):48–61.
14. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SAJR, Behjati S, Biankin A V, et al. Signatures of mutational processes in human cancer. Nature. 2013;500(7463):415–21.
15. Miao D, Margolis CA, Gao W, Voss MH, Li W, Martini DJ, et al. Genomic correlates of response to immune checkpoint therapies in clear cell renal cell carcinoma. Science. 2018;359(6377):801–6.
16. McDermott DF, Huseni MA, Atkins MB, Motzer RJ, Rini BI, Escudier B, et al. Clinical activity and molecular correlates of response to atezolizumab alone or in combination with bevacizumab versus sunitinib in renal cell carcinoma. Nat Med. 2018;24(6):749–57.

17. Smith CC, Beckermann KE, Bortone DS, De Cubas AA, Bixby LM, Lee SJ, et al. Endogenous retroviral signatures predict immunotherapy response in clear cell renal cell carcinoma. J Clin Invest. 2019;128(11):4804–20.

18. Panda A, de Cubas AA, Stein M, Riedlinger G, Kra J, Mayer T, et al. Endogenous retrovirus expression is associated with response to immune checkpoint blockade in clear cell renal cell carcinoma. JCI insight. 2018;3(16):e121522.

19. Şenbabaoğlu Y, Gejman RS, Winer AG, Liu M, Van Allen EM, de Velasco G, et al. Tumor immune microenvironment characterization in clear cell renal cell carcinoma identifies prognostic and immunotherapeutically relevant messenger RNA signatures. Genome Biol. 2016;17(1):231.

20. Remark R, Alifano M, Cremer I, Lupo A, Dieu-Nosjean MC, Riquet M, et al. Characteristics and clinical impacts of the immune environments in colorectal and renal cell carcinoma lung metastases: Influence of tumor origin. Clin Cancer Res. 2013;19(15):4079–91.

21. Giraldo NA, Becht E, Pagès F, Skliris G, Verkarre V, Vano Y, et al. Orchestration and prognostic significance of immune checkpoints in the microenvironment of primary and metastatic renal cell cancer. Clin Cancer Res. 2015;21(13):3031–40.

22. Hakimi AA, Voss MH, Kuo F, Sanchez A, Liu M, Nixon BG, et al. Transcriptomic profiling of the tumor microenvironment reveals distinct subgroups of clear cell renal cell cancer: Data from a randomized phase III trial. Cancer Discov. 2019;9(4):510–25.

23. Jansen CS, Prokhnevska N, Master VA, Sanda MG, Carlisle JW, Bilen MA, et al. An intra-tumoral niche maintains and differentiates stem-like CD8 T cells. Nature. 2019;576:465-70

24. Haas NB, Manola J, Uzzo RG, Flaherty KT, Wood CG, Kane C, et al. Adjuvant sunitinib or sorafenib for high-risk, non-metastatic renal-cell carcinoma (ECOG-ACRIN E2805): a double-blind, placebo-controlled, randomised, phase 3 trial. Lancet. 2016;387(10032):2008–16.

25. Motzer RJ, Haas NB, Donskov F, Gross-Goupil M, Varlamov S, Kopyltsov E, et al. Randomized phase III trial of adjuvant pazopanib versus placebo after nephrectomy in patients with localized or locally advanced renal cell carcinoma. J Clin Oncol. 2017;35(35):3916.

26. Ravaud A, Motzer RJ, Pandha HS, George DJ, Pantuck AJ, Patel A, et al. Adjuvant sunitinib in high-risk renal-cell carcinoma after nephrectomy. N Engl J Med. 2016;375(23):2246–54.

27. Méjean A, Ravaud A, Thezenas S, Colas S, Beauval J-B, Bensalah K, et al. Sunitinib alone or after nephrectomy in metastatic renal-cell carcinoma. N Engl J Med. 2018;379(5):417–27.

28. Bex A, Mulders P, Jewett M, Wagstaff J, van Thienen J V, Blank CU, et al. Comparison of immediate vs deferred cytoreductive nephrectomy in patients with synchronous metastatic renal cell carcinoma receiving sunitinib: the SURTIME randomized clinical trial. JAMA Oncol. 2019;5(2):164–70.

29. Dabestani S, Marconi L, Bex A. Metastasis therapies for renal cancer. Curr Opin Urol. 2016;26(6):566–72.

30. Appleman LJ, Puligandla M, Pal SK, Harris W, Agarwal N, Costello BA, et al. Randomized, double-blind phase III study of pazopanib versus placebo in patients with metastatic renal cell carcinoma who have no evidence of disease following metastasectomy: A trial of the ECOG-ACRIN cancer research group (E2810). J Clin Oncol. 2019;37(15_suppl):4502.

31. Procopio G, Cognetti F, Miceli R, Milella M, Mosca A, Chiuri VE, et al. A randomized, open label, multicenter phase 2 study, to evaluate the efficacy of sorafenib (So) in patients (pts) with metastatic renal cell carcinoma (mRCC) after a radical resection of the metastases: RESORT trial. J Clin Oncol. 2018;36(15_suppl):4502.

32. Motzer RJ, Tannir NM, McDermott DF, Arén Frontera O, Melichar B, Choueiri TK, et al. Nivolumab plus Ipilimumab versus Sunitinib in advanced renal-cell carcinoma. N Engl J Med. 2018;378(14):1277–90.

33. Heng DYC, Xie W, Regan MM, Warren MA, Golshayan AR, Sahi C, et al. Prognostic factors for overall survival in patients with metastatic renal cell carcinoma treated with vascular endothelial growth factor–targeted agents: results from a large, multicenter study. J Clin Oncol. 2009;27(34):5794–9.

34. Rini BI, Plimack ER, Stus V, Gafanov R, Hawkins R, Nosov D, et al. Pembrolizumab plus axitinib versus sunitinib for advanced renal-cell carcinoma. N Engl J Med. 2019;380(12):1116–27.

35. Motzer RJ, Penkov K, Haanen J, Rini B, Albiges L, Campbell MT, et al. Avelumab plus axitinib versus sunitinib for advanced renal-cell carcinoma. N Engl J Med. 2019;380(12):1103–15.

36. Albiges L, Powles T, Staehler M, Bensalah K, Giles RH, Hora M, et al. Updated European Association of Urology Guidelines on Renal Cell Carcinoma: Immune Checkpoint Inhibition Is the New Backbone in first line Treatment of Metastatic Clear-cell Renal Cell Carcinoma. Eur Urol. 2019;76(2):151-6.

37. Kuusk T, Albiges L, Escudier B, Grivas N, Haanen J, Powles T, et al. Antiangiogenic therapy combined with immune checkpoint blockade in renal cancer. Angiogenesis. 2017;20(2):205–15.

38. Guislain A, Gadiot J, Kaiser A, Jordanova ES, Broeks A, Sanders J, et al. Sunitinib pretreatment improves tumor-infiltrating lymphocyte expansion by reduction in intratumoral content of myeloid-derived suppressor cells in human renal cell carcinoma. Cancer Immunol Immunother. 2015;64(10):1241–50.

39. Dudani S, Graham J, Wells JC, Bakouny Z, Pal SK, Dizman N, et al. First line immuno-oncology combination therapies in metastatic renal-cell carcinoma: results from the International Metastatic Renal-cell Carcinoma Database Consortium. Eur Urol. 2019;76(6):861–7.

40. Rini BI, Pal SK, Escudier BJ, Atkins MB, Hutson TE, Porta C, et al. Tivozanib versus sorafenib in patients with advanced renal cell carcinoma (TIVO-3): a phase 3, multicentre, randomised, controlled, open-label study. Lancet Oncol. 2020;21(1):95-104.

41. Ornstein MC, Pal SK, Wood LS, Tomer JM, Hobbs BP, Jia XS, et al. Individualised axitinib regimen for patients with metastatic renal cell carcinoma after treatment with checkpoint inhibitors: a multicentre, single-arm, phase 2 study. Lancet Oncol. 2019;20(10):1386–94.

42. Powles T, Motzer RJ, Escudier B, Pal S, Kollmannsberger C, Pikiel J, et al. Outcomes based on prior therapy in the phase 3 METEOR trial of cabozantinib versus everolimus in advanced renal cell carcinoma. Br J Cancer. 2018;119(6):663–9.

43. Graham J, Shah AY, Wells JC, McKay RR, Vaishampayan U, Hansen A, et al. Outcomes of Patients with Metastatic Renal Cell Carcinoma Treated with Targeted Therapy After Immuno-oncology Checkpoint Inhibitors. Eur Urol Oncol. 2019. In press. https://doi.org/10.1016/j.euo.2019.11.001

44. Nadal R, Amin A, Geynisman DM, Voss MH, Weinstock M, Doyle J, et al. Efficacy and safety of endothelial growth factor receptor (VEGFR)-tyrosine kinase inhibitors (TKI) after programmed cell death 1 (PD-1) inhibitor treatment in patients with metastatic clear cell renal cell carcinoma (mccRCC). J Clin Oncol. 2015;33(15_suppl):4566.

45. Shah AY, Kotecha RR, Lemke EA, Chandramohan A, Chaim JL, Msaouel P, et al. Outcomes of patients with metastatic clear-cell renal cell carcinoma treated with second line VEGFR-TKI after first line immune checkpoint inhibitors. Eur J Cancer. 2019;114:67–75.

46. Auvray M, Auclin E, Barthelemy P, Bono P, Kellokumpu-Lehtinen P, Gross-Goupil M, et al. Second line targeted therapies after nivolumab-ipilimumab failure in metastatic renal cell carcinoma. Eur J Cancer. 2019;108:33–40.

47. Choueiri TK, Halabi S, Sanford BL, Hahn O, Michaelson MD, Walsh MK, et al. Cabozantinib versus sunitinib as initial targeted therapy for patients with metastatic renal cell carcinoma of poor or intermediate risk: the Alliance A031203 CABOSUN trial. J Clin Oncol. 2017;35(6):591.

48. Choueiri TK, Escudier B, Powles T, Tannir NM, Mainwaring PN, Rini BI, et al. Cabozantinib versus everolimus in advanced renal cell carcinoma (METEOR): final results from a randomised, open-label, phase 3 trial. Lancet Oncol. 2016;17(7):917–27.

49. Gan CL, Dudani S, Wells C, Donskov F, Pal SK, Dizman N, et al. Cabozantinib real-world effectiveness in the first through fourth-line settings for the treatment of metastatic renal cell carcinoma (mRCC): Results from the International mRCC Database Consortium (IMDC). J Clin Oncol. 2020;38(6_suppl):639.

50. Sternberg CN, Motzer RJ, Hutson TE, Choueiri TK, Kollmannsberger C, Bjarnason GA, et al. COMPARZ Post Hoc Analysis: Characterizing Pazopanib Responders With Advanced Renal Cell Carcinoma. Clin Genitourin Cancer. 2019;17(6):425–35.

51. Di Fiore F, Rigal O, Ménager C, Michel P, Pfister C. Severe clinical toxicities are correlated with survival in patients with advanced renal cell carcinoma treated with sunitinib and sorafenib. Br J Cancer. 2011;105(12):1811–3.

52. Poprach A, Pavlik T, Melichar B, Puzanov I, Dusek L, Bortlicek Z, et al. Skin toxicity and efficacy of sunitinib and sorafenib in metastatic renal cell carcinoma: a national registry-based study. Ann Oncol. 2012;23(12):3137–43.

53. Rini BI, Escudier B, Tomczak P, Kaprin A, Szczylik C, Hutson TE, et al. Comparative effectiveness of axitinib versus sorafenib in advanced renal cell carcinoma (AXIS): a randomised phase 3 trial. Lancet. 2011;378(9807):1931–9.

54. Sacré A, Barthélémy P, Korenbaum C, Burgy M, Wolter P, Dumez H, et al. Prognostic factors in second line targeted therapy for metastatic clear-cell renal cell carcinoma after progression on an anti-vascular endothelial growth factor receptor tyrosine kinase inhibitor. Acta Oncol. 2016;55(3):329–40.

55. McDermott DF, Lee J-L, Ziobro M, Gafanov RA, Matveev VB, Suárez C, et al. First line pembrolizumab (pembro) monotherapy for advanced non-clear cell renal cell carcinoma (nccRCC): Results from KEYNOTE-427 cohort B. J Clin Oncol. 2019;37(7_suppl):546.

56. Gupta R, Ornstein MC, Gul A, Allman KD, Ball J, Wood LS, et al. Clinical activity of ipilimumab plus nivolumab (Ipi/Nivo) in patients (pts) with metastatic non-clear cell renal cell carcinoma (nccRCC). J Clin Oncol. 2019;37(15_suppl).

57. Chanzá NM, Xie W, Bilen MA, Dzimitrowicz H, Burkart J, Geynisman DM, et al. Cabozantinib in advanced non-clear-cell renal cell carcinoma: a multicentre, retrospective, cohort study. Lancet Oncol. 2019;20(4):581–90.

58. Delafonaine B, De Backer C, Beuselinck B, Debruyne P, D'Hondt L, Gennigens C, et al. An update on the management of metastatic clear-cell renal cell carcinoma: the BSMO expert panel recommendations. BJMO. 2020;14(2):56-70.

59. Wang T, Lu R, Kapur P, Jaiswal BS, Hannan R, Zhang Z, et al. An Empirical Approach Leveraging Tumorgrafts to Dissect the Tumor Microenvironment in Renal Cell Carcinoma Identifies Missing Link to Prognostic Inflammatory Factors. Cancer Discov. 2018;8(9):1142–55.

60. Escudier B, Motzer RJ, Tannir NM, Porta C, Tomita Y, Maurer MA, et al. Efficacy of Nivolumab plus Ipilimumab According to Number of IMDC Risk Factors in CheckMate 214. Eur Urol. 2020;77(4):449–53.

61. Verbiest A, Renders I, Caruso S, Couchy G, Job S, Laenen A, et al. Clear-cell Renal Cell Carcinoma: Molecular Characterization of IMDC Risk Groups and Sarcomatoid Tumors. Clin Genitourin Cancer. 2019;17(5):e981-94.

62. Choueiri TK, Albiges L, Haanen J, Larkin J, Uemura M, Pal SK, et al. Biomarker analyses from JAVELIN Renal 101: Avelumab + axitinib (A+Ax) versus sunitinib (S) in advanced renal cell carcinoma (aRCC). J Clin Oncol. 2019;37(15_suppl):101.

63. McDermott DF, Choueiri TK, Motzer RJ, Aren OR, George S, Powles T, et al. CheckMate 214 post-hoc analyses of nivolumab plus ipilimumab or sunitinib in IMDC intermediate/poor-risk patients with previously untreated advanced renal cell carcinoma with sarcomatoid features. J Clin Oncol. 2019;37(15_suppl):4513.

64. McDermott DF, Huseni MA, Atkins MB, Motzer RJ, Rini BI, Escudier B, et al. Clinical activity and molecular correlates of response to atezolizumab alone or in combination with bevacizumab versus sunitinib in renal cell carcinoma. Nat Med. 2018;24(6):749.

65. Beuselinck B, Job S, Becht E, Karadimou A, Verkarre V, Couchy G, et al. Molecular subtypes of clear cell renal cell carcinoma are associated with sunitinib response in the metastatic setting. Clin Cancer Res. 2015;21(6):1329–39.

66. Brooks SA, Brannon AR, Parker JS, Fisher JC, Sen O, Kattan MW, et al. ClearCode34: A prognostic risk predictor for localized clear cell renal cell carcinoma. Eur Urol. 2014;66(1):77–84.

67. Gomez DR, Blumenschein Jr GR, Lee JJ, Hernandez M, Ye R, Camidge DR, et al. Local consolidative therapy versus maintenance therapy or observation for patients with oligometastatic non-small-cell lung cancer without progression after first line systemic therapy: a multicentre, randomised, controlled, phase 2 study. Lancet Oncol. 2016;17(12):1672–82.

68. Rozeman EA, Menzies AM, van Akkooi ACJ, Adhikari C, Bierman C, van de Wiel BA, et al. Identification of the optimal combination dosing schedule of neoadjuvant ipilimumab plus nivolumab in macroscopic stage III melanoma (OpACIN-neo): a multicentre, phase 2, randomised, controlled trial. Lancet Oncol. 2019;20(7):948–60.

69. Mickisch GHJ, Garin A, Van Poppel H, de Prijck L, Sylvester R. Radical nephrectomy plus interferon-alfa-based immunotherapy compared with interferon alfa alone in metastatic renal-cell carcinoma: a randomised trial. Lancet. 2001;358(9286):966–70.

70. Flanigan RC, Salmon SE, Blumenstein BA, Bearman SI, Roy V, McGrath PC, et al. Nephrectomy followed by interferon alfa-2b compared with interferon alfa-2b alone for metastatic renal-cell cancer. N Engl J Med. 2001;345(23):1655–9.

71. Pignot G, Thiery-Vuillemin A, Walz J, Lang H, Werle P, Balssa L, et al. Nephrectomy After Complete Response to Immune Checkpoint Inhibitors for Metastatic Renal Cell Carcinoma: A New Surgical Challenge? Eur Urol. 2020. In press. DOI:10.1016/j.eururo.2019.12.018.

72. Gao J, Karam JA, Tannir NM, Campbell MT, Slack Tidwell R, Ahrar K, et al. A pilot randomized study evaluating nivolumab (nivo) or nivo + bevacizumab (bev) or nivo + ipilimumab (ipi) in patients with metastatic renal cell carcinoma (MRCC) eligible for cytoreductive nephrectomy, metastasectomy or post-treatment biopsy (Bx). J Clin Oncol. 2019;37(15_suppl):4501.

73. Schmittnaegel M, Rigamonti N, Kadioglu E, Cassará A, Rmili CW, Kiialainen A, et al. Dual angiopoietin-2 and VEGFA inhibition elicits antitumor immunity that is enhanced by PD-1 checkpoint blockade. Sci Transl Med. 2017;9(385):eaak9670.

74. Dirkx AEM, oude Egbrink MGA, Kuijpers MJE, van der Niet ST, Heijnen VVT, Bouma-ter Steege JCA, et al. Tumor angiogenesis modulates leukocyte-vessel wall interactions in vivo by reducing endothelial adhesion molecule expression. Cancer Res. 2003;63(9):2322–9.

75. Allen E, Jabouille A, Rivera LB, Lodewijckx I, Missiaen R, Steri V, et al. Combined antiangiogenic and anti–PD-L1 therapy stimulates tumor immunity through HEV formation. Sci Transl Med. 2017;9(385):eaak9679.

76. McDermott DF, Lee JL, Szczylik C, Donskov F, Malik J, Alekseev BY, et al. Pembrolizumab monotherapy as first line therapy in advanced clear cell renal cell carcinoma: Results from cohort A of KEYNOTE-427. J Clin Oncol. 2018;36(15_suppl):4500.

Chapter 13

Scientific abstract

This thesis project aimed to identify novel prognostic and predictive biomarkers in clear-cell renal cell carcinoma (ccRCC), with a focus on previously established molecular subtypes. Systemic treatment paradigms for ccRCC have changed at lightning speed during the last 15 years: best supportive care and cytokine-base immune therapies have been replaced by angiogenesis inhibitors, which are now again challenged by immune checkpoint inhibitors (ICI). In Belgium, no less than 15 molecules are currently approved. However, this unprecedented luxury of options has entailed a desperate need for biomarkers to tailor treatment choices: only a subset of patients responds to any given treatment, and it is currently impossible to identify these in advance. Even more importantly, we urgently need to profoundly understand the molecular underpinnings of ccRCC and its immune microenvironment, to guide research towards optimal strategies for the use of both new and existing treatments.

Our group has recently proposed four molecular subtypes of advanced ccRCC, ccrcc1 to -4, based on unsupervised clustering of whole transcriptome data. These subtypes differ not only in terms of gene expression, but also mutation and methylation profiles, immune cell infiltration, histological features, prognosis and response to sunitinib. Briefly, the rare ccrcc3 tumors show an indolent behavior, ccrcc2 tumors have a good prognosis and high response rates to sunitinib, ccrcc1 tumors are intermediate and ccrcc4 tumors are aggressive, have a stem-cell like phenotype with upregulation of MYC-targets, and are highly inflamed with an immune suppressive microenvironment. We hypothesized that these subtypes could have a predictive value in other clinical settings besides treatment with sunitinib and could vastly improve our molecular understanding of ccRCC. Moreover, as these subtypes reflect intrinsic differences in ccRCC tumor biology instead of having been developed as a biomarker for a specific therapeutic setting, they have the potential to

retain their value in the swiftly changing landscape of ccRCC treatment.

In this thesis project, we have taken advantage of our large clinical databases and tissue bank to further unravel the molecular characteristics of the ccrcc1 to -4 subtypes and assess their value as a biomarker in different therapeutic settings. Using the established classifier algorithm on fresh-frozen tumor tissue, we have shown that ccrcc1 and -4 subtypes predict a high risk of early relapse after radical metastasectomy, and that the ccrcc2 subtype is predictive for favorable outcome on 1st line pazopanib, as well as sunitinib. Selective enrichment of the ccrcc2 subtype in IMDC Good risk patients explains the predictive value of IMDC for sunitinib and refines it, as ccrcc2 can also identify sunitinib-sensitive tumors among IMDC Intermediate risk patients. Using qRT-PCR of genes in the *VEGF*-pathway, we showed that proangiogenic gene expression is predictive for outcome on sunitinib. Moreover, the ccrcc2 subtype is a highly selective marker for angiogenic tumors, showing an almost perfect overlap with the validated "Angio high" predictive signatures from the IMmotion150 trial. To expand the use of our subtypes, which were established on fresh-frozen material, we have validated them on paraffin-embedded tissue using RNAseq. These RNAseq data have not only confirmed an angiogenic and inflamed expression profile in ccrcc2 and ccrcc4 tumors respectively, but also shed new light on the drivers of ccrcc3 and ccrcc1 tumors. Ccrcc3 tumors appear to be driven by mutations in the *FLCN* pathway, whereas arm level loss of chr14q is likely in ccrcc1 tumors. Further molecular characterization has validated the prevalence of the three predictive gene signatures from the IMmotion150 trial on RNAseq data, which differ according to molecular subtype, and has revealed vastly different microRNA expression patterns between the subtypes through miRNome analysis. In a final and ongoing part of the project, we have performed RNAseq of 174 ccRCC metastases and compared these to their primary tumors. Preliminary analyses reveal that angiogenic gene expression is highly conserved between primary tumors and their metastases, whereas immune signatures show more heterogeneity.

In conclusion, this thesis project has built on previously established molecular subtypes in ccRCC to uncover their different molecular characteristics and establish their value as a biomarker in different therapeutic settings. The resulting findings will guide us towards personalized treatment decisions and rational trial design, and provide the basis for the ongoing search of our group towards biomarkers in ccRCC.

Chapter 14

Scientific acknowledgements

We are very grateful to the Stand up to Cancer Foundation (Kom op tegen Kanker) and Research Foundation Flanders (FWO) whose funding has made this thesis project possible. Furthermore we would like to thank Thomas Van Brussel for performing the quality control and sequencing of the RNA libraries (chapter 10) and Annouschka Laenen for her statistical advice (chapter 10). Lastly we are very much indebted to the pathology department and center for human genetics of University Hospitals Leuven, who have granted us the use of their lab facilities and access to the tissue bank.

Chapter 15

Personal contribution

Detailed author contributions can be found in the separate chapters. This section will focus on the personal contributions of the thesis candidate (AV).

Chapter 3 – AV has performed RNA extraction and qRT-PCR for molecular classification together with Laure Caruana and Gabrielle Couchy. Benoit Beuselinck has collected the clinical data. AV has performed data analysis and has written the manuscript.

Chapter 4 – AV has performed the literature search, data extraction and analysis and has written the manuscript.

Chapter 5 – AV has collected the clinical data, performed data analysis and has written the manuscript. Annouschka Laenen has provided advice on statistical methodology.

Chapter 6 - AV has performed RNA extraction and qRT-PCR for molecular classification together with Laure Caruana and Gabrielle Couchy. AV has collected the clinical data, performed data analysis and has written the manuscript.

Chapter 7 – AV has performed literature review on *PBRM1* and *BAP1* mutations as well as critical revision of the manuscript.

Chapter 8 – AV has performed RNA extraction and qRT-PCR for molecular classification together with Gabrielle Couchy. Benoit Beuselinck and Inne Renders have collected the clinical data. Annouschka Laenen has calcultated the optimal cutoff for sarcomatoid dedifferentiation. AV has performed further data analysis and has written the manuscript.

Chapter 9 – The team from CNIO has performed miRNome analysis. Benoit Beuselinck has collected the clinical data. AV has directed bioinformatics and statistical analysis, which were performed by Vincent Van Hoef and Annouschka Laenen. AV has interpreted the results and has written the manuscript.

Chapter 10 – AV has performed sample selection. AV, Benoit Beuselinck and Eduard Roussel have collected the clinical data. AV has prepared tissue slides, judged tumor content, performed RNA extraction and prepared RNA libraries. For a minority of samples, extracted RNA was available from INSERM,

and another small batch was fully processed by Eduard Roussel. Thomas Van Brussel has performed library quality control and RNAseq. Vincent Van Hoef has performed preprocessing, normalization and clustering of RNAseq data. AV has performed gene expression analysis (GSEA, CIBERSORTx, CYT, IMmotion 150 signatures), survival analysis and correlation with clinical features. Annouschka Laenen has calculated R-square and intraclass correlation coefficients. AV has interpreted the data and has written the manuscript.

Chapter 11 – Benoit Beuselinck has selected SNPs of interest and collected clinical data. AV has performed DNA extraction. Thomas Van Brussel has performed SNP genotyping; other molecular data were available from previous experiments. AV has performed data analysis and has written the manuscript.

Conflict of interest statement

Co-author	Honoraria	Trial investigator	Research grant	No relevant COI
Albersen, Maarten				x
Albigès, Laurence	BMS, Pfizer, Ipsen, Peloton Therapeutics, Roche, MSD and Novartis			
Baldewijns, Marcella				x
Beuselinck, Benoit	Amgen, Ipsen, Pfizer, Bayer, Novartis	Pfizer	BMS, Ipsen	
Caruana, Laure				x
Caruso, Stefano				x
Couchy, Gabrielle				x
Grana-Castro, Osvaldo				x
De Meerleer, Gert				x
García-Donas, Jésus	Pfizer, Novartis		Pfizer	
Joniau, Steven				x
De Reyniès, Aurélien				x
Fridman, Wolf-Hervé	Adaptimmune, BMS, Curetech, Efranat, Novartis, PFM, Servier			
Job, Sylvie				x
Laenen, Annouschka				x
Lambrechts, Diether				x
Laguerre, Brigitte	Pfizer, Novartis, BMS, Ipsen			
Lerut, Evelyne				x
Machiels, Jean-Pascal	Pfizer, Roche, AstraZeneca, Bayer, Innate, Merck Serono, BristolMyers Squibb, Novartis, Janssen, Incyte, Cue Biopharma, ALX Oncology, Debio, Nanbiotix			
Meiller, Clément				x
Méjean, Arnaud	BMS, Roche, Janssen, Ferring Pharmaceuticals, Sanofi, Ipsen			
Oudard, Stéphane	Novartis, Pfizer, Roche, BMS, Ipsen, Eisai and Bayer			
Oyen, Raymond				x
Patard, Jean-Jacques	Pfizer		Pfizer	
Renders, Inne				x
Reza-Thierry, Elaidi				x
Rioux-Leclercq, Nathalie				x
Rodriguez-Antona, Cristina			Pfizer	
Roussel, Eduard				x
Sautès-Fridman, Catherine				x
Schöffski, Patrick	Merck, Novartis, GSK, Exelixis		Novartis, Exelixis	
Tosco, Lorenzo				x
Van Brussel, Thomas				x
Van Den Eynde, Kathleen				x
Van Hoef, Vincent				x
Vano, Yann				x
Van Poppel, Hendrik				x
Van Raemdonck, Dirk				x
Verbiest, Annelies	Pfizer, Novartis			
Verkarre, Virginie				x
Wozniak, Agnieszka				x
Zucman-Rossi, Jessica				x

Chapter 17

Bibliography

Peer-reviewed publications

1. **Verbiest A**, Pirenne J, Dierickx D. De novo thrombotic microangiopathy after non-renal solid organ transplantation. Blood reviews. 2014;28(6):269-79
2. Van Cann T, Loyson T, **Verbiest A**, Clement PM, Bechter O, Willems L, Spriet I, Coropciuc R, Politis C, Vandeweyer RO, Schoenaers J. Incidence of medication-related osteonecrosis of the jaw in patients treated with both bone resorption inhibitors and vascular endothelial growth factor receptor tyrosine kinase inhibitors. Supportive Care in Cancer. 2017;26(3):869-78
3. Requilé A, Clement PM, Bechter OE, Dumez H, **Verbiest A**, Sciot R, Hompes D, Sinnaeve F, Van Limbergen E, Schöffski P. Single Centre Experience of Systemic Treatment with Vincristin, Ifosfamide and Doxorubicin (VIA) alternating with Etoposide, Ifosfamide and Cisplatin (VIP) in adult patients with Ewing sarcoma. Sarcoma. 2017
4. Beuselinck B, **Verbiest A**, Couchy G, Job S, de Reynies A, Meiller C, Albersen M, Verkarre V, Lerut E, Méjean A, Patard JJ. Pro-angiogenic gene expression is associated with better outcome on sunitinib in metastatic clear-cell renal cell carcinoma. Acta Oncologica. 2018;57(4):498-508
5. **Verbiest A**, Couchy G, Job S, Zucman-Rossi J, Caruana L, Lerut E, Oyen R, de Reyniès A, Laguerre B, Rioux-Leclercq N, Wozniak A. Molecular Subtypes Of Clear-Cell Renal Cell Carcinoma Are Associated With Outcome On Pazopanib In The Metastatic Setting. Clinical Genitourinary Cancer. 2018;16(3):e605-e612
6. **Verbiest A**, Couchy G, Job S, Caruana L, Lerut E, Oyen R, de Reyniès A, Tosco L, Joniau S, Van Poppel H, Van Raemdonck D, Van Den Eynde K, Wozniak A, Zucman-Rossi J, Beuselinck B. Molecular subtypes of clear-cell renal cell carcinoma are prognostic for outcome after complete metastasectomy. European Urology. 2018;74(4):474-480
7. **Verbiest A**, De Meerleer G, Albersen M, Beuselinck B. Non-surgical ablative treatment of distant extracranial metastases for renal cell carcinoma: a systematic review. Kidney Cancer. 2018;2:57-67

8. **Verbiest A**, Lambrechts D, Van Brussel T, Couchy G, Wozniak A, Méjean A, Lerut E, Oudard S, Verkarre V, Job S, De Reynies A, Machiels JP, Patard JJ, Zucman-Rossi J, Beuselinck B. Polymorphisms in the Von Hippel Lindau gene are associated with overall survival in metastatic clear-cell renal cell carcinoma patients treated with VEGFR-tyrosine kinase inhibitors. Clinical Genitourinary Cancer. 2018;16(4):266-273

9. Bastin J, Werbrouck E, **Verbiest A**, Punie K., Bechter O, Woei-A-Jin FJ, Wolter P, Wildiers H, Lerut E, Dumez H, Decallonne B, Clement P, Vanderschueren D, Albersen M, Oyen R, Schöffski P, Beuselinck B. Prospective evaluation of hypogonadism in male metastatic renal cell carcinoma patients treated with targeted therapies. Acta Clinica Belgica. 2019;74(3):169-79

10. Werbrouck E, Bastin J, Lambrechts D, **Verbiest A**, Van Brussel T, Lerut E, Machiels JP, Verschaeve V, Richard V, Debruyne PR, Decallonne B, Schöffski P, Bechter O, Wolter P, Beuselinck B. ABCG2 polymorphism rs2231142 and hypothyroidism in metastatic renal cell carcinoma patients treated with sunitinib. Acta Clinica Belgica. 2019;74(3):180-88

11. Vanmechelen M, Lambrechts D, Van Brussel T, **Verbiest A**, Couchy G, Schöffski P, Dumez H, Debruyne PR, Lerut E, Machiels JP, Richard V, Albersen M, Verschaeve V, Oudard S, Méjean A, Wolter P, Zucman-Rossi J, Beuselinck B. Fibroblast Growth Factor Receptor-2 polymorphism rs2981582 is correlated with progression-free survival and overall survival in patients with metastatic clear-cell renal cell carcinoma treated with sunitinib. Clinical Genitourinary Cancer. 2019;17(2):e235-46

12. Haaker L, De Meue E, Wildiers H, **Verbiest A**, Dumez H, Lerut E, Pans S, Albersen M, Beuselinck B. Bone metastases and age are associated with earlier dose reductions in metastatic clear-cell renal cell carcinoma patients treated with angiogenesis inhibitors. Acta Clinica Belgica. 2019;74(6):414-23

13. **Verbiest A**, Renders I, Caruso S, Couchy G, Job S, Laenen A, Verkarre V, Rioux-Leclercq N, Schöffski P, Vano Y, Elaidi RT, Lerut E, Albersen M, Oudard S, Fridman WH, Sautès-Fridman C, Albigès L, Wozniak A, Zucman-Rossi J, Beuselinck B. Clear-cell Renal Cell Carcinoma: Molecular Characterization of IMDC Risk Groups and Sarcomatoid Tumors. Clinical Genitourinary Cancer. 2019;17(5):e981-94

14. Delafontaine B, De Backer C, Beuselinck B, Debruyne P, D'Hondt L, Gennigens C, Gil T, Vulsteke C, Martinez-Chanza N, **Verbiest A**, Strijbos M, Van Lancker G, Pelgrims G, Rottey S, on behalf of the BSMO Uro-Oncology Task Force Group. An update on the management of metastatic clear-cell renal cell carcinoma: the BSMO expert panel recommendations. Belgian Journal of Medical Oncology. 2020;14(2):56-70

15. Beuselinck B, Pans S, Bielen J, De Wever L, Noppe N, Vanderschueren G, Baldewijns M, Lerut E, Laenen A, Roussel E, **Verbiest A**, De Keyzer F, Albersen M, Vandecaveye V. Whole-body diffusion-weighted magnetic resonance imaging for the detection of bone metastases and their prognostic impact in metastatic renal cell carcinoma patients treated with angiogenesis inhibitors. Acta Oncologica. 2020. Doi: 10.1080/0284186X.2020.1750696. *In press.*

16. Haaker L, Tryssesoone L, Tamborijn J, Renders I, **Verbiest A**, Lerut E, Baldewijns M, Bourgain C, Roussel E, Van den Bulck H, Wynendaele W, Laguere B, Rioux-Leclercq N, Oudard S, Zucman-Rossi J, Laenen A, Debruyne P, Albersen M, Beuselinck B. Bone metastasis is associated with poor prognosis in metastatic papillary renal cell carcinoma patients treated with 1st line angiogenesis inhibitors. Urologic Oncology: Seminars and Original Investigations. 2020. *In press.*

17. Roussel E, Campi R, Larcher A, **Verbiest A,** Antonelli A, Palumbo C, Derweesh I, Ghali F, Bradshaw A, Musquera M, D'Anna M, Autorino R, Guruli G, Veccia A, Linares E, Van Bruwaene S, Hevia V, Porpiglia F, Checcuci E, Minervini A, Mari A, Marchioni M, Pavan N, Claps F, Capitanio U, Beuselinck B, Mir MC, Albersen M. Predictors of Perioperative Complications in Cytoreductive Nephrectomy: Analysis of the Registry for MetAstatic RCC (REMARCC). European Urology Oncology. 2020. *In press.*

18. Beuselinck B, Van Brussel T, **Verbiest A**, Vanmechelen M, Couchy G, Oudard S, Elaidi R, Roussel E, Albersen M, Debruyne PR, Baldewijns M, Machiels JP, Richard V, Verschaeve V, Pascal Wolter, Zucman-Rossi J, Lambrechts D. Validation of the correlation between single nucleotide polymorphism rs307826 in VEGFR3 and outcome in metastatic clear-cell renal cell carcinoma patients treated with sunitinib. Kidney Cancer. 2020. *In press.*

19. Verbiest A, Van Hoef V, Rodriguez-Antona C, García-Donas J, Graña-Castro O, Albersen M, Baldewijns M, Laenen A, Roussel E, Schöffski P, Wozniak A, Caruso S, Couchy G, Zucman-Rossi J, Beuselinck B. MicroRNA expression profiles in molecular subtypes of clear-cell renal cell carcinoma are associated with clinical outcome and repression of specific mRNA targets. PLOS ONE. 2020. *In press.*

Papers posted on preprint server

1. Dufies M, **Verbiest A,** Cooley LS, Ndiaye PD, Viotti J, He X, Nottet N, Souleyreau W, Hagege A, Torrino S, Parola J, Giuliano S, Borchiellini D, Schiappa R, Mograbi B, Zucman-Rossi J, Bensalah K, Ravaud A, Auberger P, Bikfalvi A, Chamorey E, Rioux-Leclercq N, Mazure N, Beuselinck B, Cao Y, Bernhard JC, Ambrosetti D, Pages G. Parola J. A novel mechanism of HIF2-dependent PLK1-mediated metastasis and drug resistance of clear cell Renal Cell Carcinoma. MedRxiv. 2020. Doi: 10.1101/2020.02.05.20020552.

Congress abstracts

1. **Verbiest A**, Couchy G, Job S, Zucman-Rossi J, Caruana L, Lerut E, Oyen R, De Reynies A, Tosco L, Joniau S, Van Poppel H, Wozniak A, Beuselinck B. Molecular subtypes of metastatic clear-cell renal cell carcinoma are associated with outcome after metastasectomy with curative intent. European Urology Supplements. 2017;16(10):e2625-7. EMUC 2017. Oral presentation, proferred abstract.

2. Beuselinck B, **Verbiest A**, Couchy G, Job S, de Reyniès A, Caruso S, Verkarre V, Rioux-Leclercq N, Schöffski P, Vano Y, Elaidi RT. 869PD Tumor molecular characteristics in patients (pts) with international metastatic renal cell carcinoma database consortium (IMDC) good (G) and intermediate/poor (I/P) risk. Annals of Oncology. 2018 Oct 1;29(suppl_8):mdy283-078. ESMO 2018. Oral presentation, proferred abstract.

3. **Verbiest A**, Beuselinck B, Couchy G, Job S, De Reynies A, Meiller C, Albersen M, Verkarre V, Lerut E, Mejean A, Patard JJ, Laguerre B, Rioux-Leclercq N, Schöffski P, Oudard S, and Zucman-Rossi J. Metastatic clear cell renal cell carcinoma: Proangiogenic gene expression and outcome on sunitinib. Journal of Clinical Oncology. 2017;35(suppl_5):e16085. ASCO 2017

4. **Verbiest A**, Beuselinck B, Couchy G, Job S, De Reynies A, Meiller C, Albersen M, Verkarre V, Lerut E, Mejean A, Patard JJ, Schöffski P, Oudard S, Zucman-Rossi J. Metastatic clear-cell renal cell carcinoma: pro-angiogenic gene expression is associated with better outcome on sunitinib. BACR 2017

5. **Verbiest A**, Zucman-Rossi J, Lambrechts D, Beuselinck B. Polymorphisms in the Von Hippel Lindau gene are associated with poor overall survival in metastatic clear-cell renal cell carcinoma patients treated with VEGFR tyrosine kinase inhibitors. BSMO 2017

6. **Verbiest A**, Van Hoef V, Lambrechts D, Rodriguez-Antona C, Grana-Castro O, Inglada-Perez L, Albersen M, Baldewijns M, Laenen A, Schöffski P, Wozniak A, Couchy G, Caruso S, Zucman-Rossi J, Beuselinck. Advanced clear-cell renal cell carcinoma (ccRCC): association of microRNAs (miRNAs) with molecular subtypes, mRNA targets and outcome. Annals of Oncology. 2019;30(suppl_5):v356-v402 ESMO 2019

7. Roussel E, Milenkovic U, **Verbiest A**, Everaerts W, Van Cleynenbreugel B, Van Poppel H, Joniau S, Beuselinck B, Albersen M. Patient selection for cytoreductive nephrectomy: Predicting factors for complications and oncological survival. European Urology Supplements. 2019;17(14):e2943. EMUC 2019

8. Roussel E, **Verbiest A**, Milenkovic U, Everaerts W, Van Cleynenbreugel B, Van Poppel H, Joniau S, Beuselinck B, Albersen M. De plaats van cytoreductieve nefrectomie in de hedendaagse behandeling van gemetastaseerd renal cel carcinoom: predictors voor complicaties en overleving. Elautprijs 2020. Best poster award.