MOLECULAR SUBTYPES OF CLEAR CELL RENAL CELL CARCINOMA ARE ASSOCIATED TO SUNITINIB RESPONSE IN THE METASTATIC SETTING


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Running Title: Transcriptomic predictor of sunitinib response in RCC

List of Key genes/proteins: MYC, PBRM1, PDCD1, VEGFR, VHL
STATEMENT OF TRANSLATIONAL RELEVANCE

After ten years of routine clinical use of tyrosine kinase inhibitors (TKIs) in metastatic clear-cell renal cell carcinoma (m-ccRCC), the selection of patients who might benefit from this treatment remains a challenge. In this translational work, based on a large series of fresh frozen kidney tumor samples combined with an extended clinical database, we have defined four robust molecular subtypes of m-ccRCC significantly associated with different responses to sunitinib. This work not only provides the first integrative genomic study of m-ccRCC, but it identifies subtypes of tumors resistant to sunitinib and associated with poor survival. These molecular subtypes of tumors are characterized by a stem-cell polycomb signature, a CpG hypermethylation, sarcomatoid differentiation and a strong inflammatory, Th1-oriented but suppressive immune microenvironment. These new molecular signatures could be used for a more personalized m-ccRCC treatment to administrate TKIs, demethylating or immunomodulatory drugs according to the molecular typing of the tumors.
ABSTRACT

Purpose: Selecting patients with metastatic clear-cell renal cell carcinoma (m-ccRCC) who might benefit from treatment with targeted tyrosine kinase inhibitors (TKIs) is a challenge. Our aim was to identify molecular markers associated with outcome in m-ccRCC patients treated with sunitinib.

Experimental Design: We performed global transcriptome analyses on 53 primary resected ccRCC tumors from patients who developed metastatic disease and were treated with first-line sunitinib. We also determined chromosome copy number aberrations, methylation status and gene mutations in VHL and PBRM1. Molecular data were analyzed in relation with response rate (RR), progression-free survival (PFS) and overall survival (OS). Validation was performed in 47 additional ccRCC samples treated in first-line metastatic setting with sunitinib.

Results: Unsupervised transcriptome analysis identified 4 robust ccRCC-subtypes (ccrcc1 to 4) related to previous molecular classifications that were associated with different responses to sunitinib treatment. ccrcc1/ccrcc4-tumors had a lower RR (p=0.005) and a shorter PFS and OS than ccrcc2/ccrcc3-tumors (p=0.001 and 0.0003, respectively). These subtypes were the only significant covariate in the multivariate cox model for PFS and OS (p=0.017 and 0.006, respectively). ccrcc1/ccrcc4-tumors were characterized by a stem-cell polycomb signature and CpG hypermethylation whereas ccrcc3-tumors, sensitive to sunitinib, did not exhibit cellular response to hypoxia. Moreover, ccrcc4-tumors exhibited sarcomatoid differentiation with a strong inflammatory, Th1-oriented but suppressive immune microenvironment, with high expression of PDCD1 (PD-1) and its ligands.

Conclusion: ccRCC molecular subtypes are predictive of sunitinib response in metastatic patients, and could be used for personalized mRCC treatment with TKIs, demethylating or immunomodulatory drugs.

KEYWORDS: clear cell renal cell carcinoma, sunitinib, outcome, gene expression profiling
INTRODUCTION

Targeted therapies (TT) have significantly improved the prognosis of m-ccRCC-patients. Sunitinib is a TKI targeting vascular-endothelial-growth-factor-receptor (VEGFR) that significantly prolongs PFS, but not OS, as compared to Interferon-alpha(1, 2). Currently, it is an approved first-line treatment option for m-ccRCC-patients. However, in the pivotal phase III trial 7% of patients experience progressive disease (PD) upon RECIST at their first evaluation and virtually all patients ultimately develop PD(2). In routine clinical practice, primary PD rate is higher and may reach 20%. Although several prognostic factors of survival in m-ccRCC-patients receiving TT have been described and several resistance mechanisms have been proposed(3), no reliable biomarkers of sunitinib sensitivity or primary/secondary resistance have been identified.

Recent unsupervised gene expression analysis of 48 ccRCCs identified two molecular ccRCC-subtypes (ccA and ccB)(4). A meta-analysis of 480 ccRCCs confirmed this classification and identified a third subtype, cluster_3, associated with a wild-type von Hippel-Lindau (VHL) gene profile(5). The Cancer Genome Atlas (TCGA) project subsequently highlighted four ccRCC-subtypes(6). In both studies, post-nephrectomy survival was related to ccRCC-subtype. A poor prognosis was also associated with somatic mutations in BAP1 and SETD2(7, 8) and with chromosome amplifications or losses at 8q,14q or 9p(9-12). However, no associations have been made between molecular features and outcome in m-ccRCC-patients on TT(13).

We hypothesized that an integrated genomic analysis of primary ccRCCs might help to identify subgroups of ccRCCs more sensitive or resistant to anti-VEGFR-TT. Thus, the aim of our study was to determine whether expression profiles, genomic abnormalities, mutational status and epigenetic changes were related to outcomes after first-line sunitinib in m-ccRCC-patients.
MATERIAL AND METHODS

**Included patients**

Primary ccRCC-specimens were collected from 121 patients undergoing nephrectomy in 19 French and one Belgian hospitals from 1994 to 2011 (patients characteristics in Table_S1). For inclusion in the study, patients had to have developed synchronous or metachronic metastases, received sunitinib (50mg/day, four weeks-on/two weeks-off) as first-line treatment in the metastatic setting (prior cytokine therapy was allowed), completed at least one 28-day cycle of sunitinib, and undergone their first CT-scan assessment.

Drug schedule and dose-reduction policy complied with local practice guidelines. Follow-up chest/abdomen CT-scans were performed every 2 cycles of treatment. Study endpoints were RR according to RECIST 1.0, PFS and OS. Four expert genitourinary pathologists blinded to patient outcome reviewed all nephrectomy pathology slides. The protocol was approved by the medical ethics review boards of all participating institutions. Signed consent was obtained from all patients in accordance with French and Belgian legislations. Frozen biological material from deceased patients was used when prior agreement for such use had been given by the institutional review board.

**Data sets and preprocessing**

**Transcriptome data:**

Transcriptomic profiling was performed using HuGene 1.0ST Affymetrix array for 53 ccRCC-samples and 6 adjacent normal tissue samples (NTs). Biotinylated single strand cDNA targets were prepared with 200ng of total RNA, using the Ambion WT Expression Kit the GeneChip® WT Terminal Labeling Kit according to Affymetrix recommendations.

**Methylome data:**

Whole-genome DNA methylation was analyzed in 102 ccRCCs and 5 adjacent NTs using the Illumina Infinium HumanMethylation450 assay that examines the DNA methylation status of 485,000 CpG sites (covering 99% of RefSeq genes and 96% of CpG islands). Genomic DNA was extracted using either the Manual-MagNa Pure LC DNA II kit (Roche) or the Manual-Gentra Puregene kit (Qiagen) and bisulfite-converted using the EZ-96 DNA Methylation Kit (Zymo Research, Orange, CA) by Integragen SA (Evry, France, [http://www.integragen.com](http://www.integragen.com)) and processed according to the Illumina protocol.
Genome data:

For chromosome gain/loss profiling, 103 ccRCCs and 5 adjacent NTs were analyzed with Illumina OmniExpress chips, containing 731,442 probes. Hybridization was performed by IntegraGen (Evry, France), according to the manufacturer’s instructions. The absolute copy numbers and genotype status of segments were determined using the Genome Alteration Print (GAP) method (14). Segments with an absolute copy number above (resp. below) the ploidy of the sample were considered as gains (resp. losses). The Genomic Identification of Significant Targets In Cancer (GISTIC) methodology (15) was used to identify significantly recurrent chromosome aberrations.

Quantitative RT PCR (qRT-PCR):

qRT-PCR reactions were performed on 98 ccRCCs and 5 NTs with Low Density Array (LDA) for 65 genes and individual probes for 5 genes as previously described (16). Genes were selected from the differential analysis of the Affymetrix data (anova q-value less than 0.05 and an absolute Fold change greater than 1.5). Primers and probes for all genes were obtained from Applied Biosystems Taqman Gene Expression Assays. Table_S2 provides the gene symbol, gene name and Applied Biosystems Assay ID-number for the genes studied.

For details on overlap between samples used for each omics, see Table_S3.

Mutation analysis

Tumors were screened for VHL and PBRM1-mutations (Table_S4) using direct sequencing (Primers and protocols available on request).

Omics Analysis

Unsupervised classification: For unsupervised class discovery within the four omics, three methods were used: the Recursively Partitioned Mixture Model (RPMM)(17) and two consensus clustering methods (18, 19). Only the results obtained with the third method were described in the paper. Nevertheless, we showed a strong association between the three methods (Fisher-exact p-values from 3.9e-19 to 2.92e-21). We used the gap statistic to determine the number of clusters (20).

Differential Analysis: We used moderate T-tests to identify genes differentially expressed between groups of samples, using limma R-package. Anova-models were used for multigroup comparison. To control for multiple testing we measured the local false discovery rate using the Benjamini and Hochberg method (R-package stats).
**Association analysis:** We evaluated the association between unsupervised or supervised subgroups and the bioclinical factors using chi-squared or Fisher-exact tests. For each clinical characterization all covariates were analyzed but only the significant covariates were shown (p<0.05). The bioclinical factors included TNM, ECOG-PS, IMDC-, Fuhrman- or MSKCC-scores, systemic treatment, hemoglobin (<11.5 g/dl in women, <13.0 g/dl in men), platelets (>400,000/mm³), LDH (>1.5x Upper Limit of Normal), neutrophils (>4,500/mm³), calcium (>10 mg/dl), pathological characteristics such as eosinophils, necrosis or inflammation, rhabdoid and sarcomatoid phenotypes.

Chi-squared or Fisher-exact tests were used to select the recurrent chromosome aberrations identified by GISTIC that are differential between groups. Criteria of sensitivity and specificity were added to select aberrations characteristic of a given subgroup. An aberration was characteristic of a given subgroup if the sensitivity and the specificity are >0.65.

**Signaling pathway analysis:** To identify biological features associated with ccRCC molecular subtypes, 17,306 pathways collected from KEGG, GO, MSigDB, SMD and Biocarta (and related genes) were tested. A hypergeometric test was used to measure the association between a gene (probe set) list –related to a given molecular subtype (see below)- and a biological pathway or a gene ontology term (GO term), as in GOstats R-package from R. Gentleman. Pathway analyses were performed on transcriptome data, methylome data and methylome data anti-correlated with transcriptome data (correlation test p<0.05 and correlation coefficient <0). In this pathways analysis, the gene lists related to each molecular subtypes corresponded to (i) genes specifically up-regulated in the subtype (resp. hypermethylated), (ii) genes specifically down-regulated in the subtype (resp. hypomethylated) and (iii) genes both up and down-regulated (resp. hyper and hypomethylated) in the subtype.

**Survival analysis:** Survival time 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. Survival curves were calculated according to the Kaplan-Meier method (function Surv, R-package survival, V2.29) and differences between curves were assessed using the log-rank test (function survdiff, R-package survival).

To find clinical criteria related to PFS or OS, univariate models were performed on all the pathological and clinical covariates (function coxph, R-package survival). Covariates showing a significant association to prognosis (logrank p<0.05) at the univariate level were selected to be analyzed in multivariate models, after the exclusion of redundant covariates (ex MSKCC- and IMDC-scores).

**Classifier building:** The training set was composed of 51 samples with Affymetrix and qRT-PCR and the validation set was composed of 47 samples with only qRT-PCR.
A first centroid based predictor was built to assign a sample to one of the three following groups ccrcc1&4/ccrcc2/ccrcc3 using a subset of the differentially expressed genes between these three groups. A gene was defined differentially expressed in a subtype when the anova p-value was <0.05 and when the absolute fold change with the other subtypes was >0.5. The optimal subset of 27 genes was obtained by a step by step strategy by optimizing the success rate. The DLDA-dissimilarity measure was then used to predict the subtype(Table_S2).

For samples predicted as ccrcc1&4 (using the predictor mentioned above), a second centroid-based predictor was built to assign each sample to one of the two subtypes ccrcc1 or ccrcc4. A subset of the differentially expressed genes between ccrcc1- and ccrcc4-subtypes was used. A gene was defined differentially expressed between the two subtypes when the anova p-value was <0.05 and when the absolute fold change was >3. The optimal subset of 8 genes was obtained by a step by step strategy by optimizing the success rate. The DLDA-dissimilarity measure was then used to predict the subtype.

The obtained centroid-based predictors were applied on two public datasets: The Brannon expression dataset (GEO-website, GSE33093)(5) and TCGA-dataset(6).

**Use of immune metagenes**

The datasets mentioned by Bindea and collaborators were downloaded and normalized separately using the fRMA Bioconductor package (21). Cancer cell lines dataset GSE5720 was also retrieved from Gene Expression Omnibus and normalized using fRMA. Samples GSM133550, GSM133594, GSM133638, GSM133657, which correspond respectively to ccRCC cell lines ACHN, SN12C, UO-31, Caki-1, were added. All probesets identified included in the five metagenes used in our work (NK CD56dim, Cytotoxic cells, T-cells, B-cells, Macrophages) were checked for expression by kidney cancer cell lines. 138/142 probesets were completely specific and sensitive to predict Immune cell-type against ccRCC cell lines samples, and 134/142 probesets had a right-tailed t-test p-value<0.05 when compared to ccRCC cell lines samples. To compute average metagene values in our dataset, log2 expression values for each probesets were first mapped to gene symbols using average expression of all the corresponding probesets, then this value was centered and scaled across our dataset. Finally, the mean of all the scaled expressions was taken to represent the signature.

**Immunohistochemistry:**

5-μm formalin-fixed and paraffin-embedded tissue sections were stained with an anti-CD8 (5.0µg/ml, clone SP16, Springbioscience) with an autostainer Link 48 (Dako) as previously described (22). Deparaffinization, rehydration and epitope retrieval was performed in a PT-Link (Dako) in a High pH-solution as described by the manufacturer. Tissue sections were first incubated with anti-CD8 (SP16,
Springbioscience) and posteriorly with a biotin-streptavidin coupled antibody. Revelation was done with 3-amino-9-ethylcarbazole substrate (Vector Laboratories).

RESULTS

Patient Characteristics and Clinical Predictors of sunitinib Response

In our series of 121 patients, after a median follow-up of 55 months (range 1.5-86), median PFS and OS were 13 and 27 months, respectively. 47% of patients experienced a complete or partial response (CR/PR), 36% Stable disease (SD) and 17% early PD following RECIST 1.0. By comparing patients with extreme phenotype (CR/PR versus PD), we identified several clinical and biological features associated with a poor sunitinib response: baseline neutrophilia, thrombocytosis, anemia, sarcomatoid differentiation, synchronous metastases at initial diagnosis, poorer ECOG-PS, Heng-(23) and MSKCC-scores (24, 25) (Figure_1A&B). However, in the overall series of patients, using all the clinical, pathological and molecular features and a variety of predictive algorithms (PAM, DLD, DQDA), supervised analyses failed to identify robust factors predictive of sunitinib response(Table_S5). As this result could be due to the molecular diversity of the tumors, we investigated the relationships between ccRCC molecular subtypes and response to sunitinib.

Molecular subtypes and sunitinib response

Using the expression profiles of the 1% most variable probe sets (n=324 representing 294 different genes) among the whole series of tissues (53 ccRCCs, 6 NTs), we performed an unsupervised consensus clustering analysis of our series. We identified four robust subgroups of tumors, called ccrc1 to ccrc4 (Figure_2). In particular, ccrc3-tumors included all NTs and showed a transcriptomic signature closed to normal samples (Figure_2B+2C).

To extend the molecular subtyping to a larger series, we built a qRT-PCR 35-genes classifier (Table_S6), which correctly classified 94% of the samples from the initial series in ccrc1-4, and was used to predict 47 additional ccRCC-tumors.

In the series of 98 patients, non-responders were enriched in ccrc1 (PD 22%) and ccrc4 (27%) versus 3 and 0% in ccrc2 and ccrc3, respectively (Figure_3A)(Table_S7). In contrast, responders were over-represented in ccrc2 (PR/CR 53%) and ccrc3 (70%) compared to 41% and 21% in ccrc1 and ccrc4, respectively (p=0.005)(Figure_3A)(Table_S7). Moreover, ccrc1 and ccrc4-tumors showed a poorer PFS (13, 8, 19 and 24 months, respectively; p=0.0003) and OS (24, 14, 35 and 50 months, respectively; p=0.001) compared to ccrc2 and ccrc3-tumors(Figure_3B)(Table_1)(Table_S7). Classification of the tumors recoded in ccrc1&4 versus ccrc2&3 was the most significant covariate in univariate cox analysis with a poorer PFS (p=0.004) and OS (p=0.0002). For the multivariate analysis, in a first step, among all the factors that were associated with PFS and OS in univariate analysis, we excluded those factors that could be overlapping(Table_1). Finally, the following factors were included in the multivariate analysis, both for PFS and for OS: IMDC-score, the presence of bone metastases, the presence of sarcomatoid dedifferentiation, 8q-amplification and the
classification recoded as ccrcc2+3 versus ccrcc1+4. It remained the only significant covariate in the multivariate cox-model for PFS (p=0.017). In the multivariate cox-model for OS, the recoded classification (p=0.0064) and the presence of bone metastasis (p=0.049) remained as significant covariates (Figure_3C).

Characterization of the m-ccrcc molecular subtypes

To better understand how each molecular subtype could influence response to sunitinib, we characterized our cohort for somatic mutations in PBRM1- and VHL-genes (n=117 tumors), methylation profiling exploring 485,000 nucleotide sites (n=102) and copy number targeting 731,442 genomic loci (n=103). Transcriptome and methylome profiles of the four subtypes were compared using pathway analysis methods and recurrent copy number aberrations were delimited from the SNP-profiles.

The ccrcc4-subtype demonstrated specific features at the pathological level with frequent sarcomatoid differentiation and inflammation (Figure_4A)(Table_2). Accordingly, pathway analysis of transcriptome profiles identified an over-expression of genes related to immune response, chemotaxis and apoptosis (Figure_4B). These pathways were also deregulated at the methylome level with hypomethylation of over-expressed genes which could be related to the inflammatory and immune microenvironments characterizing these tumors (Figure_4C)(Table_S8). The ccrcc4-subtype had a high expression of markers of inflammation, such as members of the TNF- and IRF-families. The analysis of cytokines revealed a strong expression of myeloid and T-cells homing factors and their corresponding receptors and Th-1 related factors such as Interferon-gamma and IL12. The immune suppressive IL10 as well as inhibitory receptors LAG3 and PD-1 (PDCD1) and PD-1-ligands PD-L1 and PD-L2 were also highly expressed(Figure_S1). These results suggest that ccrcc4-tumors display a strong inflammatory, Th1-oriented but suppressive immune microenvironment. We then assessed the expression of immune population-specific transcripts using gene-signatures previously published(21)(Figure_S2). ccrcc4-samples contained high amounts of B, T and cytotoxic cells-specific transcripts, but not of NK-cells-specific transcripts, suggesting that the cytotoxic signature in ccrcc4 is mostly due to CD8+T-cell infiltration. We analyzed 46 samples by immunohistochemistry and confirmed a high infiltration of CD8+cells in ccrcc4-tumors (Figure_S3). Sample contamination with diploid cells was estimated to be slightly higher in ccrcc4-tumors, which could reflect immune infiltration (Figure_S5C).

Overall, 45% of the tumors were VHL-mutated and 32% PBRM1-mutated. Whereas rare mutations in VHL and PBRM1 were found in ccrcc4-tumors they were frequent in crrcc1- and ccrcc2-tumors but without relationship with sunitinib response(Figure_4A)(Table_2).

At a global methylation level, ccrcc1/ccrcc4-tumors showed more hypermethylated probes in CpG islands compared to the other subtypes(Figure_S4A). Pathway analysis of the related hypermethylated genes showed a strong enrichment of polycomb targets (hypergeometric test p<8e-147) and corresponding genes (PRC2, SUZ12 and H3K27m3) were found down-
expressed (Figure S4B), suggesting a stem-cell phenotype for these two subtypes. The ccrcc1/ccrcc4-subtypes were indeed less differentiated displaying 76% of Fuhrman grade 4 compared to 56% in ccrcc2/ccrcc3-tumors.

The ccrcc4-subtype showed several characteristic copy number aberrations, the most significant being 2p12-, 2p22.3- and 8q21.13-amplifications (Figure 4D+S5) (Table S9) and both ccrcc1 and ccrcc4-subtypes over-expressed MYC-targets (Figure 4B). Amplification in the upstream region of MYC was found in >40% of ccrcc1- and ccrcc4-tumors compared to <22% in the other subtypes. Also, a CpG island was hypomethylated in the body gene of MYC in ccrcc1/ccrcc2/ccrcc4-tumors as compared to ccrcc3/NTs (anova p<0.0001) suggesting that both DNA copy number and methylation aberrations could contribute to MYC overexpression in the ccrcc1- and ccrcc4-subtypes (Figure S6).

Finally, the expression profile of ccrcc3-samples was similar to that of NTs concerning metabolic pathways and transporter activities, consistent with the cluster_3 described by Brannon (5) (Figure 4B). ccrcc3-tumors also showed a methylation profile similar to that of NTs (Figure S4C). Despite the ‘normal-like’ characteristics of ccrcc3-tumors, pathologic review confirmed the tumoral nature of these samples and their clear cell histology. The ccrcc2-subtype was not characterized by specific pathways; it always showed an intermediate expression signature, comprised between ccrcc3 and (ccrcc1/ccrcc4) related profiles (Figure 4B). ccrcc2-tumors showed the highest mutation rate for VHL. In ccrcc2-tumors, the “cellular response to hypoxia” pathway was less activated than in the ccrcc1/ccrcc4-subtypes (Figure 4B).

Based on these molecular characteristics, we renamed our subtypes as follows: ccrcc1="c-myc-up", ccrcc2="classical", ccrcc3="normal-like" and ccrcc4="c-myc-up and immune-up" (Table 2).

Validation using the TCGA dataset

We further predicted our four subtypes in the public TCGA-samples (Figure S7) (6) with our 35-genes classifier (Table S6). Like in our series, ccrcc3-tumors showed ‘normal-like’ transcriptome and methylome profiles. Somatic PBRM1-mutations were most frequently identified in ccrcc1/ccrcc2-tumors but rarely found in ccrcc3/ccrcc4-tumors. In both series, somatic VHL-mutations were more frequently distributed in ccrcc1/ccrcc2-tumors. The BAP1 and SETD2 mutations (data not available in our series) also showed association with the molecular subtypes: BAP1 was most mutated in the ccrcc4-tumors (p-value = 0.0098) and SETD2 was most mutated in the ccrcc1-tumors (p-value =0.06). At a methylation level, in the TCGA-samples, ccrcc1/ccrcc4-tumors also showed more hypermethylated probes in CpG-islands compared to the other subtypes. Pathways involved in immune response and mitotic cell cycle were activated in ccrcc4-tumors, meanwhile in ccrcc3-tumors, pathways involved in hypoxia were not activated. Finally, in the TCGA-cohort, 2p11.2-, 8q12.1- and 8q24.3-amplification was mostly found in ccrcc1/ccrcc4-tumors. Figure S8 shows the survival analysis according to our classification: ccrcc2/ccrcc3-tumors display the best survival, ccrcc1-tumors an intermediate survival and ccrcc4-tumors the poorest survival (p<0.0001).
DISCUSSION

Our multi-omics analysis revealed that molecular tumor subtypes are germane to predict response, PFS and OS, in m-ccRCC patients treated with sunitinib. We identified 4 robust molecular subgroups of ccRCCs based on mRNA expression data. ccrcc3- ('normal-like') and ccrcc2-tumors ('classical') showed better sunitinib responses than ccrcc1- ('c-myc-up') and ccrcc4-tumors ('c-myc-up and immune-up'). The ccrcc4-subtype was associated with the poorest sunitinib response. Subtype classification was the only significant covariate in multivariate analyses for PFS and OS. Survival was significantly longer for ccrcc2/ccrcc3 compared to ccrcc1/ccrcc4.

Comparison with previous ccRCC molecular classifications, showed a high correlation of our four ccrcc-groups with the three groups ccA, ccB and cluster_3 described by Brannon(4, 5)(Table_S10). In particular, ccrcc3-tumors shared several characteristics of cluster_3(5); it included all NTs and showed a transcriptomic signature closed to normal samples(Figure_2B+2C). Although the series of Brannon et al. and the TCGA included both patients that were cured with nephrectomy alone as well as patients that eventually reached the metastatic setting, while in our series only patients with metastatic disease were included, our subgroups are tightly related to the previously identified classification with an over-representation of the poor prognosis ccB-group and its refinement in two subgroups, ccrcc1 and ccrcc4. Subtypes were identified using a 35-gene signature that could be useful in future tumor screening. Given the important and significant difference in RR, this classification has both predictive and prognostic potential in sunitinib-treated m-ccRCC patients.

The incidence of VHL-mutations was lower than the reported incidence in literature of 55-60% in sporadic ccRCCs. VHL-disease-associated ccRCCs seem to grow more slowly and are associated with an overall better prognosis than sporadic ccRCCs. Sporadic ccRCCs that lack functional VHL-protein might, therefore, be expected to have a better prognosis than sporadic ccRCCs resulting from VHL-independent mechanisms of pathogenesis. Some studies seem to support this hypothesis(26, 27), although others have found no association between the presence or absence of VHL-alterations and prognosis or adverse clinical and pathological features.

The ccrcc1/ccrcc4-subtypes, which were more closely linked with non-responders to sunitinib, shared common molecular characteristics such as up-regulation of MYC-targets or a hypermethylated status strongly correlated with a polycomb stem-cell phenotype.

However, ccrcc4-tumors showed specific pathological features such as a more inflammatory and sarcomatoid phenotype, an up-regulation of cellular immune pathways and an omnipresent 8q21.13-amplification. These findings are consistent with several publications showing the negative impact on outcome of an elevated baseline C-reactive protein level, a marker of inflammation, and of the presence of sarcomatoid differentiation in m-ccRCC treated with anti-VEGFR-TT(28-31).

Inflammation is a double-edged sword in cancer immunology. It can both fuel tumor cells growth, and reinforce anti-tumor immunity. Consistently with our observations, extensive infiltration of CD8+T-cells in pulmonary metastases in m-ccRCC patients was associated with a shorter OS(22). Nonetheless,
infiltrating CD8+T-cells are likely suppressed in this context, due to regulatory cytokines (IL10, TGFβ1) and T-cell immunosuppressive molecules (PD-L1 (CD274)) highly expressed in ccrcc4. ccRCCs are extensively infiltrated with myeloid derived suppressor cells(32), which are likely to arise in a hypoxic environment and participate to T-cell suppression. These cells, as well as regulatory T-cells, whose markers (FOXP3, IL10 and TGFβ) are also highly expressed in ccrcc4, have also been reported to allow escape from sunitinib treatment(33, 34).

Integrative analyses of the different omic experiments suggested a possible gradient of tumor progression in the following order: normal samples/ccrcc3/ccrcc2/ccrcc1/ccrcc4. We observed a metabolism switch with increasing deregulation along the ordered subtypes; similar results are obtained for the polycomb stem-cell phenotypes, the hypermethylated profile or the MYC-target activation(Figure_S9). These features fit the transcriptional and epigenetic sequential changes responsible for cellular reprogramming leading to acquired pluripotency by fibroblasts(35): apoptosis blockade, cell cycle activation, metabolic switch, polycomb stem-cell phenotype with the involvement of MYC in this cell reprogramming. Inversely, activation of the immune pathway in ccrcc4-tumors seemed more linked to a switch on/off than to a gradient. Similarly, in ccrcc4 few VHL/PBRM1-mutations were detected, indicating that probably other gene alterations might be involved in the pathogenesis of ccrcc4-tumors.

Hypoxia pathways are not activated in ccrcc3-tumors and less activated in ccrcc2-tumors than in resistant ccrcc1/ccrcc4-tumors(Figure_4B). Hypoxia is associated with tumor aggressiveness through higher HIF-levels and expression of genes involved in tumor proliferation, vasculature, invasion, and metastatic spread leading to a poor prognosis. Through the reduction and normalization of blood vessels, anti-VEGF-TT leads to better oxygen delivery in the tumor. Thus, lowering hypoxia might be an important part of the mechanism of action of anti-VEGF-TT. However, tumor hypoxia was recently assessed before the start and after one month of sunitinib by a PET-CT-scan with 18F-fluoromisonidazole, which accumulates in hypoxic cells. Patients with initially hypoxic targets had shorter PFS than patients with non-hypoxic targets(36). Thus, the balance of neo-angiogenesis versus hypoxia could be a major trigger of response to anti-VEGF-TT(37).

In a first step, as we worked on a limited number of patients, our findings should be validated in an independent patient cohort. Once validated, this molecular subtyping of tumors could probably help treatment personalization. Since patients with ccrcc4-tumors have a short PFS under sunitinib treatment, hypomethylating agents targeting epigenetic defects(38) or immune-modulatory antibodies(39) should be preferentially tested in this subgroup of patients(40). Moreover, as even in the poor prognosis ccrcc4-subgroup, 20% of the patients experienced a PR, and as a placebo-treated subgroup of patients is not available for comparison, our classification does not permit us to preclude any subgroup of patients from treatment with sunitinib. Further validation of these findings is warranted in future clinical trials integrating molecular subtyping in their design.
AUTHOR CONTRIBUTION

Aurélien de Reynies, Jessica Zucman-Rossi, Stéphane Oudard and Benoit Beuselinck designed the study, experimental and clinical analyses. Benoit Beuselinck, Alexandra Karadimou and Gabrielle Couchy performed the molecular experiments and validations. Etienne Becht, Nicolas Giraldo, Wolf-Herman Fridman and Catherine Sautès-Fridman analyzed the immune pathways. Virginie Verkarre, Nathalie Rioux-Leclercq, Vincent Molinié and Mathilde Sibony performed the pathological reviewing. Sylvie Job and Aurélien De Reyniès performed all the biostatistical analyses. Jean-Jacques Patard, Benoit Beuselinck, Corinne Teghom, Reza Elaidi collected the clinical data and the tumors. Benoit Beuselinck constructed the database. Benoit Beuselinck, Sylvie Job, Aurélien de Reynies, Jessica Zucman-Rossi, Stéphane Oudard, Wolf-Herman Fridman, Catherine Sautès-Fridman, Etienne Becht have written the draft of the paper and ensured the overall integrity of the data. All authors critically reviewed the manuscript and approved the final version.

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**CONFLICTS OF INTEREST**

Stéphane Oudard received honorarium from Takeda, Novartis, Sanofi, Astellas, Roche and Bayer. Wolf Herman Fridman received honorarium from LFB, Sanofi-Aventis and Costim. Jean Jacques Patard received honorarium from Pfizer and GSK and research funding from Pfizer. Benoit Beuselinck is an investigator of the EudraCT: 2011-006085-40/MetaSun trial supported by Pfizer. Jessica Zucman-Rossi received honorarium from Pfizer, Astellas, Lilly and Bayer. The other authors have no conflicts of interest to declare.
REFERENCES


FIGURES: LEGEND

Figure 1: Clinical Predictors of sunitinib Response.

Barplot of the clinical covariates associated to the two extreme responders: Partial or complete response (PR) versus progressive disease (PD). Stable disease (SD) samples were not used for the statistical analysis. Stars correspond to significant anova or fisher p-values: *** p-value<0.001, ** p-value<0.01 and * p-value<0.05.

Figure 2: Identification of four robust subgroups through an unsupervised consensus clustering analysis of expression data.

(A) Co-classification matrix of the 59 RCC samples. Blue = low co-classification and red = high co-classification. (B) Sample partition for K=2 clusters to K=4 clusters. (C) Gene expression profile heatmap of the most variant probe sets ordered by subtype. Blue = low expression level and red = high expression level.

Figure 3: Correlation of the four molecular subgroups and outcome on sunitinib in the metastatic setting

(A) Association of sunitinib response with the unsupervised subgroups ccr1-ccrc4 (left) and the Brannon subgroups (right). The p-values result from Fisher-exact tests. (B) Association of the four unsupervised subgroups with progression-free survival (left) and overall survival (right). Log-rank p-values are on the top right. (C) Forest plots of the multivariate cox models for Progression-Free Survival (PFS) and Overall Survival (OS).

Figure 4: Clinical and molecular characteristics of the four clear cell RCC subgroups

(A) Barplot of the pathologic features and the incidence of VHL and PBRM1 mutations associated with the four unsupervised subgroups (ccrc1 to ccrc4). Pathologic features were analyzed through microscopy on hematoxylin and eosin stained slides. Tumors were screened for VHL and PBRM1-mutations using direct sequencing. Stars correspond to significant anova or fisher p-values: *** p-value<0.001, ** p-value<0.01 and * p-value<0.05. (B) Representation of the mean expression level of differentially regulated pathways between the four subgroups. Pathways are sorted by the difference between the ccrc4-subgroup and the normal samples (NL). For a given pathway, samples are sorted by mean expression value. (C) Representation of the rate of up-regulated genes within hypomethylated genes (black) and of the rate of down-regulated genes within hypermethylated genes (gray), for each pathway. Pathways are sorted by the difference between the rates of up and down-regulated genes in the -subgroup. (D) Barplot of the chromosomal aberrations identified by the GISTIC algorithm and associated with the ccrc4-subtype. Stars correspond to significant fisher p-values: *** p-value<0.001, ** p-value<0.01 and * p-value<0.05.
TABLES: LEGEND

Table 1: Univariate cox analyses of Overall Survival (OS) and Progression-Free Survival (PFS).

Univariate models were performed on all the pathological and clinical covariates. Only significant covariates in any of the two analyzes are indicated. Continuous variables are annotated '-' in the 'value' column.

For the multivariate analysis, in a first step, among all the factors that were associated with PFS and OS in univariate analysis, we excluded those factors that could be overlapping. For instance, IMDC (Heng) score and MSKCC score are redundant. We preferred IMDC (Heng) score, because the p-values were more significant for PFS and OS in univariate analysis. As ECOG PS and neutrophil count are part of the IMDC (Heng) score, they were excluded. As all tumors with sarcomatoid dedifferentiation were classified Fuhrman grade 4, we only retained sarcomatoid dedifferentiation. As a consequence, the following factors were included in the multivariate analysis, both for PFS and for OS: IMDC (Heng) score, the presence of bone metastases, the presence of sarcomatoid dedifferentiation, 8q-amplification and the classification recoded as ccrcc2+3 versus ccrcc 1+4.

Table 2: Summary of the molecular subtype characteristics.
FIGURE 1

This figure shows the frequency and mean values of various factors, including ECOG (PS>0), Neutrophils (>4500), HB (low), Platelets (>400000), and Synchronous metastases (Yes), in relation to PR, SD, and PD categories. The factors are compared using statistical significance markers: *** for p < 0.001, ** for p < 0.01, and * for p < 0.05. Similarly, the mean values of HENG Score, MSKCC Score, and Sarcomatoid differentiation (%) are also compared with significance markers. The data is presented in an arbitrary unit.
FIGURE 3

A

P-value = 0.005

PR SD PD

B

P-value = 0.001

Progression-free survival

P-value = 0.0003

Overall survival

C

Log-rank p-value = 0.0001

Wald p-value

Log-rank p-value = 0.00004

Wald p-value
FIGURE 4

A. Bar graphs showing the mean (arbitrary units) and frequency of inflammation, sarcomatoid differentiation, PBRM1 mutation, and VHL mutation.

B. Heat map depicting the regulation of T cell activation, immune response, apoptosis, mitotic cell cycle, cellular response to hypoxia, chemotaxis, MYC targets, metabolic pathways, PRC2 targets, fatty acid metabolism, brush border membrane, and drug metabolism (cytochrome P450).

C. Bar graphs showing the mean expression levels of ccrcc1, ccrcc2, ccrcc3, and ccrcc4 for various biological processes.

**Table 1**

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<th>Multivariate</th>
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<th>Multivariate</th>
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