Molecular Subtypes of Clear Cell Renal Cell Carcinoma Are Associated with Sunitinib Response in the Metastatic Setting

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Abstract

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

Conclusions: 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. Clin Cancer Res; 21(6); 1329–39. ©2015 AACR.
subtypes (6). In both studies, postnephrectomy 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 patients with m-ccRCC 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 patients with m-ccRCC.

Materials 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 (patient characteristics in Supplementary Table S1). For inclusion in the study, patients had to have developed synchronous or metachronous metastases, received sunitinib from 1994 to 2011 (patient characteristics in Supplementary Table S1). We 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 also 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.

Datasets and preprocessing

Dataset for transcriptome (E-MTAB-3267), SNP (E-MTAB-3269) and methylome (E-MTAB-3274) are available at ArrayExpress (https://www.ebi.ac.uk/arrayexpress/).

Transcriptome data. Transcriptomic profiling was performed using HuGene 1.0ST Affymetrix array for 53 ccRCC samples and 6 adjacent normal tissue samples (NT). Biotinylated single-strand cDNA targets were prepared with 200 ng of total RNA, using the Ambion WT Expression Kit and 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) by Integrating SA (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, 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 (respectively below) the ploidy of the sample were considered as gains (respectively 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 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. Supplementary 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 Supplementary Table S3.

Mutation analysis

Tumors were screened for VHL and PBRM1 mutations (Supplementary Table S4) using direct sequencing (primers and protocols available on request).

Omens analysis

Unsupervised classification. For unsupervised class discovery within the four omics, three methods were used: the recursively partitioned mixture model (RPMM; ref. 17) and two consensus clustering methods (18, 19). Only the results obtained with the third method were described in the article. 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 FDR 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 \( \chi^2 \) or Fisher exact test. For each clinical characterization, all covariates were analyzed but only the significant covariates were shown \((P \leq 0.05)\). The bioclinical factors included tumor–node–metastasis, Eastern Cooperative Oncology Group Performance Status (ECOG–PS), International Metastatic Renal Cell Carcinoma Database Consortium (IMDC), Fuhrman, or Memorial Sloan Kettering Cancer Center (MSKCC) scores, systemic treatment, Kettering Cancer Center (MSKCC) scores, systemic treatment, metastasis, Eastern Cooperative Oncology Group Performance Status were added. Criteria of sensitivity and specificity were tested. A hypergeometric test was used to measure the association between a gene (probeset) list and a biologic pathway or a gene ontology term (GO term), as in GOstats R-package from R. Significance was characteristic of a given subgroup if the sensitivity and specificity were >0.65.

**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 survdif, R-package survival).

**Immunohistochemistry**
Formalin-fixed and paraffin-embedded tissue sections (5 \( \mu m \)) were stained with an anti-CD8 (5.0 \( \mu g/mL; \) clone SP16; Spring-bioscience) with an autostainer Link 48 (Dako) as previously described (22). Deparaffinization, rehydration, and epitope retrieval were performed in a PT-Link (Dako) in a high \( \mathrm{pH} \) 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. Forty-seven percent 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 vs. PD), we identified several clinical and biologic 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; Fig. 1A and B). However, in the overall series of patients, using all the clinical, pathologic, and molecular features and a variety of predictive algorithms (PAM, DLDA, DQDA), supervised analyses failed to identify robust factors predictive of sunitinib response (Supplementary 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 ccrcc1 to ccrcc4 (Fig. 2). In particular, ccrcc3 tumors included all NTs and showed a transcriptomic signature closed to normal samples (Fig. 2B and C).

To extend the molecular subtyping to a larger series, we built a qRT-PCR 35-gene classifier (Supplementary Table S6), which correctly classified 94% of the samples from the initial series in ccrcc1–4, and was used to predict 47 additional ccRCC tumors. In the series of 98 patients, nonresponders were enriched in ccrcc1 (PD 22%) and ccrcc4 (27%) versus 3% and 0% in ccrcc2 and ccrcc3, respectively (Fig. 3A; Supplementary Table S7). In contrast, responders were over-represented in ccrcc2 (PR/CR, 53%) and ccrcc3 (70%) compared with 41% and 21% in ccrcc1 and ccrcc4, respectively ($P = 0.005$; Fig. 3A; Supplementary Table S7). Moreover, ccrcc1 and ccrcc4 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 with ccrcc2 and ccrcc3 tumors (Fig. 3B; Table 1; Supplementary Table S7).

Classification of the tumors recoded in ccrcc1&4 versus ccrcc2&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 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 (Fig. 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 delimitated from the SNP profiles.

The ccrcc4 subtype demonstrated specific features at the pathologic level with frequent sarcomatoid differentiation and inflammation (Fig. 4A; Table 2). Accordingly, pathway analysis of transcriptome profiles identified an overexpression of genes related to immune response, chemotaxis, and apoptosis (Fig. 4B). These pathways were also deregulated at the methyolome level with hypomethylation of overexpressed genes which could be related to the inflammatory and immune microenvironments characterizing these tumors (Fig. 4C and Supplementary 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 Th1-related factors such as IFNγ 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 (Supplementary Fig. 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 (Supplementary Fig. S2; ref. 21). ccrcc4 samples contained high amounts of B, T, and cytotoxic cells-specific transcripts, but not of natural killer 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 (Supplementary Fig. S3). Sample contamination with diploid cells was estimated to be slightly higher in ccrcc4 tumors, which could reflect immune infiltration (Supplementary Fig. 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 ccrcc1 and ccrcc2 tumors but without relationship with sunitinib response (Fig. 4A and Table 2).

At a global methylation level, ccrcc1/ccrcc4 tumors showed more hypermethylated probes in CpG islands compared with the other subtypes (Supplementary Fig. S4A). Pathway analysis of the related hypermethylated genes showed a strong enrichment of polycomb targets (hypergeometric test, $P < 8 \times 10^{-147}$), and corresponding genes (PRC2, SUZ12, and H3K27m3) were found downexpressed (Supplementary Fig. S4B), suggesting a stem-cell phenotype for these two subtypes. The ccc1/ccrcc4 subtypes were indeed less differentiated, displaying 76% of Fuhrman grade 4 compared with 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 (Fig. 4D; Supplementary Fig. S5; Supplementary Table S9), and both ccrcc1 and ccrcc4 subtypes overexpressed MYC targets (Fig. 4B). Amplification in the upstream region of MYC was found in >40% of ccrcc1 and ccrcc4 tumors compared with <22% in the other subtypes. Also, a CpG island was hypomethylated in the body gene of MYC in ccrcc1/ccrcc2/ccrcc4 tumors as compared with 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 (Supplementary Fig. 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 and colleagues (ref. 5; Fig. 4B). ccrcc3 tumors also showed a methylation profile similar to that of NTs (Supplementary Fig. 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 (Fig. 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 (Fig. 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 (Supplementary Fig. S7; ref. 6) with our 35-gene classifier (Supplementary 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 with 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 amplifications were mostly found in ccrcc1/ccrcc4 tumors. Supplementary Fig. S8 shows the survival analysis according to our classification: ccrcc2/ccrcc3 tumors display the best survival, ccrcc1 tumors an
Table 1. Univariate Cox analyses of OS and PFS

<table>
<thead>
<tr>
<th>Annotation</th>
<th>Value</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HR (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td><strong>PFS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classification</td>
<td>ccrcc2</td>
<td>0.60 (0.34–1.1)</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>ccrcc3</td>
<td>0.56 (0.24–1.3)</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>ccrcc4</td>
<td>2.31 (1.1–4.7)</td>
<td>0.02</td>
</tr>
<tr>
<td>Recoded classification</td>
<td>ccrcc1&amp;4 vs. ccrcc2&amp;3</td>
<td>0.49 (0.3–0.8)</td>
<td>0.004</td>
</tr>
<tr>
<td>Baseline neutrophil levels</td>
<td>&gt;4,500/mm³</td>
<td>2.14 (1.4–3.3)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Sarcomatoid dedifferentiation</td>
<td>Poor</td>
<td>1.029</td>
<td>0.007</td>
</tr>
<tr>
<td>in tumor (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fuhrman grade</td>
<td>—</td>
<td>1.55 (0.99–2.4)</td>
<td>0.05</td>
</tr>
<tr>
<td>ECOG-PS</td>
<td>—</td>
<td>1.54 (1.2–2)</td>
<td>0.001</td>
</tr>
<tr>
<td>Amp_Bq2113</td>
<td>Y</td>
<td>1.99 (1.2–2)</td>
<td>0.004</td>
</tr>
<tr>
<td>Bone metastasis</td>
<td>Intermediate</td>
<td>1.42 (0.92–2.2)</td>
<td>0.1</td>
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<tr>
<td>IMDC (HENG) score</td>
<td>Poor</td>
<td>2.46 (1.3–4.4)</td>
<td>0.03</td>
</tr>
<tr>
<td>MSKCC score</td>
<td>Intermediate</td>
<td>3.83 (1.7–8.9)</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>2.62 (1.2–5.8)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.65 (1.1–6.2)</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>OS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Classification</td>
<td>ccrcc2</td>
<td>0.45 (0.25–0.8)</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>ccrcc3</td>
<td>0.55 (0.24–1.3)</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>ccrcc4</td>
<td>1.93 (0.97–3.8)</td>
<td>0.06</td>
</tr>
<tr>
<td>Recoded classification</td>
<td>ccrcc1&amp;4 vs. ccrcc2&amp;3</td>
<td>0.40 (0.24–0.66)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Baseline neutrophil levels</td>
<td>&gt;4,500/mm³</td>
<td>1.60 (1–2.5)</td>
<td>0.04</td>
</tr>
<tr>
<td>Sarcomatoid dedifferentiation</td>
<td>Poor</td>
<td>1.103</td>
<td>0.0009</td>
</tr>
<tr>
<td>in tumor (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fuhrman grade</td>
<td>—</td>
<td>1.95 (1.2–3.2)</td>
<td>0.008</td>
</tr>
<tr>
<td>ECOG-PS</td>
<td>—</td>
<td>1.41 (1.1–1.8)</td>
<td>0.01</td>
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<tr>
<td>Amp_Bq2113</td>
<td>Y</td>
<td>1.99 (1.2–2)</td>
<td>0.004</td>
</tr>
<tr>
<td>Bone metastasis</td>
<td>Intermediate</td>
<td>1.94 (1.2–2)</td>
<td>0.004</td>
</tr>
<tr>
<td>IMDC (HENG) score</td>
<td>Poor</td>
<td>2.67 (1.1–6.7)</td>
<td>0.04</td>
</tr>
<tr>
<td>MSKCC score</td>
<td>Intermediate</td>
<td>4.34 (1.6–12)</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Poor</td>
<td>3.64 (1.4–9.3)</td>
<td>0.007</td>
</tr>
</tbody>
</table>

**NOTE:** Univariate models were performed on all the pathologic and clinical covariates. Only significant covariates in any of the two analyses 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 vs. ccrcc1 & 4. Abbreviation: CI, confidence interval.

Baseline neutrophil levels >4,500/mm³ were associated with the poorest sunitinib response. Subtype classification was the only significant covariate in multivariate analyses for OS and PFS. Survival was significantly longer for ccrcc2/ccrcc3 compared with ccrcc1/ccrcc4.

Comparison with previous ccRCC molecular classifications showed a high correlation of our four ccrc groups with the three groups ccA, ccB, and cluster_3 described by Brannon and colleagues (4, 5; Supplementary 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 (Fig. 2B and C). Although the series of Brannon and colleagues and the TCGA included both patients that were cured with nephrectomy alone as well as patients that eventually reached the metastatic setting, whereas 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 patients with m-ccRCC.

The incidence of VHL mutations was lower than the reported incidence in literature of 55% to 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 pathologic features. The ccrcc1/ccrcc4 subtypes, which were more closely linked with nonresponders to sunitinib, shared common molecular characteristics such as upregulation of MYC targets or a hypermethylated status strongly correlated with a polycomb stem-cell phenotype. However, ccrcc4 tumors showed specific pathologic features such as a more inflammatory and sarcomatoid phenotype, an upregulation 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 mRCC treated with anti-VEGFR TT (28–31).

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 (Supplementary Fig. 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/
Table 2. Summary of the molecular subtype characteristics

<table>
<thead>
<tr>
<th>Subgroup (frequency)</th>
<th>ccrcc1 (33%)</th>
<th>ccrcc2 (41%)</th>
<th>ccrcc3 (11%)</th>
<th>ccrcc4 (15%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outcome under sunitinib</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early PD</td>
<td>22.2%</td>
<td>2.78%</td>
<td>0.00%</td>
<td>26.67%</td>
</tr>
<tr>
<td>PR</td>
<td>40.74%</td>
<td>52.78%</td>
<td>70.00%</td>
<td>20.00%</td>
</tr>
<tr>
<td>Median OS (month)</td>
<td>24</td>
<td>35</td>
<td>50</td>
<td>14</td>
</tr>
<tr>
<td>Median PFS (month)</td>
<td>13</td>
<td>19</td>
<td>24</td>
<td>8</td>
</tr>
</tbody>
</table>

**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: 46.67%, 62.50%, 20.00%, 20.00%
- PBRM1: 46.67%, 37.50%, 20.00%, 0.00%

**Upregulated pathways**

- MYC targets: Immunity
- Glycolysis: Apoptosis
- Hypoxia: Chemoattraction

**MYC expression level**

- + + + +
- + + + +

**Methylation status**

- Hypomethylated
- Hypermethylated

**Polycomb stem-cell phenotype**

- + + + +
- 2p12/2p22.3/8q21.3

**Copy number amplification**

- 2p12/2p22.3/8q21.3

**Proposal for names**

- MYC.UP
- Classical
- Normal like

**Translation**

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 (Fig. 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 nonhypoxic targets (36). Thus, the balance of neoangiogenesis 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. Because patients with ccrcc4 tumors have a short PFS under sunitinib treatment, hypomethylating agents targeting epigenetic defects (38) or immunomodulatory 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.

**Disclosure of Potential Conflicts of Interest**

B. Beuselinck reports receiving a commercial research grant from Pfizer. E. Becht is a consultant/advisory board member for SOTIO. S.M. Oudard is a consultant/advisory board member for Pfizer. No potential conflicts of interest were disclosed by the other authors.

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