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Circulating tumor cells as a predictor and prognostic tool for metastatic clear cell renal carcinoma: An immunocytochemistry and genomic analysis

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ABSTRACT

Background: Treatment of metastatic clear cell renal carcinoma (mccRCC) has changed dramatically over the past 20 years, without improvement in the development of biomarkers. Recently, circulating tumor cells (CTCs) have been validated as a prognostic and predictive tool for many solid tumors.
Objective: We evaluated CTCs in blood samples obtained from patients diagnosed with mccRCC. Comparisons of CTC counts, protein expression profiling, and DNA mutants were made in relation to overall survival and progression-free survival.
Methods: CTCs were isolated from 10 mL blood samples using the ISET® system (Isolation by SizE of Tumor Cells; Rarecells, France) and counted. Protein expression was evaluated in immunocytochemistry assays. DNA mutations were identified with next generation sequencing (NGS).
Results: Blood samples (10 mL) were collected from 12 patients with mccRCC before the start of first-line systemic therapy, and again 30 and 60 days after the start of treatment. All 12 patients had CTCs detected at baseline (median, 1.5 CTCs/mL; range: 0.25–7.75). Patients with CTC counts greater than the median had two or more metastatic sites and exhibited worse progression-free survival (19.7 months) compared to those with CTC counts less than the median (31.1 months). Disease progression was observed in 7/12 patients during the study. Five of these patients had baseline CTC counts greater than the median, one had higher CTC levels at the second blood collection, and one patient had CTCs present at 1 CTC/mL which positively stained for PD-L1, N-cadherin, VEGF, and SETD2. CTC DNA from six patients with worse outcomes was subjected to NGS. However, no conclusions could be made due to the low variant allele frequencies.
Conclusion: Detection of CTCs in patients with mccRCC receiving first-line treatment is a feasible tool with prognostic potential since increased numbers of CTCs were found to be associated with metastasis and disease progression.

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1. Introduction

Currently, there are approximately 430 new cases of renal cell carcinoma (RCC) diagnosed worldwide each year [1]. In recent years, the incidence rate for this rare kidney disease has increased, while the mortality rate has decreased, mainly due to early detection [2,3]. However, RCC is often detected during investigations or screenings performed for other diseases, since symptoms of early stage RCC are difficult to detect. When diagnosed as a localized disease, treatment of RCC is usually successful. In approximately 30% of RCC patients, progression to metastatic disease occurs [4].

Management of advanced RCC varies from a "watch and wait" approach to invasive metastasectomy and systemic treatment with multiple drugs. In order to optimize treatment, appropriate timing and selection of intervention need to be determined on a case-by-case basis. When a metastatic stage of disease is considered, a very wide prognostic spectrum is involved, consistent with the complexity of advanced tumor biology [5]. Systemic treatment of RCC has achieved overall survival (OS) that ranges from 32.53 months to 74.1 months, with progression-free survival (PFS) ranging from 12.3 months to 23.9 months [6–8].

Ten years ago, single anti-angiogenic drug therapy was administered for treatment of RCC. More recently, a combination of immune checkpoint inhibitors (i.e., nivolumab, ipilimumab) [7] or a combination of immune checkpoint inhibitors and anti-angiogenic agents (i.e., pembrolizumab and axitinib, pembrolizumab and lenvatinib, cabozantinib with nivolumab) [6,8,9] has become a first-line treatment [10]. In a molecular study conducted of different tumor types, the immunological and angiogenic profiles obtained were found to be predictive of treatment response [11]. In another important study, clear cell carcinoma was classified into seven molecular subtypes based on transcription and gene alteration profiling [12]. This type of classification had treatment implications.

Clear cell renal carcinoma (ccRCC) develops in the tubules of the kidney and is the most common type of RCC. Key differences between ccRCC and RCC include chromosomal phenotypes and gene mutations associated with the two diseases. However, to facilitate early detection, less invasive approaches are needed. For example, research regarding liquid biopsy, a method with straightforward collection that can detect circulating tumor cells (CTCs), is of particular interest. Multiple studies of various advanced tumor types have shown that CTCs can dynamically indicate tumor burden and treatment response [13–16]. In addition, the DNA content of CTCs can be examined [17]. In cases of advanced RCC, identification of CTCs has been shown to be very useful, yet also challenging since surface expression profiling has revealed different subtypes of CTCs [18,19]. Characterization of these subtypes has been achieved with use of both immunocytochemistry (ICC) and gene expression/mutation analyses [20]. Therefore, the aim of this study was to evaluate CTC counts and kinetics at various time points before and during treatment of patients with metastatic ccRCC (mccRCC). Specifically, protein expression profiling by immunocytochemistry (ICC) and gene analysis studies were performed. These data were then correlated with patient prognosis according to OS and PFS.

2. Patients and methods

This prospective study was conducted at the Oncology Department of A.C. Camargo Cancer Center between November 2017 and February 2021 and was approved by the institutional review board (nº 2855/20). Patients with mccRCC were invited to participate in the study prior to starting a first-line therapy (i.e., immunotherapy or targeted therapy with tyrosine kinase inhibitors or mTOR inhibitors). Inclusion criteria were: histological diagnosis of mccRCC; aged over 18 years (elderly were also included); first-line treatment prescribed; metastatic disease confirmed by pathological and/or radiological evaluation; extension of disease confirmed by clinical examination and imaging; and disease measurable by RECIST, version 1.1. Patients who received previous oncological treatment and those undergoing any type of surgical intervention within the previous two weeks were excluded. All of the participating patients signed an informed consent.

2.1. Isolation and purification of CTCs

Blood samples were collected at three time points: prior to the start of first-line therapy (C1), and 30 days (C2) and 60 days (C3) after the start of therapy. Each blood sample (10 mL) was collected in an EDTA tube (BD Vacutainer, Franklin Lakes, NJ, USA) and processed within 4 h of collection. Briefly, each blood sample was diluted 1:10 in erythrocyte lysis buffer and filtered using the ISET® system (Isolation by SizE of Tumor Cells; Rarecells Diagnostics, SAS, Paris, France), according to the manufacturer's instructions. CTCs isolated on membranes were subsequently washed with PBS, dried at room temperature, protected from light, and stored at − 20 ºC until analyzed.

For DNA mutation analysis, DNA was extracted from the CTCs isolated on polycarbonate membranes (ISET), as previously described [17]. Briefly, four spots of ISET membrane were stored in RNAlater Solution (Thermo Fisher Scientific, Waltham, MA, USA) at − 20 ºC for up to a week. Each membrane spot was cut into small pieces and transferred into lysis buffer and proteinase K supplied by a QIAamp DNA Micro Kit (Qiagen, Valencia, CA, USA). Extractions were performed according to the manufacturer's protocol.

2.2. ICC reactions performed for protein analysis of CTCs

For ICC and protein expression analysis, a dual color reaction was performed. Briefly, 6 fixed membrane spots (with 37% formaldehyde solution) were cut and placed in 24-well plates containing Tris-buffered saline (TBS). After 20 min, cells were permeabilized with TBS/0.2% Triton X-100 for 5 min at room temperature. After a wash with TBS, the membrane spots were incubated in the dark with 3% hydrogen peroxide solution for 15 min. After another wash with TBS, a primary antibody was added and incubated for 1 h. For a negative control, cell lines not expressing the antibodies of interest were stained in parallel. Conversely, positive controls included tumor cell lines that expressed the antibodies of interest. Cell lines were selected based on The Human Protein Atlas (https://www.proteinatlas.org/) (Supp. Table 1). Antibodies recognizing the following proteins were used: SETD2, PD-L1, CD133, VEGF, BAP, PBRM1 and N-cadherin. Dilutions and antibody product information are provided in Supplementary Table 1.

Visualization of reaction products was achieved using the Dual Link System-HRP (Dako™) and the chromogen, diaminobenzidine 3,3′ (DAB) (Dako™). Briefly, after using the DAB, samples were incubated with a second primary antibody for 1 h. Rabbit/Mouse (LINK) reagent (Kit Envision™ G/2 System/AP) was added for an additional 20 min. The samples were then washed with TBS and incubated with AP Enzyme (Enhancer) for 30 min. Antibody binding was visualized with Permanent Red. To exclude the possibility of a non-specific reaction, leukocytes were incubated with an anti-CD45 antibody. This staining also confirmed that the cells labeled by the antibodies of interest were not leukocytes [13].

To evaluate phenotype and CTC count, the spots were stained with hematoxylin for 1 min before being adhered to a slide with aqueous mounting medium. CTCs were characterized according to the following criteria: nuclear size equal to or greater than 16 μm, nuclear contour irregularity, presence of visible cytoplasm, and high nucleus-cytoplasm ratio (> 0.8), as described by [21]. If any of the described criteria were absent, the cells were classified as atypical. Slides were examined under an optical Research System Microscope BX61 (Olympus, Tokyo, Japan) coupled to a digital camera (SC100, Olympus). Number of CTCs per mL of blood was recorded for each slide.

2.3. Gene panel sequencing

Next generation sequencing (NGS) was performed for 28 genes frequently mutated in RCC. The entire coding region was evaluated for 21 of the 28 genes (*VHL*, *PBRM1*, *BAP1*, *SETD2*, *KDM5C*, *PTEN*, *TP53*, *ARID1A*, *SMARCA4*, *PCF11*, *AR*, *MET*, *FAT1*, *NF2*, *KDM6A*, *SMARCB1*, *NFE2L2*, *STAG2*, *CDKN2A*, *FH*, and *FLCN*). Only hotspot regions for mutations were analyzed in the remaining seven genes (*MTOR*, *PIK3CA*, *NRAS*, *BRAF*, *KRAS*, *EGFR*, and *TCEB1*).

CTC DNA libraries were constructed from the extracted DNA samples by using the QIAseq Targeted DNA Custom Panel and the QIAseq 96-Index I Set A kit (Qiagen), according to the manufacturer's protocols. Briefly, 40 ng of genomic DNA was enzymatically fragmented, amplified with the gene panel, and ligated into barcodes containing unique molecular indices and identifiers. Sequencing was performed on the NextSeq 500 platform (Illumina, San Diego, CA, USA) with use of the Mid Output 300 cycles kit (Illumina).

The Data Analysis Center Qiagen platform (https://www.qiagen.com/dk/shop/genes-and-pathways/data-analysis-center-overview-page/) was used to analyze the data generated with the NextSeq 500 platform (Illumina). Using the Varseq tool (Golden Helix), the variants identified were annotated according to information available in multiple public databases: dbSNP, COSMIC, ExAc, ESP, and dbNSFP. Relevant information included: population allele frequency, presence in tumor and/or normal tissues, and prediction of functional effect. The parameters used for an initial selection of somatic mutations were: minimum coverage $\geq$ 100 and mutation allele frequency $\geq$ 2%. Additional filters based on the presence of mutations in tumor databases (COSMIC) and the general population (GnomAd), as well as predicted functional effects of proteins (Revel), were used to prioritize the variants according to greatest interest.

2.4. Validation by amplicon sequencing

To validate the changes in expression detected in the sequencing panel, sequencing of amplicons targeted to the variants detected in each patient was performed, followed by NGS sequencing (Target Amplicon NGS). For each variant that was detected, primer pairs from 80 to 170 bp were designed using the Primer3 tool (http://bioinfo.ut.ee/primer3-0.4.0) to amplify a specific region of each mutation. For each sample, these primers were used with the Multiplex PCR Kit (Qiagen). Barcodes were associated with the results by using the Ion Xpress Plus Fragment Library Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. The samples were sequenced using the Ion Proton platform (Thermo Fischer Scientific) and the Ion PI™ ChipV3 chip.

Target sequencing readings were analyzed using TMAP and TVC tools for alignment and variant calling, respectively (Thermo Fischer Scientific). As an evaluation criterion, we considered a minimum coverage of 5000 sequences, to provide the possibility of detecting with high reliability above 0.5% mutation. The test samples (DNA isolated from CTCs) were sequenced in parallel with control DNA samples in order to evaluate the noise of the sequencing reaction in the detection of low-frequency variants. The allele frequencies of the reference base and the altered base in the test sample and in the control sample were evaluated by visual evaluation of the BAM mapping file using the Integrative Genomics Viewer (IGV) tool. According to the allele frequencies of the variants (AVF) observed in the case and in the control, we classified the mutations detected in the DNA CTC samples as: high confidence (allele frequency $>$ 0.5% and $>$ 2 $\times$ the noise of the control reaction); low confidence (allele frequency $>$ 0.5% and $<$ 2 $\times$ the control reaction noise or allelic frequency $>$ 0.05% and $>$ 2 $\times$ the control reaction noise); or negative (allele frequency $<$ 0.5% and $<$ 2 $\times$ the noise of the control reaction).

2.5. Statistical analysis

Patient characteristics were reported as absolute and relative frequencies for qualitative variables. Kaplan-Meier survival curves were generated and differences between them were compared according to log-rank testing. PFS was assessed from the date of first collection of blood sample to the date of imaging which detected disease progression. OS was assessed from the date of first collection of blood sample to the date of death or last follow-up. The level of significance was 5% for all of the tests performed. All statistics were performed in SPSS version 24.0 (IBM, Armonk, NY, USA), with the significance criterion set at 5%.

3. Results

3.1. Clinical features

Fifteen patients with mccRCC were considered for this study. However, two patients had a second primary tumor detected and a third patient was diagnosed with non-ccRCC. Therefore, 12 patients were examined in this study and their median age was 59.91 y (range: 35.00–84.97). Additional patient characteristics are summarized in Table 1.

3.2. CTC counts and protein expression

All 12 patients had CTCs detected at baseline (C1; before the start of treatment) (Fig. 1). The median CTC count at C1 was 1.5 CTCs/mL (range: 0.25–7.75). At the first follow-up time point (C2; 30 days after the start of treatment), only 8 patients were evaluated and their median CTC count was 0.9 CTC/mL (range: 0.25–2.25). At the second follow-up (C3; 60 days after the start of treatment), five patients were evaluated. Three had no CTCs detected, one patient had a CTC count of 0.25 CTC/mL, and the other patient's CTC count was 1.5 CTC/mL.

At C1, the proteins expressed at the highest levels were SETD2 and PD-L1, and they were detected in 50% of the patients (6/12). The next highest levels of expression were detected for CD133 (5/12), VEGF (4/12), BAP 1 (3/12), PBRM1 (2/12), and NCAD (1/12). At C2, the most highly expressed proteins included VEGF and HIF, both detected in 2/12 patients (16.7%). Increased expression of CD133, NCAD, PD-L1, and BAP 1 was detected in another patient (8.3%), while PBRM1 and SETD2 were not detected in any of the patients. At C3, a patient had CTCs at 1.5 CTC/mL that expressed CD133, SETD2, and BAP1 (Table 2).

3.3. CTC counts and clinical outcomes

Due to the small size of the cohort examined, no statistical analysis could be conducted. However, when we plotted the results obtained, we observed that all five of the patients with CTC counts $>$ 1.5 CTC/mL had two or more sites of metastasis (Table 3).

The median PFS for our cohort was 16.15 months (range: 6.64–45.63). Among 12 patients, 7 exhibited disease progression. Patients with CTC counts higher than the median ($>$ 1.5 CTC/mL) exhibited poor PFS in relation to those with CTC counts less than the median. Among the 7 patients exhibiting disease progression, 5 had CTC counts at C1 that were greater than the median (Table 1). In one patient, higher CTCs levels were detected at C2, and one patient had a CTC count of 1 CTC/mL, with cells stained for PD-L1, N-cadherin, VEGF, and SETD2. When the patients were examined based on kinetics, we observed that those with CTC counts that diminished from C1 to C2 (i.e., favorable kinetics) had a better PFS than those with CTC counts that increased after baseline (i.e., unfavorable kinetics) (24.76 months vs. 6.65 months, respectively).

The mean OS for our cohort was 52.7 months (range: 11.6–61.0). Median OS was not reached. Among our 12 patients, two progressed to death. Patients with CTC counts greater than the median at baseline exhibited poor OS. A similar observation was made at C1. It was also

Table 1
CTC levels and clinical characteristics of tumors.

Patient	Base line (C1)	First follow-up (C2)	Second follow-up (C3)	Gender	Age (y)	T	N	IMDC	ISUP scale	NPY	MSY	No. treatment lines	1st-line treatment	DP
1	7.75	0.75	0.25	M	64	1b	-	0	3	-	+	4	TKI	+
2	5.25	1.5	1.5	M	39	4	1	2	-	+	+	3	IO+IO	+
3	4.25	1.0	0.0	M	49	3 A	1	1	4	-	+	2	IO+IO	+
4	0.46	0.0	*	M	88	1 A	1	1	-	+	+	1	IO+IO	-
5	0.25	2.25	0.0	M	62	1B	-	0	2	+	-	3	TKI	+
6	4.5	*	*	M	69	1B	-	0	4	+	+	4	TKI	+
7	2.0	*	0.0	M	68	4	1	3	3	+	-	1	TKI	-
8	2.25	2.25	*	F	69	3a	-	1	2	-	+	2	IO+IO	+
9	0.75	0.75	*	F	57	3a	-	1	4	-	-	1	IO+IO	-
10	0.5	0.25	*	M	74	1a	-	2	4	-	-	2	IO+IO	-
11	1.0	0.0	*	M	63	3a	-	2	3	-	+	2	IO+IO	+
12	0.58	0.5	*	F	69	1b	-	0	-	-	+	2	TKI	-
Median values	1.5	0.75	0.35		66									

Abbreviations: *not collected; -: not determined; M: male; F: female; T: T stage; N: N stage; IMDC: International Metastatic RCC Database Consortium System; ISUP: International Society of Urological Pathology Scale; NPY: nephrectomy; MSY: metastasectomy; IO= immunotherapy; TKI= tyrosine kinase inhibitors; DP: disease progression.

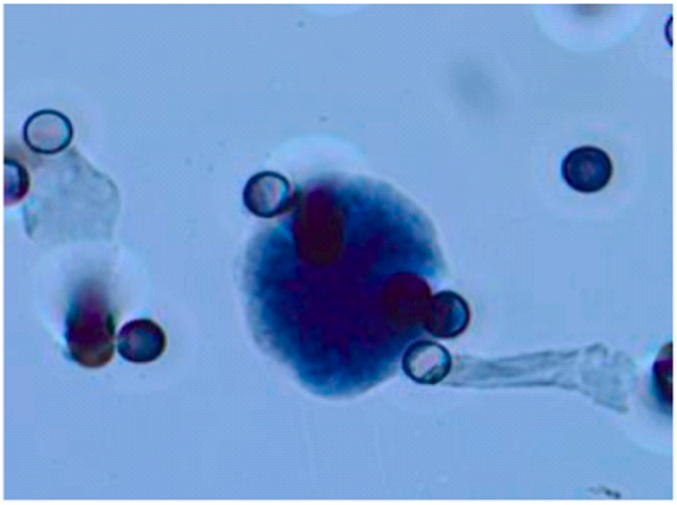

Fig. 1. CTC of a patient with advanced renal carcinoma stained with hematoxylin, photomicrographed, at 40x magnification, with a light microscope (Research System Microscope BX61 - Olympus, Tokyo, Japan) coupled to a digital camera (SC100 - Olympus, Tokyo, Japan). The image was analyzed using a Research System Microscope BX61 (Olympus, Tokyo, Japan) coupled to a digital camera (SC100–Olympus). Morphological characteristics of CTCs are observed: cells with diameter more than 8 μm; hyperchromatic nucleus and cytoplasm scarcity.

observed that the patients with CTCs that expressed PBRM-1 and BAP-1 at baseline exhibited a better OS (median, 12.46 months) than the patients without this type of CTCs (median, 28.05 months). In contrast, CTCs expressing PD-L1 and CD133 at baseline (C1) exhibited worse OS (19.69 months and 29.22 months, respectively; data not shown).

Table 3
CTC levels and metastasis.

Patient	C1 CTC/mL	C2 CTC/mL	C3 CTC/mL	≥ 2 Metastatic sites	Sites of metastasis	DP
1	7.75	0.75	0.25	+	LN / SP / LU / BO	+
2	5.25	1.5	1.5	+	LN / LI	+
3	4.25	1.0	0.0	+	LN / LU / B0	+
4	0.46	0.0	*	-	LN	-
5	0.25	2.25	0.0	-	SP	+
6	4.5	*	*	-	LU	+
7	2.0	*	0.0	+	LN / LU / LI	-
8	2.25	2.25	*	+	SP / LU / CNS	+
9	0.75	0.75	*	-	CNS	-
10	0.5	0.25	*	-	SP	-
11	1.0	0.0	*	-	LU	+
12	0.58	0.5	*	-	SP	-
Median	1.5	0.75	0.35			

Abbreviations: *Not collected; LN: lymph nodes; SP: soft parts; LU: lung; LI: liver; BO: bones; CNS: central nervous system; DP: disease progression.

Table 2
CTC levels and protein expression according to collection time point.

	CTC/mL			Staining of: C1 / C2 / C3 - CTC Samples							
Patient	C1	C2	C3	CD 133	HIF-1	PBRM1	N-CAD	PDL1	SETD 2	VEGF	BAP1
1	7.75	0.75	0.25	+ / + / -	- / - / -	+ / - / -	- / - / -	+ / - / -	- / - / -	+ / + / +	+ / - / -
2	5.25	1.5	1.5	+ / - / +	- / - / -	- / - / -	- / - / -	- / - / -	+ / - / +	- / - / -	- / - / +
3	4.25	1.0	0	+ / - / -	- / - / -	- / - / -	- / - / -	- / - / -	+ / - / -	- / - / -	- / - / -
4	0.46	0.0	*	+ / * / *	- / * / *	- / * / *	- / * / *	+ / * / *	+ / * / *	- / * / *	- / * / *
5	0.25	2.25	0	- / - / -	- / - / -	- / - / -	- / - / -	+ / - / -	- / - / -	- / + / +	- / - / -
6	4.5	*	*	- / * / *	- / * / *	- / * / *	- / * / *	- / * / *	- / * / *	- / * / *	+ / * / *
7	2.0	*	0	- / * / -	- / * / -	+ / * / -	- / * / -	+ / * / -	+ / * / -	- / * / -	- / * / *
8	2.25	2.25	*	- / - / *	- / + / *	- / - / *	- / - / *	+ / - / *	+ / - / *	+ / - / *	- / + / *
9	0.75	0.75	*	- / - / *	- / + / *	- / - / *	- / - / *	- / - / *	- / - / *	- / - / *	+ / - / *
10	0.5	0.25	*	- / - / *	- / - / *	- / - / *	- / - / *	- / - / *	- / - / *	- / - / *	- / - / *
11	1.0	0.0	*	- / * / *	- / * / *	- / * / *	+ / * / *	+ / * / *	+ / * / *	+ / * / *	- / * / *
12	0.58	0.5	*	+ / - / *	- / - / *	- / - / *	- / + / *	- / + / *	- / - / *	+ / - / *	- / - / *
Median	1.5	0.75	0.35								

Abbreviations: *Not collected; + : positive staining; -: negative staining; N-CAD: N-cadherin

3.4. Analysis of mutations identified in CTC DNA

CTC DNA from six patients was sequenced. With two CTC samples analyzed per patient, the average coverage of the samples was 3009 times, with 95% of the panel bases covered over 900 × . Only two of the six patients had rare variants (frequency <1% in the GnomAd database) detected. Seven of these nine variants were confirmed to be germline alterations based on amplicon sequencing in leukocyte DNA. A variant with 6% variable allele frequency (VAF) (PBRM1) detected in patient number 1/collection 1 was not confirmed in the validation of amplicons in the CTC DNA samples. The origin of the other variant could not be determined due to an absence of germline DNA.

For patient number 2, prior sequencing of the primary tumor had been performed that detected somatic mutations in *VHL*, *PBRM1*, and *KDM5C*. These mutations were also detected in the amplicon sequencing performed in the present study. Furthermore, the VHL change for patient 2 was evaluated. It was detected with low confidence in the CTC sample from collection 2 (VAF = 1.9%) and was not detected in the CTC sample from collection 1.

4. Discussion

In the present study, collection of CTCs was feasible and compatible with first-line systemic treatment for patients with mccRCC. The former advantage also facilitated monitoring of CTC dynamics at various time points. The use of an isolation technique based on membrane exclusion according to size allows cells to be captured independent of their cell surface expression profile. This is important for cells with epithelial-to-mesenchymal transitional features and a lack of epithelial cell adhesion molecule (EpCAM) expression. The shortcomings of EpCAM-based enrichment methodologies have previously been described [22,23]. Another advantage of using a size exclusion method is that circulating rare cells which can include circulating stem cells and non-tumor cells of epithelial and/or endothelial origin [20] can be isolated. However, the ability to isolate CTCs from other cell types remains an ongoing challenge.

Baseline CTC count has been shown to be a prognostic tool for a number of solid advanced tumors [24–30], including RCC where it has been shown to reflect tumor burden [31]. For example, in cases of localized disease that were treated with curative intent, an increase in CTCs at follow-up was found to correlate with worse prognosis [10,32]. Number of CTCs at baseline also appeared to correlate with prognosis in the present study. For five of the twelve patients with CTC levels above the median (> 1.5 CTCs/mL), they had two or more metastasis sites detected and a markedly lower median PFS than those with CTC counts less than the median (19.7 months versus 31.1 months, respectively). This observation is consistent with the results of previous studies [31, 33]. OS was also worse for the patients with higher CTCs counts.

We observed that both number of CTCs and their kinetics during treatment were predicting factors of outcome, consistent with the results of other studies [18,31,34]. Specifically, patients that exhibited a decrease in CTC counts after two months of systemic treatment had a > 4-fold increase in PFS compared with the patients exhibiting unfavorable kinetics (24.76 months versus 6.65 months, respectively).

In our small cohort, patients with CTCs expressing PD-L1 exhibited poor OS compared with patients without CTCs expressing PD-L1 (9.77 months vs. 14.3 months, respectively). The same conclusion was obtained in a meta-analysis that included twenty studies and reported a pooled hazard ratio for OS among patients with CTCs expressing PD-L1 of 1.85 months (p = .001) [35]. Additionally, PFS was 1.50 months (p = .007) [35]. It is well-known that PD-L1 expression by primary tumors represents a prognostic indicator [36,37]. Thus, it is possible that PD-L1 expression by CTCs may reflect the same aggressive phenotype of primary tumors expressing PD-L1. It was further observed that patients in the present study with CTCs expressing CD133 exhibited a worse outcome compared with the patients with CD133-negative CTCs. Similarly, in a study conducted by Nel and colleagues, both CD133 and NCAD expression in CTCs correlated with inferior PFS [20]. Since CD133 is an indicator of stem-cell like features, and possibly of drug resistance, this may account for the poorer performance observed for the patients with CD133-positive CTCs in the present study.

Genes that encode histone/chromatin regulators, such as BAP1, SETD2, and PBRM1, play important roles in carcinogenesis and in the phenotype of ccRCC [38,39]. Mutations in these genes can lead to low expression of these biomarkers and indicate lower PFS and OS. In the present cohort, expression of BAP-1 and PBRM-1 appears to be related to improved outcome. We further examined this issue by evaluating the mutations identified from NGS performed for six of the patients in our cohort. However, no solid conclusion could be made based on the low VAF of the data. Intratumoral heterogeneity is also an important consideration when evaluating genomic profiling of ccRCC cases. Correspondingly, heterogeneity has been reflected in exome sequencing of CTCs [40].

5. Conclusion

Treatment of metastatic ccRCC has changed dramatically over the past 20 years. In order to optimize patient treatment and OS, evaluation and prediction of possible treatment responses needs to be considered when selecting an appropriate treatment strategy. Liquid biopsy has the potential to serve as an important predictive and prognostic tool for ccRCC, although few studies have directly applied it to ccRCC. In the present study, a high frequency of CTCs was identified prior to the start of first-line treatment in patients with mccRCC. Furthermore, a possible association between number of CTCs at baseline and patient outcome was observed. With use of the ISET method for CTC collection, examination of CTC kinetics during treatment may also indicate whether treatment strategies need to be modified. Additional studies of possible protein- or genomic-based biomarkers to classify renal cell populations and patterns could further optimize treatment. The preliminary findings we report here demonstrate the value of this approach, its feasibility, and the need for further study of this prognostic approach.

Author Contributions

M.S.T: selection of patients, data analysis and interpretation, collection and/or assembly of data, manuscript writing; C.C.G.B: data analysis and interpretation; J.A.T: data analysis and interpretation, collection and/or assembly of data, manuscript writing; A.P.C.R. and A. C.B. data analysis and interpretation; VSA: collection and/or assembly of data; D.J.F.C: data analysis and interpretation; S.C.Z, W.H.C. and A.P.F: manuscript review; G.T. and D.M.C.: NGS analysis and interpretation; L. T.D.C: conception/design, data analysis and interpretation, manuscript writing, final approval of manuscript.

Institutional Review Board Statement

Approved by the ethics committee: (CEP 2855/20).

Informed Consent Statement

Not applicable.

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Declaration of Competing Interest

All authors declared that there are no conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.prp.2023.154918.

References

[1] Kidney, (2020). https://gco.iarc.fr/today (accessed May 22, 2023).
[2] J.M. Hollingsworth, D.C. Miller, S. Daignault, et al., Rising incidence of small renal masses: a need to reassess treatment effect, J. Natl. Cancer Inst. 98 (2006) 1331–1334, https://doi.org/10.1093/jnci/djj362.
[3] A. Znaor, J. Lortet-Tieulent, M. Laversanne, et al., International variations and trends in renal cell carcinoma incidence and mortality, Eur. Urol. 67 (2015) 519–530, https://doi.org/10.1016/j.eururo.2014.10.002.
[4] T. Osawa, A. Takeuchi, T. Kojima, et al., Overview of current and future systemic therapy for metastatic renal cell carcinoma, Jpn J. Clin. Oncol. 49 (2019) 395–403, https://doi.org/10.1093/JJCO/HYZ013.
[5] D.Y.C. Heng, W. Xie, M.M. Regan, et al., External validation and comparison with other models of the international metastatic renal-cell carcinoma database consortium prognostic model: a population-based study, Lancet Oncol. 14 (2013) 141, https://doi.org/10.1016/S1470-2045(12)70559-4.
[6] T.K. Choueiri, M. Eto, E. Kopyltsov, et al., 660P Phase III CLEAR trial in advanced renal cell carcinoma (aRCC): Outcomes in subgroups and toxicity update, Ann. Oncol. 32 (2021) S683–S685, https://doi.org/10.1016/j.annonc.2021.08.056.
[7] R.J. Motzer, D.F. McDermott, B. Escudier, et al., Conditional survival and long-term efficacy with nivolumab plus ipilimumab versus sunitinib in patients with advanced renal cell carcinoma, Cancer 128 (2022) 2085, https://doi.org/10.1002/CNCR.34180.
[8] R.J. Motzer, T. Powles, M. Burotto, et al., Nivolumab plus cabozantinib versus sunitinib in first-line treatment for advanced renal cell carcinoma (CheckMate 9ER): long-term follow-up results from an open-label, randomised, phase 3 trial, Lancet Oncol. 23 (2022) 888–898, https://doi.org/10.1016/S1470-2045(22)00290-X.
[9] B.I. Rini, E.R. Plimack, V. Stus, et al., Pembrolizumab (pembro) plus axitinib (axi) versus sunitinib as first-line therapy for advanced clear cell renal cell carcinoma (ccRCC): results from 42-month follow-up of KEYNOTE-426, J. Clin. Oncol. 39 (2021), https://doi.org/10.1200/JCO.2021.39.15_SUPPL.4500, 4500–4500.
[10] Y. Guan, F. Xu, J. Tian, et al., The prognostic value of circulating tumour cells (CTCs) and CTC white blood cell clusters in patients with renal cell carcinoma, BMC Cancer 21 (2021) 1–10, https://doi.org/10.1186/S12885-021-08463-7/FIGURES/5.
[11] R.J. Motzer, T. Powles, M.B. Atkins, et al., Final overall survival and molecular analysis in IMmotion151, a phase 3 trial comparing atezolizumab plus bevacizumab vs sunitinib in patients with previously untreated metastatic renal cell carcinoma, JAMA Oncol. 8 (2022) 275–280, https://doi.org/10.1001/JAMAONCOL.2021.5981.
[12] R.J. Motzer, R. Banchereau, H. Hamidi, et al., Molecular subsets in renal cancer determine outcome to checkpoint and angiogenesis blockade, Cancer Cell 38 (2020) 803, https://doi.org/10.1016/J.CCELL.2020.10.011.
[13] T. Bueno de Oliveira, A. Camila Braun, U. Ribaldo Nicolau, et al., Prognostic impact and potential predictive role of baseline circulating tumor cells in locally advanced head and neck squamous cell carcinoma, Oral. Oncol. 121 (2021), 105480, https://doi.org/10.1016/j.oraloncology.2021.105480.
[14] V.S.E. Silva, E.A. Abdallah, A.B.C. de Brito, et al., Baseline and kinetic circulating tumor cell counts are prognostic factors in a prospective study of metastatic colorectal cancer, Diagnostics 11 (2021), https://doi.org/10.3390/DIAGNOSTICS11030502.
[15] M.F. Fanelli, T.B. Oliveira, A.C. Braun, et al., Evaluation of incidence, significance, and prognostic role of circulating tumor microemboli and transforming growth factor-β receptor I in head and neck cancer, Head. Neck 39 (2017) 2283–2292, https://doi.org/10.1002/HED.24899.
[16] E.A. Abdallah, A.C. Braun, B.P. Flores, et al., The potential clinical implications of circulating tumor cells and circulating tumor microemboli in gastric cancer, Oncologist 24 (2019) e854–e863, https://doi.org/10.1634/THEONCOLOGIST.2018-0741/-/DC1.
[17] M.E.C. Buim, M.F. Fanelli, V.S. Souza, et al., Detection of KRAS mutations in circulating tumor cells from patients with metastatic colorectal cancer, Cancer Biol. Ther. 16 (2015) 1289, https://doi.org/10.1080/15384047.2015.1070991.
[18] M. Bootsma, R.R. McKay, H. Emamekhoo, et al., Longitudinal molecular profiling of circulating tumor cells in metastatic renal cell carcinoma, J. Clin. Oncol. 40 (2022) 3633–3641, https://doi.org/10.1200/JCO.22.00219.
[19] S. Kruck, G. Gakis, A. Stenzl, Disseminated and circulating tumor cells for monitoring chemotherapy in urological tumors, Anticancer Res 31 (2011).
[20] I. Nel, T.C. Gauler, K. Bublitz, et al., Circulating tumor cell composition in renal cell carcinoma, PLoS One 11 (2016), 153018, https://doi.org/10.1371/JOURNAL.PONE.0153018.
[21] M.G. Krebs, J.M. Hou, R. Sloane, et al., Analysis of circulating tumor cells in patients with non-small cell lung cancer using epithelial marker-dependent and -independent approaches, J. Thorac. Oncol. 7 (2012) 306–315, https://doi.org/10.1097/JTO.0b013e31823c5c16.
[22] A. Gradilone, R. Iacovelli, E. Cortesi, et al., Circulating tumor cells and "suspicious objects" evaluated through cellsearch® in metastatic renal cell carcinoma, Anticancer Res. (31) (2011).
[23] P.K. Grover, A.G. Cummins, T.J. Price, et al., Circulating tumour cells: the evolving concept and the inadequacy of their enrichment by EpCAM-based methodology for basic and clinical cancer research, Ann. Oncol. 25 (2014) 1506–1516, https://doi.org/10.1093/ANNONC/MDU018.
[24] H.J. Chalfin, T. Pramparo, A. Mortazavi, et al., Circulating tumor cell subtypes and t-cell population as prognostic biomarkers to combination immunotherapy in metastatic genitourinary cancer patients, Clin. Cancer Res. 27 (2021) 1391, https://doi.org/10.1158/1078-0432.CCR-20-2891.
[25] B. Tong, Y. Xu, J. Zhao, et al., Prognostic significance of circulating tumor cells in non-small cell lung cancer patients undergoing chemotherapy, Oncotarget 8 (2017) 86615, https://doi.org/10.18632/ONCOTARGET.21255.
[26] S. Pang, H. Li, S. Xu, et al., Circulating tumour cells at baseline and late phase of treatment provide prognostic value in breast cancer, Sci. Rep. 11 (2021), 13441, https://doi.org/10.1038/S41598-021-92876-8.
[27] A. Goldkorn, C. Tangen, M. Plets, et al., Baseline circulating tumor cell count as a prognostic marker of PSA response and disease progression in metastatic castrate sensitive prostate cancer (SWOG S1216), Clin. Cancer Res. 27 (2021) 1967, https://doi.org/10.1158/1078-0432.CCR-20-3587.
[28] X. Huang, P. Gao, Y. Song, et al., Meta-analysis of the prognostic value of circulating tumor cells detected with the CellSearch System in colorectal cancer, BMC Cancer 15 (2015) 1–12, https://doi.org/10.1186/S12885-015-1218-9/FIGURES/5.
[29] S. Pernot, C. Badoual, M. Terme, et al., Dynamic evaluation of circulating tumour cells in patients with advanced gastric and oesogastric junction adenocarcinoma: prognostic value and early assessment of therapeutic effects, Eur. J. Cancer 79 (2017) 15–22, https://doi.org/10.1016/j.ejca.2017.03.036.
[30] D. Olmos, R.D. Baird, T.A. Yap, et al., Baseline circulating tumor cell counts significantly enhance a prognostic score for patients participating in phase I oncology trials, Clin. Cancer Res. 17 (2011) 5188–5196, https://doi.org/10.1158/1078-0432.CCR-10-3019/84228/AM/BASELINE-CIRCULATING-TUMOR-CELL-COUNTS.
[31] U. Basso, A. Facchinetti, E. Rossi, et al., Prognostic role of circulating tumor cells in metastatic renal cell carcinoma: a large, multicenter, prospective trial, Oncologist 26 (2021) 740, https://doi.org/10.1002/ONCO.13842.
[32] J. Bialek, A. Wencker, F. Kawan, et al., Potential use of CTCs as biomarkers in renal cancer patients, Life 12 (2022) 89, https://doi.org/10.3390/LIFE12010089.
[33] B. Nayak, S. Panaiyadiyan, P. Singh, et al., Role of circulating tumor cells in patients with metastatic clear-cell renal cell carcinoma, Urol. Oncol. Semin Orig. Invest. 39 (2021) 135.e9–135.e15, https://doi.org/10.1016/J.UROLONC.2020.10.077.
[34] E. Rossi, M. Fassan, M. Aieta, et al., Dynamic changes of live/apoptotic circulating tumour cells as predictive marker of response to Sunitinib in metastatic renal cancer, Br. J. Cancer 107 (2012) 1286, https://doi.org/10.1038/BJC.2012.388.
[35] D. Kong, W. Zhang, Z. Yang, et al., Correlation between PD-L1 expression ON CTCs and prognosis of patients with cancer: a systematic review and meta-analysis, Oncoimmunology 10 (2021), https://doi.org/10.1080/2162402X.2021.1938476.
[36] R. Iacovelli, F. Nolè, E. Verri, et al., Prognostic role of PD-L1 expression in renal cell carcinoma. A systematic review and meta-analysis, Target Oncol. 11 (2016) 143–148, https://doi.org/10.1007/S11523-015-0392-7/METRICS.
[37] A. Carretero−gonzález, D. Lora, I.M. Sobrino, et al., The value of PD-L1 expression as predictive biomarker in metastatic renal cell carcinoma patients: a meta-analysis of randomized clinical trials, Cancers 12 (2020) 1945, https://doi.org/10.3390/CANCERS12071945.
[38] L. Liu, R. Guo, X. Zhang, et al., Loss of SETD2, but not H3K36me3, correlates with aggressive clinicopathological features of clear cell renal cell carcinoma patients, Biosci. Trends 11 (2017) 214–220, https://doi.org/10.5582/BST.2016.01228.
[39] S. Bihr, R. Ohashi, A.L. Moore, et al., Expression and mutation patterns of PBRM1, BAP1 and SETD2 mirror specific evolutionary subtypes in clear cell renal cell carcinoma, Neoplasia 21 (2019) 247, https://doi.org/10.1016/J.NEO.2018.12.006.
[40] X. Xu, Y. Hou, X. Yin, et al., Single-cell exome sequencing reveals single-nucleotide mutation characteristics of a kidney tumor, Cell 148 (2012) 886, https://doi.org/10.1016/J.CELL.2012.02.025.