Hilar fat infiltration: a new prognostic factor in metastatic clear cell renal cell carcinoma with first-line sunitinib treatment

Running title: Hilar fat infiltration in metastatic renal carcinoma

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ABSTRACT

Introduction. The selection of patients with metastatic clear-cell renal cell carcinoma (ccRCC) who may benefit from targeted tyrosine kinase inhibitors has been a challenge, even more so now with the advent of new therapies. Hilar fat infiltration (HFI) is a validated prognostic factor in non-metastatic ccRCC (TNM 2009 staging system) but has never been studied in metastatic patients. We aimed to assess its phenotype and prognostic impact in patients with metastatic ccRCC treated with first-line sunitinib.

Materials and methods: In a multicentric study, we retrospectively included 90 patients and studied the corresponding ccRCC at the pathological, immunohistochemical and molecular levels. Patient and tumour characteristics were compared using univariate and multivariate analysis. All the features were then studied by Cox models for prognostic impact.

Results: HFI was found in 42 patients (46.7%), who had worse prognosis (Heng criteria) (p=0.003), liver metastases (p=0.036) and progressive diseases at first radiological evaluation (p=0.024). The corresponding ccRCC were associated with poor pathological prognostic factors that are well known in non-metastatic ccRCC. For these patients, median progression-free survival (PFS) was 4 months versus 13 months (p=0.02), and median overall survival (OS) was 14 months versus 29 months (p=0.006). In a multivariate Cox model integrating all the variables, only poor prognosis according to the Heng criteria and HFI remained independently associated with both PFS and OS.

Conclusion: HFI was demonstrated for the first time to be an independent poor prognostic factor. Its potential role in predicting resistance to anti-angiogenic therapy warrants further investigation.

Key-words: clear-cell renal cell carcinoma, sunitinib, prognostic, hilar fat infiltration

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INTRODUCTION

Renal cell carcinoma (RCC) accounts for 3% of all adult cancers, with approximately 270 000 new cases and 110 000 deaths per year worldwide [1]. Clear cell renal cell carcinoma (ccRCC) is the most frequent histological subtype of renal cancer, estimated to account for approximately 70%, with a poor prognosis [2]. Indeed, 20% of patients are metastatic at the time of diagnosis, and 30% will develop metastases. The 5-year overall survival is estimated to be 40% [1].

A better understanding of the oncogenic pathways involved in the oncogenesis of ccRCC, such as VHL/HIF/VEGF and PI3K/AKT/mTOR, led to the development of targeted therapies for metastatic patients. Sunitinib is an anti-angiogenic tyrosine kinase inhibitor (TKI) approved for use as a first-line treatment of metastatic ccRCC that is currently the most frequently administered [3, 4]. However, approximately 20% of patients are inherently resistant to this therapy because they progress according to response evaluation criteria in solid tumours (RECIST) at first evaluation [5].

A new approach is based on targeted immunotherapy using checkpoint inhibitors (antiPD-1, antiPD-L1 or anti-CTLA4 antibodies) since ccRCC is considered an immunogenic tumour [6]. Recently, nivolumab, an anti-PD1 antibody, has demonstrated efficiency as a second-line treatment versus everolimus (mTOR inhibitor) [7]. Most notably, a phase 3 study comparing anti-PD1 and anti-CTLA4 antibody association versus sunitinib in first-line treatment is ongoing, highlighting questions about chronologic treatment strategies. Oncologists currently face the challenge of drug selection to treat their patients but lack reliable predictors of response or prognostic factors that may help choose the most appropriate therapy.

Hilar fat infiltration, also known as renal sinus fat infiltration, was previously described in several studies as a poor prognostic factor in non-metastatic ccRCC [8]. Consequently, it was integrated in the TNM (Tumour Node Metastasis) staging system 2009, classifying tumours at least T3a [9]. This stage also includes peri-renal fat and venal invasion. Nevertheless, the impact of hilar fat infiltration has not been individually studied in metastatic ccRCC (m-ccRCC) thus far.

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In a retrospective cohort of metastatic patients with ccRCC treated by first-line sunitinib therapy, we aimed to correlate hilar fat infiltration with clinicopathological and molecular characteristics as well as clinical outcomes.

PATIENTS AND METHODS

Patient selection and classification

The study population included metastatic patients who were at least 18 years old with histologically proven ccRCC. Primary ccRCC-specimens were collected from patients undergoing nephrectomy in two French University Hospitals (Rennes and Bordeaux, from UroCCR data base). Patients with metastatic ccRCC received sunitinib (50 mg/day, four weeks on/two weeks off) as first-line treatment (prior cytokine therapy was allowed) and completed at least one 28-day cycle of sunitinib. Tumour evaluation was realised according to Response Evaluation Criteria In Solid Tumours (RECIST 1.1) [10]. Follow-up chest/abdomen CT-scans were performed every 2 cycles of treatment (3 months). For each patient, the following clinical and pathologic information was gathered: age, sex, pTNM stage, tumour size, and nucleolar ISUP grade. Hilar fat infiltration was suspected during gross examination and was confirmed by microscopy. Histopathologic assessment was performed by three experienced pathologists (SFKJ, MY and NRL). For each patient, formalin-fixed paraffin-embedded (FFPE) ccRCC were available. Informed consent was obtained from each patient, and institutional review board approval was obtained for this study (CNIL authorization receipt 1812601v0).

Immunohistochemical study

Protein expression patterns were assessed by immunohistochemistry using the following antibodies: anti-VEGFA (monoclonal, clone sp28, dilution, Spring Bioscience, California, USA), anti-CAIX (polyclonal, ab15086, dilution 1:1500, Abcam, Cambridge, UK), anti-PD1 (anti-PD-1 antibody, clone NAT105, dilution 1:50; Abcam, Cambridge, UK) and anti-PDL1 (anti-PD-L1 antibody, clone 130021, dilution 1:200; R&D Systems, Minneapolis, USA) [11-13]. The reactivity of antibodies was revealed with HRP-labelled polymer-conjugated secondary antibodies using diaminobenzidine (DAB) as the chromogen (Sigma-Aldrich, France). Negative controls were performed by omitting the primary antibody. The tumour expression for each antibody was independently evaluated (SFKJ and NRL) without

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knowledge of the case. The cut-off for positive cases was 30% of tumour cells for VEGF and 85% for CAIX as previously described [12, 14]. For PDL1, absent (0), weak (1), moderate (2) and strong expression (3) were reported and cases were then subdivided into negative (score 0–1) or positive (score 2–3) subgroups [15]. For PD1, immunostaining density was evaluated in tumour-infiltrating lymphocytes and was semi-quantified as absent, rare, moderate or dense as previously reported [15].

VHL status

Next-generation sequencing (NGS)

For the *VHL* gene, the entire coding sequence and exon-intron junctions of exons 1, 2 and 3 were analysed. Genomic DNA was extracted using Magtration System 12GC (Bionobis) according to the manufacturer's instructions. Genomic DNA from all samples was quantitated with the Quan-iT PicoGreen dsDNA assay kit (Thermo Fisher Scientific). DNA target preparation and enrichment were performed by amplification using the Access Array® system (Fluidigm, San Francisco, USA). A 10-nucleotide "barcode" tag, specific to each sample and Illumina-specific sequencing adaptors were attached using secondary PCR. Purified products were then pooled and sequenced on an Illumina MiSeq NGS instrument (Illumina Inc., San Diego, California).

Multiplex Ligation-dependent Probe Amplification (MLPA)

Methylation-specific-MLPA (MS-MLPA) was used to detect CpG methylation islands in the *VHL* gene promotor. The SALSA MS-MLPA kit ME001B Tumour suppressor-1 allows detection of aberrant methylation of CpG-Islands located in the promoter region of the *VHL* gene [16]. The unmethylated DNA does not generate a signal, and a normal probe signal is detected if the site is methylated.

Statistical analysis

The focus of the study was to evaluate the relationship between HFI and clinicopathologic characteristics, molecular characteristics, and clinical outcomes. Associations were analysed with univariate analysis (χ^2, Fisher's and Mann–Whitney tests) and multivariate analysis (logistic regression). The second focus was to assess its clinical relevance by calculating

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progression-free survival (PFS) from the date of sunitinib introduction to the date of progression and overall survival (OS) from the date of sunitinib introduction to the date of death. PFS and OS were represented by Kaplan–Meier curves and compared by log-rank test. The prognostic impact of all the variables was then evaluated using univariate Cox regression models in PFS and OS. Therefore, we used a backward stepwise selection with the likelihood ratio criterion (inclusion/exclusion criteria: $p<0.05$) for a multivariate Cox regression model. All statistical tests were two sided and performed at a significance level of 0.05 using StataCorp version 14 and R version 3.3.0.

RESULTS

Patient and tumour characteristics

Patient and tumour characteristics are presented in Table 1. The study retrospectively included 90 metastatic patients with primary ccRCC who underwent nephrectomy from 1997 to 2013. Men were more represented (62.2% versus 37.8%). According to the Heng criteria, most of patients were in the intermediate group (47.7%). Locally advanced tumours were most represented (stage T3-T4 in 75.5% of patients) and showed a high nucleolar ISUP grade 3 or 4 (n=88, 92.3%). Forty-two patients (46.7%) had ccRCC with hilar fat infiltration. Metastases were present at initial diagnostic for 55.6% of patients. With time, non-metastatic patients at diagnosis developed metastases on average 10.5 months (0-144 months) with a median follow-up was 43 months (1-171 months) from nephrectomy. More than two-thirds of all patients presented multiple metastatic sites (n=69; 76.7%). The most common metastatic sites were the lung (75.6%), bone (53.3%) and liver (23.3%). Eighty-five patients (94.4%) experienced progression and 71 (78.9%) died from their cancer. The mean follow-up period was 25 months (range 1-76 months) from sunitinib introduction.

Correlation between the clinical, pathological, immunohistochemical and molecular phenotype of tumours and hilar fat infiltration

Hilar fat infiltration was associated with poor prognosis according to both the Heng and MSKCC criteria (p=0.003 and 0.006, respectively) and was also associated with liver metastasis (p=0.036), Table 2. Hilar fat infiltration was also associated with early resistance to

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sunitinib treatment, defined as disease progression at the first radiologic evaluation (p=0.024). Among pathological characteristics, hilar fat infiltration was associated with the sarcomatoid component (p=0.008), microvascular invasion (p=0.013) and tumour necrosis (p=0.012). Peri-renal fat infiltration (p=0.035) and local node metastasis (p=0.005) were also significantly associated with hilar fat infiltration. No association with CAIX, VEGFA, PD1 and PDL1 expression by immunohistochemistry were identified. Fewer *VHL* gene mutations were found in ccRCC with hilar fat infiltration (p=0.023). After logistic regression, three factors remained significantly associated with hilar fat infiltration: peri-renal fat infiltration (p=0.037), poor prognosis (Heng score) (p=0.002) and local node metastasis (p=0.006).

Correlation between hilar fat infiltration and survival

Kaplan–Meier curves for PFS and OS are presented in Figures 1 and 2, respectively. Metastatic patients with hilar fat infiltration had a median PFS of 4 months versus 13 months for patients without hilar fat infiltration (p=0.020). Similarly, patients with hilar fat infiltration had a significantly shorter survival, with a median OS of 14 months versus 29 months for patients without hilar fat infiltration (p=0.006).

Correlation between clinicopathological features and survival outcome

In univariate Cox model, clinicopathological factors associated with worse PFS were age ≥ 65 years (p=0.044), poor prognosis according to the Heng and MSKCC criteria (p=0.001 and p=0.004, respectively), liver metastasis (p=0.004), and hilar fat infiltration (p=0.027), Table 3. A multivariate Cox model showed that age ≥ 65 years, poor prognosis according to the Heng criteria and hilar fat infiltration remained significantly associated with poor PFS.

In univariate Cox model, OS was associated with poor prognosis according to the Heng and MSKCC criteria (p=0.001), liver metastasis (p=0.017) and hilar fat infiltration (p=0.007), Table 4. In a multivariate Cox model, poor prognosis according to the Heng criteria and hilar fat infiltration were the only two factors that remained significantly associated with poor OS.

DISCUSSION

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To our knowledge, this is the first study to assess the phenotype of ccRCC with hilar fat infiltration in metastatic patients who were treated by first-line sunitinib and to evaluate its prognostic value. Hilar fat infiltration was associated with aggressive tumours with worse outcomes.

Hilar fat infiltration was associated with poor prognostic factors already identified in non-metastatic ccRCC, such as nucleolar ISUP grade 4, sarcomatoid component, tumour necrosis, microvascular invasion, higher tumour stage and local node extension [17]. Among pathological factors, local node extension and peri-renal fat infiltration remained independently associated with hilar fat infiltration in metastatic patients. Hilar fat area is characterised by dense lymphatic vessels favouring metastatic spread through lymph nodes. Moreover, ccRCC with hilar fat infiltration are larger and have a more frequent peri-renal extent, as we observed in our series. Finally, they were associated with fewer *VHL* mutations, previously associated with poor prognosis factors [18]. The phenotype of these ccRCC may be completed by further molecular studies to understand the biological mechanisms underlying these results.

In the literature, predictors of response and prognostic factors for patients with metastatic renal cell carcinoma treated with anti-VEGF therapies have been extensively studied [19, 20]. However, these studies are often retrospective with a relative limited number of patients. In addition, the histology data include not only ccRCC but also papillary and chromophobe renal cell carcinoma, although their carcinogenesis is different [21]. Finally, anti-VEGF therapies comprise sunitinib and other TKI, whose targets are different. Although our study shared retrospective and size limits impacting multivariate analysis, we included 90 metastatic patients with sunitinib first-line therapy and histologically proven ccRCC.

Few predictive and prognostic markers are described in the literature in metastatic ccRCC with first-line sunitinib. Single-nucleotide polymorphisms and transcriptomic clusters have shown interesting results but were not investigated in this study [22, 23]. Moreover, consistent with our results, up to now, no immunohistochemical markers were associated with both PFS and OS. In accordance with other studies, we confirmed the prognostic impact of the Heng score [24].

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Hilar fat is continuous with visceral fat infiltration. The latter characteristic was previously described as an independent prognostic factor in metastatic ccRCC treated by anti-angiogenic therapies[25]. Adipose tissue may be involved through the production of adipokines, such as IL-6 and TNFα, leading to cancer growth and dysregulated angiogenesis [26]. Indeed, insulin-like growth factors produced by adipocytes and the release of inflammatory cytokines by infiltrating lymphocytes have cancer-promoting and angiogenic effects, by stimulating VEGF production [27]. This particular microenvironment may promote the emergence of aggressive clones favouring tumour dissemination and treatment resistance.

We have demonstrated, for the first time in metastatic ccRCC, a significant association between hilar fat infiltration and disease progression in RECIST at first evaluation even if association did not remain significant after logistic regression. If validated on an external cohort, it may constitute a potential candidate for predictor of early resistance to sunitinib therapy. Otherwise, hilar fat infiltration along with the Heng criteria were the only two variables independently associated with both poor PFS and OS although the multivariate Cox model was limited by the size of our cohort. Liver metastases were associated with worse PFS and OS but did not remain significant after multivariate analysis. Age over 65 years was also noteworthy but was limited to PFS. Among all the pathological variables we studied, hilar fat infiltration was the only one to have a poor prognostic impact. It is not known if the worse survival outcomes in patients with HFI is due to sunitinib resistance, especially since RECIST progression was not significant on multivariate analysis, however; it would be interesting to further investigate.

In the TNM staging system, T3a stage combines tumours with hilar, peri-renal fat infiltration or renal vein invasion without discriminating them [9]. Our study demonstrated the particularly worse prognosis of ccRCC with hilar fat infiltration in metastatic patients. Indeed, peri-renal fat and renal vein invasion were not associated with PFS and OS. These results suggest that, in future studies, it would be worthwhile to individualise and report hilar fat infiltration.

To conclude, our study demonstrated hilar fat infiltration as a new independent prognostic factor in metastatic patients with ccRCC treated by first-line sunitinib therapy. This result, if confirmed in larger and prospective studies, could have a substantial impact in predicting

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prognosis under sunitinib therapy, which has become an even more crucial issue with the advent of targeted immunotherapies.

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CONFLICT OF INTEREST

Authors indicated no conflict of interest in relation to this study

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Figure 1: Kaplan Meier curve representing progression-free survival according to hilar fat infiltration.

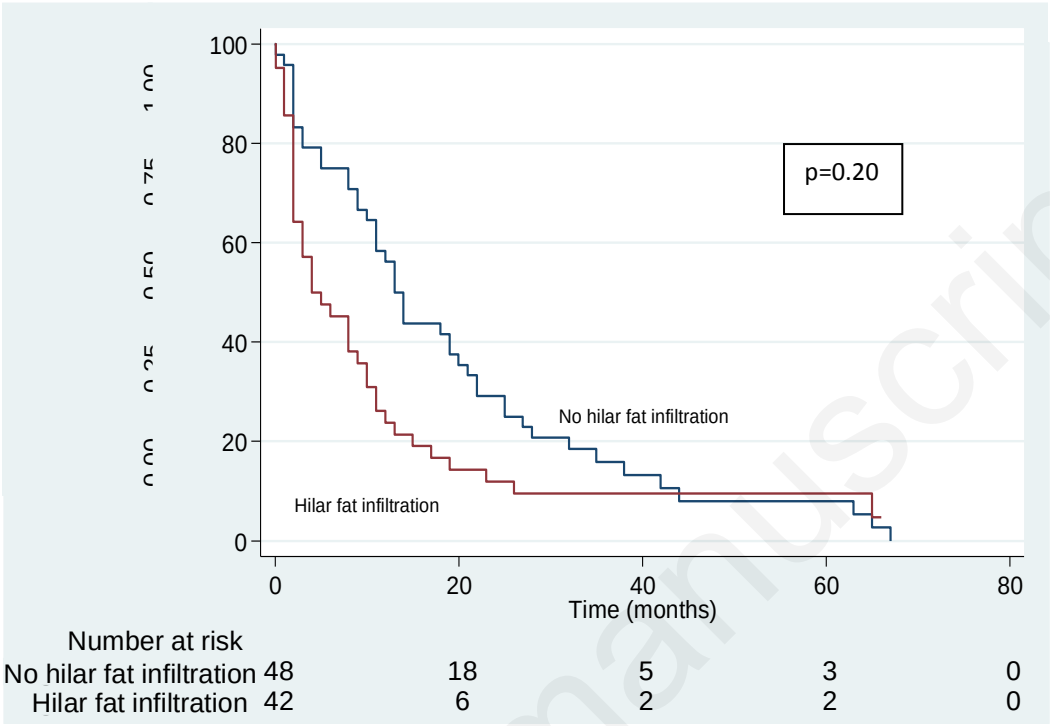
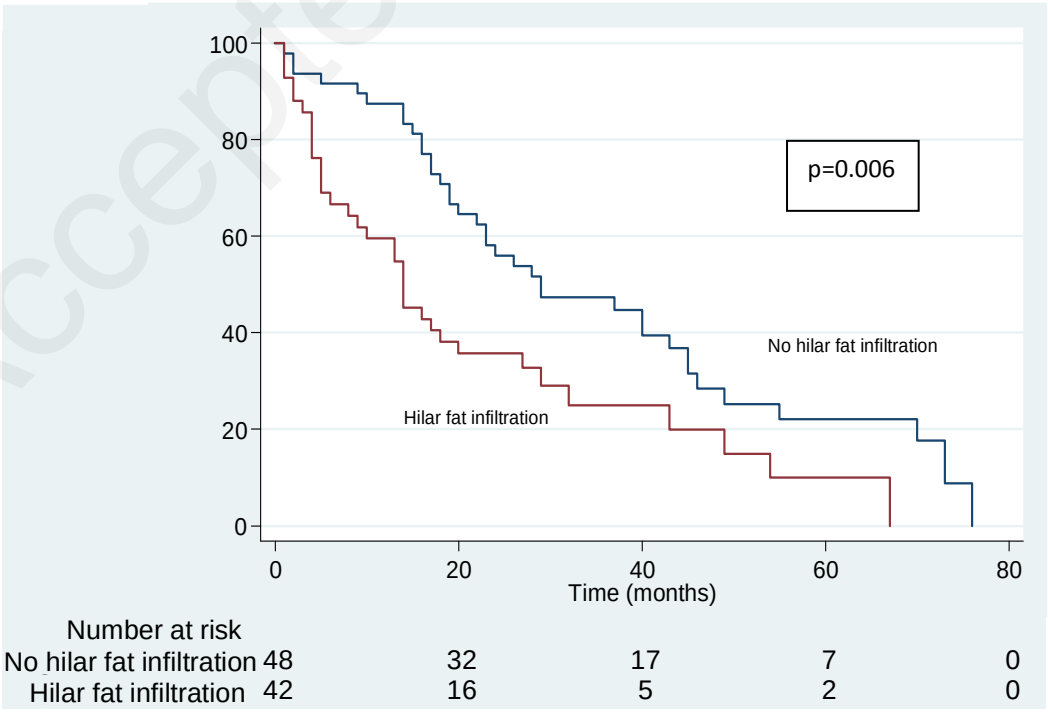


Figure 2: Kaplan Meier curve representing overall survival according to hilar fat infiltration.



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Table 1: Patient and tumour characteristics of 90 metastatic clear cell renal cell carcinoma

Characteristics	Number	%
Age (years)		
Mean	61.3	
Range	37-85	
Sexe		
Male	56	62.2%
Female	34	37.8%
Heng score		
Favourable	20	22.2%
Intermediate	43	47.8%
Poor	27	30.0%
T stage		
T1	14	15.6%
T2	8	8.9%
T3	63	70.0%
T4	5	5.6%
N stage		
N0	75	83.3%
N1	15	16.7%
M stage		
M0	40	44.4%
M1	50	55.6%
Time to develop metastasis (months)		
Mean	10.5	
Range	0-144	
Metastatic localisations		
Lung	68	75.6%
Bone	48	53.3%
Liver	21	23.3%
Cerebral	19	21.1%
Multiple localisations	69	76.7%
Tumor size (cm)		
Mean	9.6	
Range	2-9.5	
Nucleolar ISUP grade		
Grade 2	7	7.8%

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	Grade 3	33	36.7%
	Grade 4	50	55.6%
RECIST 1			
	Complete response	1	1.1%
	Partial response	27	30.0%
	Stable disease	34	37.8%
	Progression disease	28	31.1%

Abbreviations: TNM: Tumor Node Metastasis, RECIST 1: first evaluation according to response evaluation criteria in solid tumours (RECIST) 1.1 criteria, ISUP: International Society of Uropathology

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Table 2: Clinicopathological, radiological and molecular comparisons between metastatic clear cell renal cell carcinoma according to hilar fat infiltration.

Variables	No hilar fat infiltration (n=48)		Hilar fat infiltration (n=42)		Univariate analysis p-value	Rank	Multivariate analysis p-value
Clinical data							
Male	31	64.6%	25	59.5%	0.621		
Age >65	14	29.2%	20	47.6%	0.24		
Prior cytokine therapy	8	16.7%	3	7.1%	0.17		
Poor prognosis (Heng)	8	16.7%	19	45.2%	**0.003**		**0.002**
Poor prognosis (MSKCC)	4	8.3%	13	31.0%	**0.006**		
Pulmonary metastasis	35	72.9%	33	78.6%	0.53		
Bone metastasis	29	60.4%	19	45.2%	0.15		
Liver metastasis	7	14.6%	14	33.3%	**0.036**	5	0.064
Cerebral metastasis	10	20.8%	9	21.4%	0.945		
Multiple metastasis	37	77.1%	32	76.2%	0.92		
Progression (RECIST 1)	10	20.8%	18	42.9%	**0.024**	2	0.352
Pathological analysis							
Size >7cm	27	56.3%	31	73.8%	0.083		
Nucleolar ISUP grade 4	22	45.8%	28	66.7%	**0.047**		
Sarcomatoid component	5	10.4%	14	33.3%	**0.008**	3	0.207
Tumor necrosis	33	68.8%	38	90.5%	**0.012**	4	0.141
Microvascular invasion	16	33.3%	25	59.5%	**0.013**	1	0.684
T3-T4 stage	26	54.2%	42	100.0%	**0.001**		
Peri-renal fat invasion	19	39.6%	26	61.9%	**0.035**		**0.037**
Venal invasion	18	37.5%	23	54.8%	0.101		
N1 stage	3	6.3%	12	28.6%	**0.005**		**0.006**
M1 stage	24	50.0%	26	61.9%	0.257		
CAIX >85%	14	29.2%	14	33.3%	0.67		
VEGFA >30%	28	58.3%	22	52.4%	0.571		
MET 2-3 intensity	31	64.6%	31	73.8%	0.346		
PD-L1 2-3 intensity	32	66.7%	34	81.0%	0.126		
PD1	25	52.1%	23	54.8%	0.799		
VHL status							
VHL mutation	39	81.3%	25	59.5%	**0.023**	6	0.108
Promoter methylation	3	6.3%	7	16.7%	0,179 ‡		
VHL inactivation	42	87.5%	32	76.2%	0.162		

‡ fisher exact test

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Table 3: Cox model for progression-free survival

Variables	Univariate Cox PH Model			Multivariate Cox PH Model			
	HR	95% CI	*P*	Rank	HR	95% CI	*P*
Clinical data							
Male	0.74	0.47-1.15	0.176				
Age >65	1.56	1.01-2.41	**0.044**		1.663	1.06-0.58	**0.024**
Prior cytokine therapy	1.01	0.53-1.91	0.981				
Poor prognosis (Heng)	2.59	1.59-4.24	**0.001**		2.501	1.51-4.12	**0.001**
Poor prognosis (MSKCC)	2.26	1.30-3.93	**0.004**				
Liver metastasis	1.72	1.04-2.85	**0.035**	1	1.300	0.78-2.18	**0.316**
Bone metastasis	1.16	0.75-1.80	0.49				
Cerebral metastasis	1.50	0.89-2.52	0.123				
Multiple metastasis	1.17	0.70-.96	0.549				
Pathological analysis							
Size >7cm	1.11	0.71-1.73	0.641				
Nucleolar ISUP grade 4	1.25	0.81-1,93	0.305				
Sarcomatoid component	1.31	0.76-2.25	0.33				
Tumor necrosis	1.53	0.89-2.62	0.121				
Microvascular invasion	1.12	0.73-1.73	0.599				
T3-T4 stage	1.29	0.79-2,12	0.307				
Hilar invasion	1.63	1.06-2.53	**0.027**		1.580	1.01-2.47	**0.045**
Peri-renal fat invasion	1.05	0.68-1.61	0.821				
Venal invasion	0.87	0.56-1.35	0.54				
N1 stage	1.45	0.81-2.60	0.21				
M1 stage	1.12	0.73-1.73	0.595				
CAIX >85%	0.86	0.54-1.37	0.539				
VEGFA >30%	1.37	0.88-2.12	0.161				
MET 2-3 intensity	1.05	0.66-1.67	0.835				
PD1	1.07	0.70-1.65	0.749				
PD-L1 2-3 intensity	1.29	0.80-2.09	0.29				
VHL status							
VHL mutation	1.01	0.63-1.63	0.957				
Promoter methylation	1.05	0.53-2.11	0.88				
VHL inactivation	1.05	0.60-1.85	0.857				

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Table 4: Cox model for overall survival

Variables	Univariate Cox PH Model			Multivariate Cox PH Model			
	HR	95% CI	*P*	Rank	HR	95% CI	*P*
Clinical data							
Male	0.74	0.45-1.19	0.22				
Age >65	1.42	0.88-2.29	0.143				
Prior cytokine therapy	1.40	0.74-2.68	0.301				
Poor prognosis (Heng)	3.93	2.34-6.61	**0.001**		3.646	2.15-6.18	**0.001**
Poor prognosis (MSKCC)	3.41	1.92-6.05	**0.001**				
Liver metastasis	1.91	1.12-3.25	**0.017**	**1**	1.512	0.88-2.59	0.133
Bone metastasis	1.32	0.82-2.13	0.255				
Cerebral metastasis	1.09	0.61-1.95	0.757				
Multiple metastasis	1.11	0.63-1.95	0.708				
Pathological analysis							
Size >7cm	1.23	0.75-2.01	0.412				
Nucleolar ISUP grade 4	1.37	0.84-2.21	0.204				
Sarcomatoid component	1.31	0.72-2.38	0.368				
Tumor necrosis	1.67	0.89-3.10	0.109				
Microvascular invasion	1.44	0.90-2.31	0.127				
T3-T4 stage	1.55	0.88-2.75	0.132				
Hilar invasion	1.93	1.19-3.13	**0.007**		1.666	1.017-2.728	**0.042**
Peri-renal fat invasion	1.12	0.70-1.80	0.634				
Venal invasion	1.06	0.66-1.70	0.792				
N1 stage	1.48	0.82-2.66	0.192				
M1 stage	1.42	0.87-2.30	0.162				
CAIX >85%	0.75	0.45-1.27	0.288				
VEGFA >30%	1.04	0.65-1.66	0.879				
MET 2-3 intensity	0.96	0.58-1.57	0.862				
PD1							
PD-L1 2-3 intensity	1.00	0.99-1.00	0.483				
VHL status							
VHL mutation	0.88	0.52-1.47	0.624				
Promoter methylation	1.27	0.60-2.66	0.524				
VHL inactivation	0.97	0.53-1.78	0.930				

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