Correlation of c-MET Expression with PD-L1 Expression in Metastatic Clear Cell Renal Cell Carcinoma Treated by Sunitinib First-Line Therapy

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Correlation of c-MET expression with PD-L1 expression in metastatic clear cell renal cell carcinoma treated by sunitinib first-line therapy

Short title: c-MET in metastatic renal carcinoma

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ABSTRACT

Background: Clear cell renal cell carcinoma (ccRCC) are highly metastatic. Cabozantinib, an antiangiogenic tyrosine kinases inhibitor, targeting c-MET, proved interesting results in metastatic ccRCC treatment.

Objective: To better understand c-MET role, we assessed its status in a population of patients with metastatic ccRCC.

Patients and methods: For this purpose, c-MET expression was studied by immunohistochemistry (IHC), *c-MET* copy number alteration, by fluorescence in situ hybridization (FISH) and *c-MET* mutation, by next generation sequencing (NGS) in a retrospective cohort of 90 primary ccRCC of patients with metastases treated by first-line sunitinib. The expression of c-MET was correlated with pathological, immunohistochemical (VEGFA, CAIX, PD-L1), clinical and molecular criteria (*VHL* status) by univariate and multivariate analyses and to clinical outcome using Kaplan Meier curves compared by log-rank test.

Results: 31.1% of ccRCC had c-MET low expression (absent to weak intensity by IHC) versus 68.9% with high expression (moderate to strong intensity). The high expression of c-MET was associated with a gain in FISH analyses ($p = 0.0284$) without amplification. No mutation was detected in NGS. Moreover, high c-MET expression was associated with lymph node metastases ($p=0.004$), sarcomatoid component ($p=0.029$), VEGFA overexpression ($p=0.037$) and PD-L1 ($p=0.001$), the only factor that remained independently associated ($p<0.001$) after logistic regression. No difference was observed in clinical outcome.

Conclusion: This study is the first to analyse c-MET status in metastatic ccRCC. The high expression of c-MET in the majority of ccRCC and its independent association with PD-L1 expression, may suggest a potential benefit from c-MET inhibitors and targeted immunotherapy in combination.

Key points:

- This study is the first to assess c-MET status, in metastatic renal carcinoma treated with anti-angiogenic therapy.
- The high expression of c-MET was observed in the majority of patients and independently associated with the overexpression of PD-L1.
- These findings may suggest a potential benefit from the combination of c-MET inhibitors and targeted immunotherapy.

1. INTRODUCTION

Renal cell carcinoma (RCC) is the most common form of kidney cancer with more than 270 000 cases diagnosed worldwide and an estimated of 110 000 deaths from this tumour annually [1]. The most frequent histological subtype is clear cell RCC (ccRCC), representing 70% of all renal cancers [2]. This subtype is a highly aggressive tumour, as 40 to 50% of patients with ccRCC develop metastases: 20 to 30% of patients have metastatic disease at diagnosis, while 30% of patients who have local RCC will develop metastases during follow-up [3].

Inactivation of the von Hippel–Lindau (*VHL*) tumour-suppressor protein has been described in more than 80% of ccRCC, resulting in the stabilization of Hypoxia Inducible Factor (HIF) and the up-regulation of vascular endothelial growth factor (VEGF) production [4]. A better understanding of the molecular pathways involved in ccRCC contributed to the development of anti-angiogenic drugs, such as sunitinib or pazopanib, but long-standing responses are rare [5, 6].

New strategies to treat metastatic ccRCC are in development. The first strategy is based on targeted immunotherapy using checkpoint inhibitors (such as anti-PD-1/PD-L1). A recent clinical trial demonstrated nivolumab (an anti-PD-1) superiority over everolimus, an inhibitor of mammalian target of rapamycin (mTOR), in second-line treatment [7]. Another approach uses a novel antiangiogenic tyrosine kinase inhibitor (TKI), cabozantinib, which has the particularity to target c-MET and also showed interesting results in second-line treatment compared to everolimus [8].

The tyrosine kinase receptor c-MET is a proto-oncogene involved in tumour angiogenesis, proliferation and metastasis. The dysregulation of c-MET and its ligand, hepatocyte growth factor (HGF), has been implicated in a range of malignancies including renal cancers [9-11]. c-MET can be activated through several mechanisms such as the mutation of the tyrosine kinase receptor domain or gene amplification and protein overexpression [6]. Although cabozantinib is a promising therapy, c-MET status has never been assessed in metastatic ccRCC.

The aim of the present study was to verify whether c-MET is activated in metastatic ccRCC in order to optimize the use of c-MET inhibitors in such tumours. For this purpose, we assessed c-MET status in a retrospective cohort of 90 patients with metastatic ccRCC treated by first-line sunitinib therapy.

2. MATERIALS AND METHODS

2.1 Patients and pathological analysis

Primary ccRCC-specimens were collected from 90 patients undergoing nephrectomy in 2 French University Hospitals (Rennes, n=46 and Bordeaux, n=44 from UroCCR database) from 1997 to 2013 with metastases that appeared between 2005 and 2013. When the metastases appeared, these patients were treated with antiangiogenic sunitinib (Sutent®) therapy, as a first-line treatment. For each case, clinical, biological and radiological features were obtained from referring physicians and medical records. The response to treatment was assessed using response evaluation criteria in solid tumours (RECIST), the first evaluation was assessed after 2 cycles of sunitinib (3 months). The study protocol was approved by the local advisory board (CNIL declaration receipt 1812601v0) and informed consent was obtained from each patient. The macroscopic and histological parameters analysed were tumour size, nucleolar grade according to the International Society of

Urological Pathology (ISUP) grading system, sarcomatoid component, tumour necrosis and microvessel invasion. Tumour stage was defined according to the latest International Union Against Cancer 2009 classification. For each tumour, formalin-fixed paraffin-embedded (FFPE) and frozen samples were available.

2.2 Immunohistochemistry

For each ccRCC case, a representative slide of the tumour with the highest nucleolar grade and the corresponding paraffin block was selected. Four µm-thick whole tissue sections were cut and mounted on glass slides (Superfrost+, Menzel Glazer). The preparations were dried for 1 hour at 58°C, and then overnight at 37°C. The sections were deparaffinized with toluene and rehydrated with ethanol. The preparations were pretreated and immunostained using Ventana Benchmark XT. c-MET (Anti-Total c-MET, SP44, Rabbit Monoclonal Primary Antibody, ready-diluted, Ventana, Roche, Switzerland), VEGFA (Anti-VEGF antibody, sc-152, dilution 1/100 ; Santa Cruz Biotechnology, Santa Cruz, CA, USA), CAIX (Anti-CAIX antibody, ab15086, dilution 1/1500, Abcam, Cambridge, UK), PD-L1 (Anti-PD-L1 antibody, clone 130021, dilution 1/200, RD System, Minneapolis, USA) and PD1 (anti-PD-1 antibody, clone NAT105, dilution 1:50; Abcam, Cambridge, UK) expressions were assessed by immunohistochemistry as previously described [12-15]. The reactivity of antibody was revealed with HRP-labeled polymer conjugated secondary antibody using diaminobenzidine (DAB) as chromogen (Sigma-Aldrich, France). Appropriate positive and negative controls were used. Two pathologists (SM, SFKJ) and one uropathology expert (NRL) independently scored immunomarkers expression. The cut-off for positive cases was 30% of tumour cells for VEGF and 85% for CAIX as previously described [16, 17]. 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 (0), rare (1), moderate (2) or dense (3); cases were then subdivided into negative (score 0–1) or positive (score 2–3) subgroups [18, 14]. For c-MET IHC, membranous and cytoplasmic staining were considered. The intensity of the staining was scored as absent (0), weak (1), moderate (2) or strong (3) as previously assessed [15]. For statistical analysis, c-MET expression was considered high when moderate or strong staining was observed and low when the staining was absent or weak.

2.3 Fluorescent *in situ* hybridization

FISH analyses were performed on the Rennes cohort (n=46). FISH analyses were performed on 4 µm sections of FFPE tumour tissue with the Zyto*Light*® SPEC c-MET (7q31, Spectrum Green®) / CEN 7 (Spectrum Orange®) Dual Color Probe (Zytovision, Bremerhaven, Germany). One cytogeneticist (MABR) independently analysed FISH. The slides were deparaffinized with xylene using a VP2000processor (Abbott, Wiesbaden, Germany). The tissue was digested with pepsin (Dako, Les Ulis, France) for 6 min. The target DNA and probe were co-denatured for 10 min at 75 °C using a programmable system (Thermobrite, Abbott Laboratories, North Chicago, Illinois, USA) and probe hybridization was performed overnight in a humidified atmosphere at 37 °C. The slides were analysed using a fluorescence microscope (BX61, Olympus, Rungis, France) and Isisimaging software (Metasystems, Altlussheim, Germany) [19]. The entire hybridized surface was screened using a double band-pass filter with an X63 objective and 50 non-overlapping tumour nuclei were examined. For the c-MET analysis,

gain (copy number alteration) was defined by strictly more than 2 c-MET signals and amplification was defined according to UCCC (University of Colorado Cancer Center) scoring system [20].

2.4 Next generation sequencing

Small samples were collected from surgical specimens, frozen in liquid nitrogen and stored at -80°C until DNA extraction. Genomic DNA was extracted using QIAamp DNA minikit (Qiagen, Courtaboeuf, France). DNA quantity and quality were estimated by optical density (OD 260/280) measurement and 0,8% agarose gel electrophoresis using standard protocols. Sequencing amplicon libraries were prepared using the TruSeq Amplicon Cancer Panel (Illumina Inc., San Diego, California), according to the manufacturer's instructions. DNA target preparation and enrichment were performed using the Access Array® polymerase chain reaction (PCR) system (Fluidigm, San Francisco, USA). A 6-nucleotide "barcode" tag, specific to each sample and Illumina-specific sequencing adaptors were attached using secondary PCR. Purified products were subsequently pooled and sequenced on the Illumina MiSeq NGS instrument (Illumina Inc., San Diego, California). For the *c-MET* gene, exons 2, 14, and 16 to 20 were fully sequenced to identify mutations in the tyrosine kinase domain. For the *VHL* gene, the entire coding sequence and exon-intron junctions of exons 1, 2 and 3 were analysed.

2.5 Multiplex Ligation-dependant Probe Amplification

Methylation-Specific-MLPA (MS-MLPA) was used to detect CpG islands methylation in the *VHL* gene promoter [21]. The SALSA MS-MLPA kit ME001B Tumour suppressor-1 was used to detect the aberrant methylation of CpG-islands located in the promoter region of the *VHL* gene. The DNA (50–200 ng) was denatured and the probes were allowed to hybridize (16h at 60°C). The VHL probes used for the methylation quantification analysis contained one methylation-sensitive restriction site (HhaI) in the target recognition sequence. Following hybridization, the samples were divided in half and one half of the samples was ligated, whereas ligation was combined with HhaI digestion enzyme for the other part of the sample. This digestion resulted in the ligation of only the methylated sequences. PCR was performed on all the samples and the products were then analysed by electrophoresis. Reference unmethylated DNA samples, isolated from blood from healthy volunteers, were included in each set of MLPA experiments. Unmethylated DNA will not generate a signal, and a normal probe signal will be detected if the site is methylated.

2.6 Statistical analysis

Chi2 (or Fisher exact) and Mann-Whitney tests were performed to compare qualitative and quantitative parameters respectively between groups. For logistic regression, we used a backward stepwise selection with $p<0.05$ inclusion criteria. For clinical outcome, we calculated PFS from the date of sunitinib introduction to the date of progression and OS from the date of sunitinib introduction to the date of specific death or last contact. All p-values were 2-sided, and p-values less than 0.05 were considered statistically significant. All statistical analyses were performed using Stata 14.1 software (College Station, TX).

3. RESULTS

3.1 Patients and histological parameters

The mean age at diagnosis was 61 years (37-85). The mean tumour size was 8.7 cm with tumours ranging from 2

cm to 20 cm. A total of 69 patients (76.6%) had an ECOG performance status of 0. In 50 cases (55.5%), metastases were present at diagnosis. Non-metastatic tumours at diagnosis developed metastases on average after 10.5 months (0-144 months). The mean follow-up was 43.9 months (1-171 months) from nephrectomy. Eighty-five patients (94.4%) experienced progression and 71 patients (78.9%) died from their cancer. The mean follow-up period was 25.9 months (range 1-76 months) from sunitinib introduction. The population characteristics and pathological parameters are summarized in Table 1.

3.2 VHL status

VHL status was assessed in the entire cohort (n=90). All patients were negative for germ-line mutations. A *VHL* gene mutation was observed in 64 cases (71.1%). Mutations occurred in exons 1, 2 and 3 in 28 (43.8%), 21 (32.8%) and 15 cases (23.4%) respectively. Stop, frameshift, missense, and splice site mutations were detected in 8 (12.5%), 34 (53.1%), 18 (28.1%) and 4 (6.3%) cases respectively. *VHL* promoter methylation occurred in 10 cases (11.1%). At least one or more *VHL* abnormalities (*VHL* inactivation) were observed in 74 cases (82.2%).

3.3 c-MET status

For c-MET immunostaining, 11.1% (n=10) of ccRCC cases were negative, while 20.0% (n=18) of ccRCC cases had weak staining. Moderate staining was observed in 26.7% (n=24) of cases and strong staining was observed in 42.2% (n=38) of cases. Overall, 31.1% (n=28) of tumours were considered low and 68.9% (n=62) of cases were considered high for c-MET expression. In the NGS analysis for the 90 patients, *c-MET* status was assessed in 90 tumours. DNA quality was acceptable for 75 samples and no mutation was identified. *c-MET* FISH status was assessed in 39 out of 46 interpretable cases. Gains were observed without amplification of *c-MET*. Two copies were observed. The mean percentage of cells with gains was significantly associated with c-MET high expression (p=0.0284).

3.4 Correlation of c-MET expression with pathological, immunohistochemical and molecular characteristics and clinical outcomes

Tumours with low or high c-MET expression and patients characteristics were summarized and compared in Table 2. Overall, high c-MET expression was not associated with progression at first radiologic evaluation. High c-MET expression was also associated with high ISUP nucleolar grade (p=0.037), lymph node status (p=0.004) and sarcomatoid component (p=0.029). VEGFA and PD-L1 overexpression were also associated with high c-MET expression (p=0.037 and p=0.001 respectively). CAIX expression was not associated with c-Met expression. PD-L1 overexpression remained significantly associated after logistic regression (p<0.001, [2.3-18.7]). The high expression of c-MET was not associated with VHL status (p=0.239). No differences were observed between progression-free and overall survival according to c-MET expression (p=0.94 and p=0.73 respectively, Figure 4).

4. DISCUSSION

Antiangiogenic VEGF-targeted therapies have been approved as first-line treatment in metastatic ccRCC. However, despite showing efficacy and survival benefit, almost 20 to 30% of patients develop early disease progression [5]. Thus, tumour recurrence in metastatic ccRCC has been one of the major challenges in patients

treated with angiogenesis inhibitors, and explains the emergence of new strategies such as targeted immunotherapy and TKI targeting new pathways such as HGF/cMET [10].

It is important to acknowledge the limitations of our study. Firstly, this a retrospective study with patients included from 2 centers. The sample size is limited and partially explained by our rigorous inclusion criteria such as homogeneous histology (ccRCC) and sunitinib as first line treatment. Secondly, as recognized by the litterature, immunohistochemistry usually experienced poor standardization; however, in the case of c-Met antibody, this statement is limited as the same clone for c-MET antibody is widely used [20, 15, 9].

In the present study, we assessed c-MET status in a series of 90 patients with m-ccRCC treated by sunitinib as first line treatment with a median follow-up of 43 months. High c-MET expression was found in 69% of tumours. The FISH analysis revealed gains without *c-MET* amplification as previously found by Macher-Goeppinger *et al* [22]. We observed a significant correlation between the percentage of gains by FISH and c-MET expression by IHC, suggesting a potential mechanism for overexpression of the protein detected by IHC that could be inherited to metastatic tumour cells. Another explanation for c-MET overexpression may be related to post-translational modification, as previously described for tyrosine kinase receptors [23]. NGS analysis revealed no mutation on the c-MET gene, consistent with a previous study,, unlike papillary RCC, particularly type 1, which shows nearly 20% c-MET mutations [11, 22].

As previously described, c-MET expression has been associated with poor prognostic factors such as nucleolar grade, sarcomatoid component and the presence of lymph nodes metastases [24]. A recent study by Peltola *et al.* assessed that c-MET high expression was also associated with poor survival in patients with metastatic RCC treated with sunitinib [25]. This difference may be explained by the non-homogeneous histology of the renal cell carcinoma included. Indeed, in their population (n=137), n=107 were clear cell renal cell carcinoma, whereas n=15 were non ccRCC and for 15 tumors, the histology was missing. Non ccRCC have different carcinogenesis and there is no evidence whether first-line sunitinib is the optimal treatment in this subgroup [26, 27].

In our study, high c-MET expression was independently associated with PD-L1 by IHC. Shin *et al* first reported an association between c-MET and PD-L1 expressions in non-selected ccRCC [15]. In the present cohort, this association was observed in the primary ccRCC of patients with metastases. This cohort is more representative of patients likely to benefit from the corresponding inhibitors. Moreover, the independent association we identified, although limited by the sample size, gives argument for the combined use of c-MET inhibitors and targeted immunotherapy. Indeed, cabozantinib and nivolumab combination is currently investigated (NCT02496208) [28].

The HGF/cMET pathway has been described as an alternative antiangiogenic pathway that could be responsible for early resistance to anti-VEGF therapy [29]. In addition, chronic sunitinib treatment was demonstrated to lead to c-MET up regulation *in vitro* [30]. Consequently, we hypothesize that the response could be improved targeting c-MET pathway as well. Nevertheless, MET overexpression was not associated with progressive disease at first radiological evaluation, PFS and OS, in patients uniformly first-treated by sunitinib.

With *VHL* inactivation, HIF stabilization leads to the transcription of targeted genes, such as CAIX, VEGFA and c-MET whose expression and suggesting a correlation between *VHL* status and HIF-targeted gene expression [31, 32]. However, we did not observe a correlation between *VHL* inactivation (*VHL* mutation or promoter

methylation) and the expression of these targets genes suggesting the involvement of alternative pathways, such as the MAP kinase and PI3K-AKT-mTOR pathways, in ccRCC oncogenesis, independent of *VHL* mechanisms [33, 34].

In conclusion, the present study confirmed a high proportion of metastatic ccRCC showing a high c-Met protein expression without association with sunitinib response. The independent association of c-MET expression with PD-L1 expression, may suggest a potential benefit to the use of c-MET inhibitors with targeted immunotherapy for these patients.

COMPLIANCE WITH ETHICAL STANDARDS SECTION

Informed consent

Informed consent was obtained from all individuals participants included in the study.

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Conflict of interest

The authors have no conflict of interest to declare.

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FIGURE LEGENDS

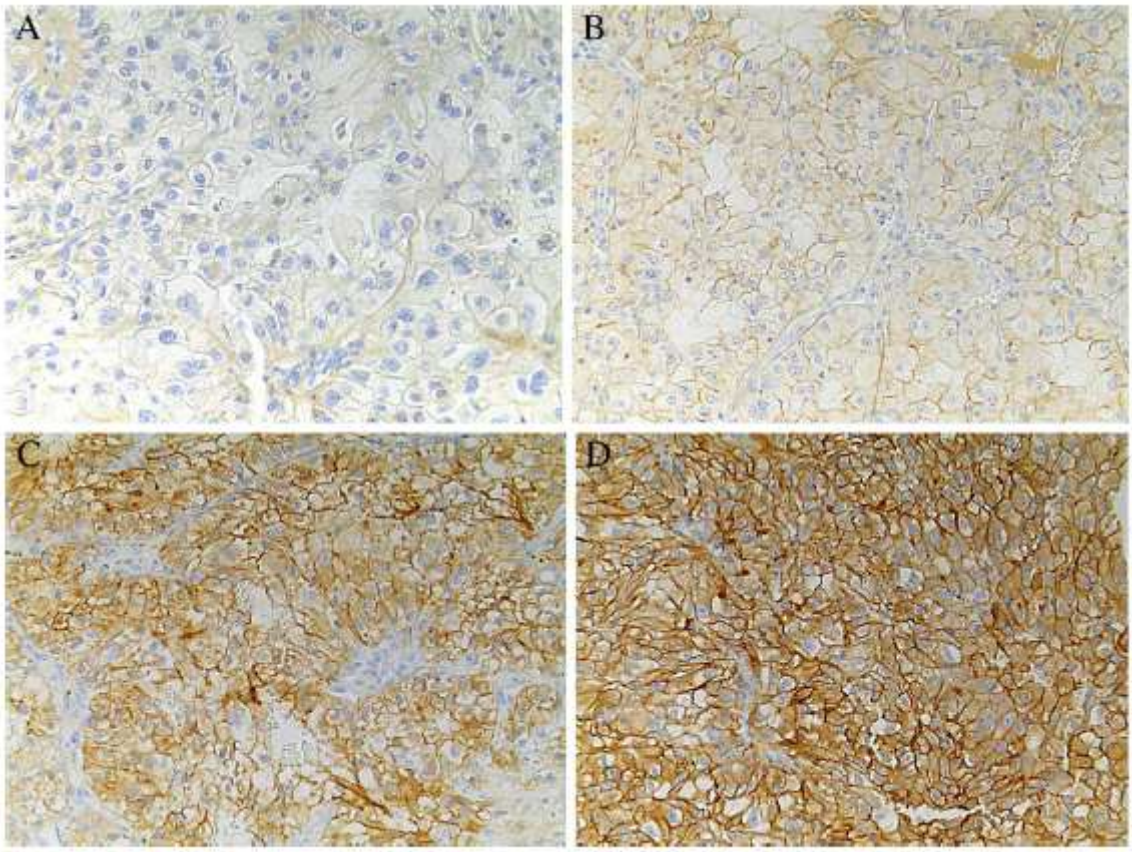

Figure 1: Intensity of c-MET expression:

A) Absence of c-MET expression, IHC x100

B) Low expression of c-MET in tumor cells, IHC x100

C) Moderate expression of c-MET in tumor cells, IHC x100

D) High expression of c-MET in tumor cells, IHC x100

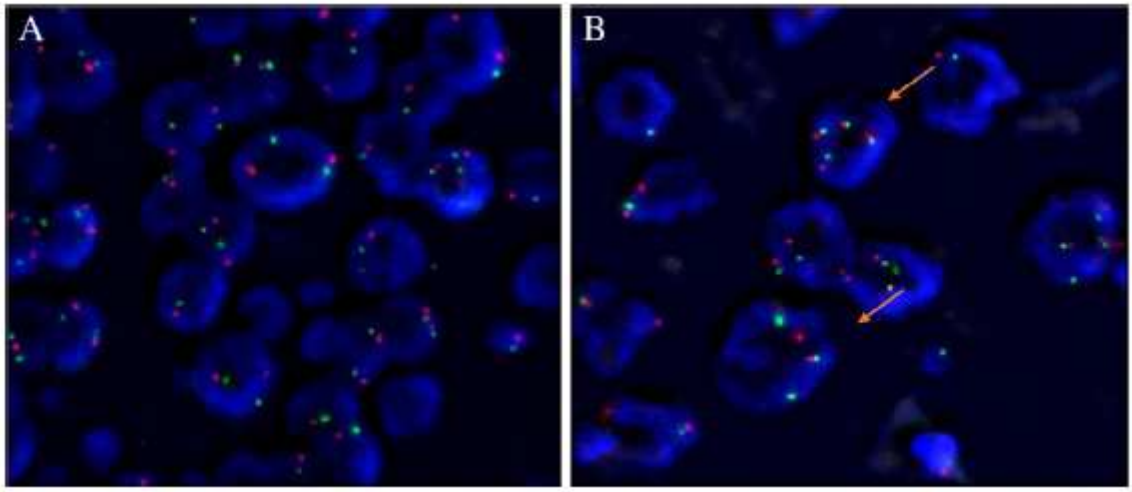

Figure 2: FISH analysis

A) Nuclei with a normal hybridization pattern (2 green and 2 orange signals)

B) Nuclei with a c-MET gain exhibit more than 2 green signals (arrows).

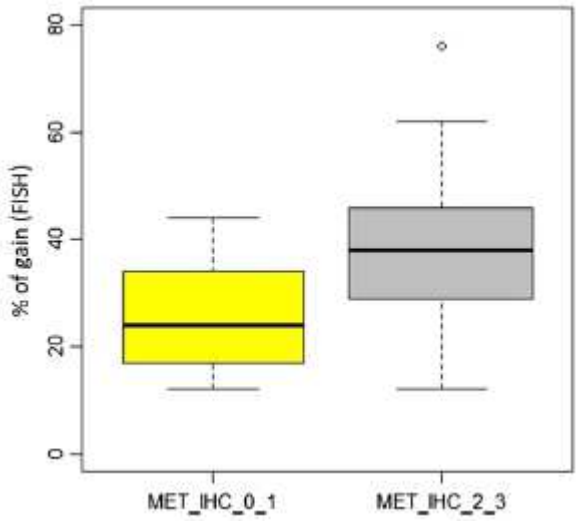

Figure 3: Percentage of gains (FISH) according to c-MET expression (IHC).

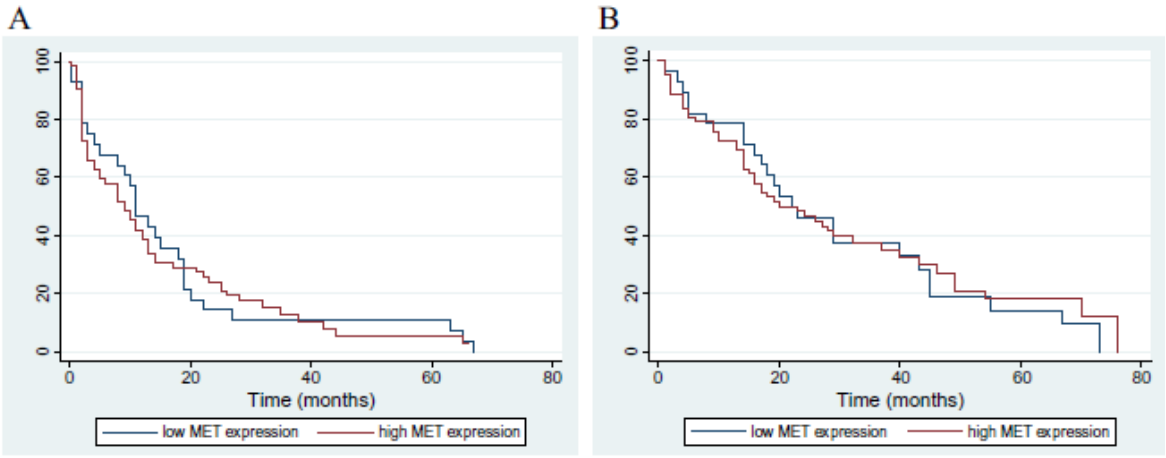

Figure 4: Progression-free survival (PFS) and overall survival (OS) after sunitinib according to c-MET expression in metastatic ccRCC:

A) PFS: median survival: low: 11months, high: 9 months (p=0.828)

B) OS: median survival: low: 22 months, high: 20 months (p=0.860)

449 **TABLE LEGENDS**

Characteristics	No	%
Age (years)		
Mean	61.3	
Range	37–85	
Sex		
Male	34	37.8%
Female	56	62.2%
Heng score		
Favorable	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-N2	15	16,7%
M stage		
M0	40	44.4%
M1	50	55.6%
Tumor size (cm)		
Mean	9.6	
Range	2–20	
ISUP nucleolar grade		
Grade 2	7	7.8%
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%
Progressive disease	28	31.1%

450
451
452 **Table 1. Summary of the clinical and histopathological characteristics of 90 patients with metastatic**
453 **ccRCC treated by first line sunitinib.**

Variables	MET low (n = 28)	%	MET high (n = 62)	%	p-value
Clinical and radiological data					
Age > 65 years	14	50.0%	23	37.1%	0.249†
Good/intermediate prognosis (Heng score)	21	75.0%	42	67.7%	0.487†
Distant lymph node metastasis	11	39.3%	28	45.2%	0.603†
Pulmonary metastasis	22	78.6%	46	74.2%	0.655†
Bone metastasis	17	60.7%	31	50.0%	0.346†
Liver metastasis	8	28.6%	13	21.0%	0.430†
Cerebral metastasis	7	25.0%	12	19.4%	0.544†
Multiple metastasis	22	78.6%	47	75.8%	0.774†
Progressive disease (RECIST)	7	25.0%	21	33.9%	0.400†
Pathological analysis					
Size >7 cm	19	67.9%	39	62.9%	0.649†
Nucleolar ISUP grade 4	11	39.3%	39	62.9%	0.037†
Sarcomatoid component	2	7.1%	17	27.4%	0.029†
Tumor necrosis	20	71.4%	51	82.3%	0.244†
Microvascular invasion	13	46.4%	28	45.2%	0.911†
T3-T4 stage	22	78.6%	46	74.2%	0.655†
Hilar fat infiltration	11	39.3%	31	50.0%	0.346†
Peri-renal fat infiltration	16	57.1%	29	46.8%	0.362†
Venal invasion	12	42.9%	29	46.8%	0.730†
N1-N2 stage	0	0.0%	15	24.2%	0.004‡
M1 stage	13	46.4%	37	59.7%	0.242†
Immunohistochemistry					
CAIX >85%	7	25.0%	21	33.9%	0.400†
VEGFA >30%	11	39.3%	39	62.9%	0.037†
PD-L1 (2–3 intensity) *	13	46.4%	53	85.5%	0.001†
PD-1 (2–3 density)	11	39.3%	37	59.7%	0.073†
VHL status					
VHL mutation	23	82.1%	41	66.1%	0.121†
Promoter methylation	2	7.1%	8	12.9%	0.718‡
VHL inactivation	25	89.3%	49	79.0%	0.239†

†, χ^2 test; ‡, Fisher exact test

RECIST 1: First RECIST evaluation, CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease

*Independent variable after logistic regression

Table 2. Summary of histopathological and immunohistochemical characteristics and VHL status of tumors according to c-MET expression.