Angiogenic, inflammatory and immunologic markers in predicting response to sunitinib in metastatic renal cell carcinoma

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The objective of this prospective study was to identify baseline angiogenic and inflammatory markers in serum as well as the baseline levels of immune cells in whole blood to predict progression-free survival in patients with metastatic renal cell carcinoma treated with sunitinib. Blood samples were collected at baseline in all 90 patients to analyze serum angiogenic and inflammatory markers together with peripheral blood immunological marker. The association between each marker and sunitinib efficacy was analyzed. Univariate and multivariate Cox proportional model analyses were used to assess the correlation between those markers with survival. Baseline levels of interleukin-6, interleukin-8, high sensitivity C-reactive protein and myeloid-derived suppressor cells were significantly higher in patients who progressed when compared with those with clinical benefit. Analysis by the Cox regression model showed that baseline interleukin-8, high sensitivity C-reactive protein and percentage of T helper type 1 cells were significantly associated with progression-free survival in univariate analysis. Furthermore, in multivariate analysis, those three markers were independent indices to predict progression-free survival. In conclusion, angiogenic (interleukin-8), inflammatory (interleukin-6, high sensitivity C-reactive) and immunologic (myeloid-derived suppressor cells, percentage of T helper type 1 cells) markers at baseline would predict the response to sunitinib therapy and/or disease progression in patients with metastatic renal cell carcinoma.

Kidney cancer, or renal cell carcinoma (RCC), arises from renal tubular epithelium. Complete surgical resection remains the only known curative treatment in the early stages; however, up to 40% of patients eventually experience disease recurrence after curative resection and one-third of patients already have metastasis at initial presentation.[1] With the introduction of molecular targeted therapies which inhibit tumor angiogenesis, the outlook of metastatic RCC (mRCC) has been changing.[2] Drugs that inhibit vascular endothelial growth factor (VEGF) pathway, such as bevacizumab or tyrosine kinase inhibitors (TKI), are currently considered standard care for the treatment of mRCC.

Sunitinib malate is an oral TKI that inhibits VEGF pathway. This drug is approved multinationally for the first-line treatment of mRCC. In a phase III trial, sunitinib achieved better progression-free survival (PFS) than interferon alpha in treatment-naïve patients.[3] In this trial, up to 50% of patients receiving sunitinib obtained clinical benefits in objective response (31%) or disease stabilization (48%).[3] However, some patients experience disease progression at first

evaluation, possibly owing to intrinsic resistance. Moreover, even when an initial response is obtained, nearly all patients develop resistance, known as acquired resistance.

Currently sunitinib is recommended in treatment guidelines for the first-line treatment of mRCC. The identification of patients who may obtain benefit from sunitinib has the potential to avoid unnecessary costs and adverse events. By linking aspects of the biology of RCC, many investigators have explored the use of angiogenic factors as prognostic and predictive biomarkers in mRCC;[4–6] however, validated baseline predictive molecular markers to identify individuals who might benefit from sunitinib have not been found. The aim of this prospective study was to evaluate the utility of baseline immunological and inflammatory markers, besides angiogenic molecules, to predict the efficacy of sunitinib in patients with mRCC. Because RCC is considered to be an immunological and inflammatory tumor, this approach may be appropriate. For example, non-specific immunotherapy has been the mainstay of mRCC treatments and increases in immune parameters have been correlated with poor outcomes.[7] Tumor-mediated

inflammation is considered to play an important role in the host tumor defense response.

We investigated the capacity of baseline angiogenic and inflammatory markers in serum as well as the baseline levels of immune cells in whole blood to predict responses to sunitinib. The availability of markers that predict responses may accelerate drug selection.

Materials and Methods

Patients and methods. In this prospective multicenter study, 90 consecutive patients with favorable or intermediate Memorial Sloan-Kettering Cancer Center (MSKCC) risk features were enrolled from 18 institutions between November 2009 and August 2012.[8] Patients 18 years of age and older were eligible for this study, if they had good Eastern Cooperative Oncology Group performance status, a life expectancy of ≥12 weeks, a histological diagnosis of predominantly clear cell carcinoma, received no systemic therapy or one regimen of cytokine therapy, had measurable metastatic disease, and adequate hematological, hepatic, renal and cardiac functions. All patients voluntarily consented to participate in the clinical and biological studies. The present study was approved by the independent ethics committee of Keio University Hospital (No 2009-94). All procedures performed in studies involving human participants were in accordance with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. All patients gave written informed consent before the study.

Study design and treatment. Sunitinib was given at a starting dose of 50 mg/day in a 4-weeks-on/2-weeks-off regimen. Blood samples were collected prior to sunitinib treatment. Dose reductions were permitted based on individual safety and tolerability. Sunitinib treatment was discontinued with progression, unacceptable adverse events or physicians' discretion. Patients were clinically assessed every 4 weeks during the treatment until week 24, and every 6 weeks thereafter. Based on Response Evaluation Criteria in Solid Tumors (RECIST) 1.1, tumor response was assessed 6 and 12 weeks after treatment.[9] After week 12, response assessments were performed every 12 weeks. The primary endpoint of the current study was identification of biomarkers to predict PFS for mRCC patients treated with first-line sunitinib. Secondary endpoints of this study were to assess PFS, overall survival (OS) and tumor response as evaluated based on RECIST.

Analysis of serum biomarkers. Prior to the initiation of sunitinib, blood samples were collected from every patient and centrifuged at the speed of 400 *g* for 5 min. All serum samples were stored at −80°C until analyzed. Validated enzyme-linked immunosorbent assays were used to determine serum protein levels of VEGF-C, soluble VEGF receptor (sVEGFR)-2, sVEGFR-3, hepcidin, basic fibroblast growth factor (bFGF), high-sensitivity C reactive protein (hs-CRP), interleukin (IL)-6 and IL-8. All assays were performed at SRL (Tokyo, Japan).

Flow cytometry -based analyses of immune cell population. We collected peripheral blood mononuclear cells (PBMC) from 90 patients. We calculated the percentage of T helper type 1 (Th1), T helper type 2 (Th2), T helper type 0 (Th0) cells, and the Th1:Th2 cell ratio of peripheral blood. The capacity for IFN-γ and IL-4 production within the CD4 subsets were analyzed by flow cytometry. The CD4 subsets were divided based on cytokine expression pattern into IFN-γ+/IL-4− (Th1) cells and IFN-γ−/IL-4 + (Th2) cells. We also calculated the percentage of regulatory T (Treg) cells and

myeloid-derived suppressor cells (MDSC). Treg cells were identified as CD4 + CD25 + FoxP3 + cells. PBMC were stained with antibodies to human CD4, CD25 and FoxP3 to identify Treg cells. MDSC were identified as CD14-CD15 + CD33 + HLA-DR^{LOW} cells. Cells were stained with antibodies to human CD14, CD15, CD33 and HLA-DR to identify MDSC. All results are shown as the percentage of positive cells in all PBMC. All assays were performed at SRL.

Statistical analysis. Statistical analyses focused on the investigation of baseline angiogenic, immunological and inflammatory markers as possible biomarkers. The relationship between each marker and efficacy (clinical benefit/progression) was tested using a logistic regression analysis. A receiver operating characteristics (ROC) analysis was used to see the relationships between tumor response and possible biomarkers, as previously described.[10] The Kaplan–Meier method estimates the probability of survival. The prognostic effect of each marker on PFS or OS was investigated with Cox's proportional-hazards regression model. In all analyses, a *P*-value of <0.05 were considered significant.

Results

This study group comprised 90 patients. The patient characteristics are summarized in Table 1. The median follow-up was 21 months (range 2–86 months). Among 90 patients treated with sunitinib, 56 (62.2%) patients received subsequent therapy. A total of 24 patients (42.8%) subsequently received everolimus; 15 (26.8%), 8 (14.3%) and 6 (8.9%) patients received axitinib, temsirolimus and sorafenib, respectively. The remaining patients received pazopanib (*n* = 1; 1.8%), interferon (*n* = 1; 1.8%) or sorafenib plus interferon (*n* = 1; 1.8%). During sunitinib treatment, 4 (4.6%) and 17 (19.6%) patients were assessed as complete response (CR) and partial response (PR), respectively. Twenty-four (27.6%) patients achieved stable disease (SD) for more than 6 months. Forty-two patients (48.3%) were assessed as PD or SD for less than 6 months. Three patients were considered not assessable for efficacy. The objective response rate (CR and PR) and clinical benefit rate (CR, PR and SD ≥ 6Mo) for sunitinib treatment were 24.2 and 51.8%, respectively. During subsequent treatment, 1 (1.8%), 4 (7.1%), 36 (64.3%) and 13 (23.2%) patients were assessed as CR, PR, SD and PD, respectively. Two patients were considered not assessable for efficacy of subsequent treatment. The estimated median PFS for sunitinib and subsequent therapy were 8.4 (95% CI 6.5–13.1) and 5.1 (95% CI 2.8–6.5) months, respectively (Fig. 1a). The estimated median OS was 31.1 months (95% CI 17.4–42.8), respectively (Fig. 1b).

Table 1. Patient characteristics

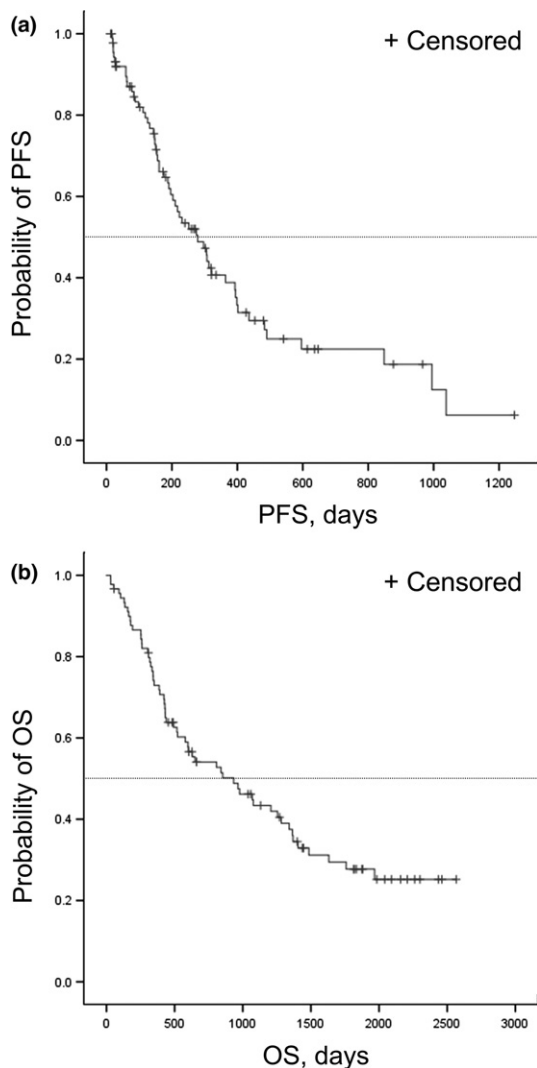| Characteristics	
Patients (number)	90
Age (year, median range)	65 (31–79)
Gender (number, male/female)	71/19
ECOG performance status (number)	
0	77
1	11
2	2
MSKCC risk group	
Favorable	21
Intermediate	69

(a)

(b)

Fig. 1. (a) Progression-free survival (PFS) in 90 mRCC patients treated with sunitinib. The median PFS was 9.3 months (95% CI 6.5–13.1). (b) Overall survival (OS) in 90 mRCC patients treated with sunitinib. The median OS was 31.1 months (95% CI 17.4–42.8).

Among the angiogenic, inflammatory and immunological markers analyzed, baseline levels of IL-6, IL-8, hs-CRP and MDSC were significantly lower in patients with clinical benefits when compared with its counterparts, respectively. However, no significant differences were found for the other markers tested (Table 2). The areas under the ROC curves for hs-CRP, IL-6, IL-8 and MDSC as predictive markers of sunitinib were 0.603, 0.612, 0.591 and 0.558, respectively (Fig. 2).

In univariate Cox regression model analysis using the continuous value for each marker, baseline IL-8, hs-CRP and %Th1 cells significantly correlated with PFS, respectively (Table 3). On-treatment hypertension ($P < 0.0001$, hazard ratio = 0.173, 95% confidence interval [CI] 0.091–0.337) was significantly correlated with better PFS. A multivariate analysis identified following baseline indices: IL-8 ($P = 0.0470$, unit risk = 1.011, 95% CI 1.000-1.022), hs-CRP ($P = 0.0075$, unit risk = 1.00009, 95% CI 1.00003–1.00016), and %Th1 cells ($P = 0.0329$, unit risk = 0.960, 95% CI 0.924–0.997), as independent indices to predict PFS for sunitinib treatment (Table 3). When baseline markers and on-treatment markers

Table 2. Association between each marker and efficacy of sunitinib using a logistic regression analysis

Markers	Clinical benefit	PD+SD<6Mo	P value
VEGF-C	2209 ± 1195	2048 ± 1185	0.4647
VEGF-R2	9618 ± 2285	8824 ± 2179	0.2059
VEGF-R3	48.5 ± 18.5	52.6 ± 18.9	0.2898
Hepcidine	112 ± 84	121 ± 67	0.2467
bFGF	11.1 ± 3.9	11.4 ± 3.2	0.0695
hs-CRP	14 181 ± 31 030	35 499 ± 40 256	0.0023*
IL-6	13.8 ± 41.2	17.4 ± 22.8	0.0012*
IL-8	8.5 ± 10.1	23.6 ± 38.4	0.0039*
%Th1 T cells	24.6 ± 9.5	21.5 ± 9.3	0.0593
%Th2 T cells	3.7 ± 1.8	4.2 ± 2.4	0.3914
Th1/Th2 ratio	8.9 ± 7.8	7.5 ± 7.3	0.1628
MDSC	5.8 ± 5.1	11.6 ± 12.5	0.0260*
Treg	2.9 ± 1.2	2.9 ± 1.0	0.7602

*Statistically significant.

were analyzed together, on-treatment hypertension ($P < 0.0001$, hazard ratio = 0.161, 95% CI 0.084–0.319) and %Th1 cells ($P = 0.0081$, unit risk = 0.954, 95% CI 0.920–0.988) independently predicted PFS for sunitinib treatment.

In univariate Cox regression model analysis, baseline sVEGFR-3, hs-CRP and MDSC significantly correlated with OS, respectively (Table 4). On-treatment hypertension, longer sunitinib treatment period and longer subsequent treatment period predicted better OS, respectively (Table 4). A multivariate analysis identified MDSC ($P < 0.0001$, unit risk = 1.062, 95% CI 1.033–1.087) independently predicted OS (Table 4). When baseline markers and on-treatment markers were analyzed together, MDSC ($P = 0.0024$, unit risk = 1.045, 95% CI 1.019–1.075) and longer sunitinib treatment period ($P < 0.0001$, unit risk =0 .876, 95% CI 0.820–0.927) independently predicted OS.

Discussion

With increases in the number of treatment options currently available for mRCC, researchers and clinicians now face the question of how to maximize patient benefits based on the drugs available. Although treatment guidelines that recommend appropriate drugs according to evidence from the findings of clinical trials have been established (http://www.nccn.org/),[11] biomarkers to recommend the most appropriate agent for each patient are warranted to maximize efficacy and to avoid unnecessary toxicities. In the present study, patients with low or intermediate risk features were evaluated for multiple baseline serum factors as well as immunological markers with the aim of identifying an informative and potentially prognostic patient profile. The present results strongly indicate baseline hs-CRP and IL-8 could predict poor PFS. We also demonstrated that some immunological markers, like MDSC or %Th1 cells, possess the ability to predict prognoses and responses.

C reactive protein, which is an acute phase protein, is mainly produced by hepatocytes and has been widely used as a marker of systemic inflammation.[12] Elevation in CRP is often observed in advanced cancers. Furthermore, high pretreatment levels of CRP often predict poor responses to systemic treatment in advanced cancer patients. This implies that an underlying inflammatory mechanism would play some role in forming resistance to systemic therapy.[13,14] The reasons for CRP elevations in cancer patients are not clearly
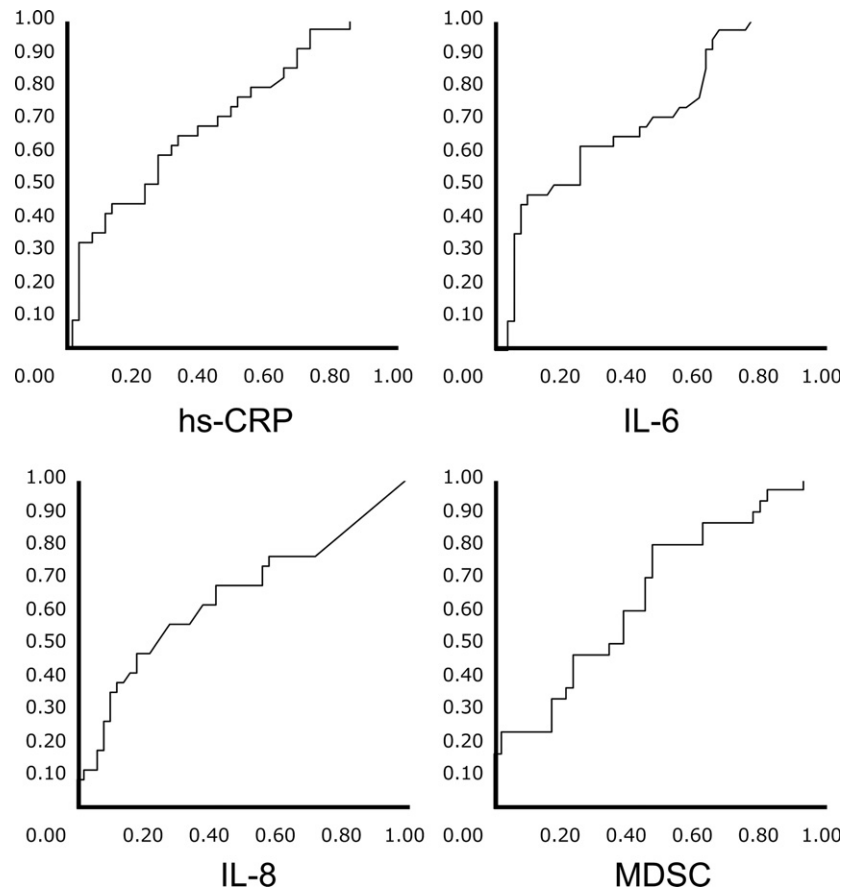
Fig. 2. Receiver operating characteristics (ROC) curves for prediction of clinical benefit in 90 mRCC patients treated with sunitinib. Sensitivity and specificity were plotted for all possible cut-off values. The area under the curve is shown (1.000 = a perfect test). The area under the curves (AUC) were 0.603, 0.612, 0.591 and 0.558, for hs-CRP, IL-6, IL-8 and MDSC, respectively.

Table 3. Univariate and multivariate Cox regression analyses of potential baseline factors affecting progression-free survival

Markers (continuous)	Univariate	Multivariate		
	P value	P value	Unit risk	95% CI
VEGF-C	0.3289			
VEGF-R2	0.5206			
VEGF-R3	0.1754			
Hepcidine	0.1321			
bFGF	0.5446			
hs-CRP	0.0125*	0.0075	1.000	1.000–1.000
IL-6	0.8825			
IL-8	0.0388*	0.0470	1.011	1.000–1.022
%Th1 T cells	0.0172*	0.0329	0.960	0.924–0.997
%Th2 T cells	0.2743			
Th1/Th2 ratio	0.1248			
MDSC	0.2255			
Treg	0.9051			

*Statistically significant. CI, confidence interval.

Table 4. Univariate and multivariate Cox regression analyses of potential baseline factors affecting overall survival

Markers (continuous)	Univariate	Multivariate		
	P value	P value	Unit risk	95% CI
VEGF-C	0.0506			
VEGF-R2	0.2055			
VEGF-R3	0.0464*			
Hepcidine	0.2219			
bFGF	0.5446			
hs-CRP	0.0011 *			
IL-6	0.7330			
IL-8	0.0964			
%Th1 T cells	0.1800			
%Th2 T cells	0.1694			
Th1/Th2 ratio	0.2107			
MDSC	<0.0001 *	<0.0001 *	1.062	1.033–1.087
Treg	0.9051			

*Statistically significant. CI, confidence interval.

understood; however, several possible mechanisms have been suggested to explain the association between cancer and increased CRP levels.[15] One possible explanation might be that tumor growth causes inflammation around the tumor, thereby elevating the level of CRP.[16,17] Alternatively, chronic inflammation, for which CRP is useful in monitoring, may cause cancer progression. In addition, inflammatory cytokines may facilitate cancer progression by promoting cancer cell growth and proliferation. The whole family of IL-6 type

cytokine is known to induce production of acute phase proteins, including CRP, in hepatocytes. Because IL-6 acts as an intracrine growth factor in RCC, an increase in serum CRP might be induced by IL-6 produced from RCC. Among the large number of inflammatory markers, hs-CRP, which accurately measures lower levels of serum CRP than traditional CRP assays, might be the most extensively studied biomarker of low grade inflammation, especially in cardiovascular diseases.[18] More recently, hs-CRP elevations have been

associated with cancer progression and an increased risk of cancer mortality.[19] Indeed, several studies have tried to reveal the correlation between chronic low grade systemic inflammation and cancer. The present study investigated the correlation of baseline hs-CRP, which can detect low-grade inflammation, with prognosis and found that this inflammatory marker has prognostic value in mRCC patients treated with sunitinib. Moreover, multivariate Cox analysis revealed that hs-CRP and IL-8 were independent indices to predict shorter PFS, which suggests that, besides inflammation-based resistance reflected by hs-CRP, IL-8 signaling system would independently contribute to formation of resistance to sunitinib.

IL-8, also known as CXCL8, is a potent pro-inflammatory cytokine. Besides its central role in inflammation, IL-8 induces angiogenesis by directly interacting with endothelial cells.[20,21] IL-8 is also known to be an autocrine growth factor for cancer cells, including RCC.[22,23] IL-8 is one of the most frequently detected cytokines in RCC. Thus, IL-8 produced from RCC cells might play a crucial role in developing clinical features of RCC, such as positive inflammatory reactions, hypervascularity and resistance to immunotherapy.[24] In addition, a previous study reported that neutralizing antibodies to IL-8 increased the anti-angiogenic effect of sunitinib in RCC xenograft models, which implies roles of IL-8 in alternative pathways utilized by cancers to escape from sunitinib.[25] Thus, it was not surprising when increased baseline serum IL-8 level predicted resistance to sunitinib. Several relationships have been detected between polymorphisms in IL-8 genes and the anti-angiogenic therapeutic outcomes of mRCC patients. A relationship has also been reported between the variant genotype of IL-8 and reduced OS in mRCC patients treated with pazopanib or sunitinib.[26]

Some immunological markers may predict responses to sunitinib because RCC are known to have immunological features. Cytokine therapy was the standard of care before the advent of targeted drugs and a novel class of immunotherapeutic agents called checkpoint inhibitors is currently being tested for their application to the treatment of mRCC. In the present study we demonstrated that baseline MDSC, which can suppress T cell and natural killer cell function, could predict poor OS in mRCC patients; however, this was not surprising because previous studies proposed that MDSC would play some role in developing resistance to sunitinib treatment.[27] We also demonstrated potential relationships between the baseline proportions of T cell subsets and the efficacy of sunitinib in mRCC patients. Th cells are classified functionally into two main types, Th1 and Th2 cells, which are antagonistic to each other. Th1 cells can secrete interferon-gamma, IL-2, tumor necrosis factor-alpha, to promote macrophages toward an M1 phenotype, further promoting cell-mediated immunity through cytotoxic T lymphocyte activation as well as Th1 responses.[28] Th2 cells mainly secrete IL-4, IL-5, IL-9, IL-10 and IL-13, and are involved in humoral immunity.[28] In numerous cancers, including RCC, activated Th1 has been thought to enhance anti-tumor immune responses, in contrast to the inhibitory mechanisms of the Th2 system on innate immunity.[28,29] Therefore, patients with elevated level of Th1 cells as well as Th1/Th2 ratio exhibit productive anti-cancer immune responses. In those cases, sunitinib might affect cancer cells with innate anticancer immunity synergistically. The combination of antiangiogenic therapy with immunotherapy based on an immune checkpoint blockade has been proposed as a potential new therapeutic approach for mRCC patients.[30] Because our study demonstrated that some immunological markers possess the ability to predict the efficacy of sunitinib before treatment, these immunological markers may assist in the selection of antiangiogenic drugs in combination with immune checkpoint inhibitors before treatment.

Previous studies have demonstrated that on-treatment hypertension, thrombocytopenia and relative dose intensity are useful biomarkers to predict sunitinib efficacy.[31,32] Indeed, in our cohort, on-treatment hypertension and longer sunitinib treatment period predicted better survival. The prognostic value of those on-treatment markers seems promising; however, unfortunately, on-treatment markers have little impact on drug choice in the first-line setting. Therefore, we further evaluated baseline markers as prognostic indices of sunitinib treatment and found that some baseline markers have the potential to predict survival in patients with mRCC.

In summary, our results show that some angiogenic, inflammatory and immunological markers at baseline may have the potential to predict treatment response to first-line sunitinib for mRCC. The predictive ability of each marker is considerable, and these results have important implications for optimizing the care of mRCC patients.

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