

Tumour cell expression of interleukin 6 receptor α is associated with response rates in patients treated with sunitinib for metastatic clear cell renal cell carcinoma

Martin Pilskog^{1,2}, Leif Bostad³, Reidunn J Edelman¹, Lars A Akslen^{1,3}, Christian Beisland^{4,5} and Oddbjørn Straume^{1,2*}

¹ Centre for Cancer Biomarkers CCBIO, University of Bergen, Bergen, Norway

² Department of Oncology, Haukeland University Hospital, Bergen, Norway

³ Department of Pathology, Haukeland University Hospital, Bergen, Norway

⁴ Department of Urology, Haukeland University Hospital, Bergen, Norway

⁵ Department of Clinical Medicine, University of Bergen, Bergen, Norway

*Correspondence to: Oddbjørn Straume, Department of Oncology, Haukeland University Hospital, 5021 Bergen, Norway. E-mail: Oddbjorn.straume@helse-bergen.no

Abstract

Clear cell renal cell carcinoma (ccRCC) is the most common type of renal cell carcinoma, and anti-angiogenic treatment is currently first line therapy for metastatic ccRCC (mccRCC). Response rates and duration of response show considerable variation, and adverse events have a major influence on patient quality of life. The need for predictive biomarkers to select responders to receptor tyrosine kinase inhibitors upfront is urgent. We investigated the predictive value of immunohistochemical biomarkers associated with angiogenesis and systemic inflammation in mccRCC. Forty-six patients with metastatic or non-resectable ccRCC treated with sunitinib were included. Metastatic and/or primary tumour tissue was stained by immunohistochemistry for selected markers related to angiogenesis [vascular endothelial growth factor A (VEGF-A), VEGF receptor 2 (VEGFR2), platelet-derived growth factor receptor β (PDGFR β), and heat shock protein 27 (HSP27)] and immune responses [Interleukin 6 receptor α (IL6R α), interleukin-6 (IL6), and jagged1 (JAG1)]. The predictive potential of the candidate markers was assessed by correlations with response rates (RECIST). In addition, progression free survival (PFS) and overall survival (OS) were analysed. Low tumour cell expression of IL6R α was significantly associated with improved response to sunitinib (Fisher's exact test, $p = 0.03$), but not with PFS or OS. Median/high expression of IL6R α showed significant association with median/high expression of VEGF-A and HSP27. Furthermore, low expression of IL6 was significantly associated with improved PFS, but not OS or response rates. High expression of IL6 was significantly associated with high expression of JAG1, VEGF-A, VEGFR2, and PDGFR β . Loss of tumour cell expression of IL6R α in mccRCC patients treated with sunitinib predicts improved treatment response, and might represent a candidate predictive marker.

Keywords: clear cell renal cell carcinoma; sunitinib; interleukin 6; interleukin 6 receptor α ; response rates; biomarker; anti-angiogenesis; inflammation; immunohistochemistry; treatment efficacy

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Introduction

Clear cell renal cell carcinoma (ccRCC) is known to be an immunogenic cancer [1]. Recently, nivolumab, an immune checkpoint inhibitor, was shown to improve overall survival (OS) in second line treatment of metastatic disease [2]. Immunotherapy, such as interferon and interleukin-2 therapy, was the only

treatment choice up until 2007 when anti-angiogenic receptor tyrosine kinase inhibitors (rTKI) showed superior efficacy [3]. rTKIs are currently first line treatment options for metastatic disease. Still, response rates and duration of response show considerable variation among patients, and adverse events have a major influence on quality of life [4]. Despite scientific efforts to identify clinically useful

predictive markers of response to anti-angiogenic treatment, no such indicators have currently been successful. Among many, the focus has been on angiogenesis markers [5,6], markers of hypoxia [5–7], clinical markers [8–10], *VHL* mutation status [11], and single nucleotide polymorphisms [12,13], but immune response-related markers are less studied.

Vascular endothelial growth factor (VEGF) is the most important mediator of tumour-associated angiogenesis in renal cell carcinoma (RCC), and VEGF receptor 2 (VEGFR2) is the main target of sunitinib. Some reports suggest a role of systemic inflammation in development and progression of RCC [1,14,15]. Along with a stimulating effect on tumour-associated angiogenesis, VEGF also plays an important role in the local immune response during wound healing as well as in tumours by inducing accumulation of immature dendritic cells, myeloid-derived suppressor cells, regulatory T cells, and VEGF inhibits the migration of T lymphocytes to the tumour [16].

In a recent study, we investigated the role of systemic inflammation in metastatic ccRCC (mccRCC) treated with sunitinib [4]. We found a significant correlation between low serum C-reactive protein (CRP) and objective response (OR). CRP is a relevant biomarker for systemic inflammation [17]. Tissue and serum levels of interleukin-6 (IL6) are elevated in RCC, and high levels of IL6 are associated with elevated CRP in RCC patients [18,19]. IL6 has a role in inflammation, infection responses, and the regulation of metabolic, regenerative, and neural processes [20–23]. In RCC, IL6 is secreted when cells are exposed to hypoxia, and enhanced levels of IL6 result in RCC cell invasion [23,24]. IL6 has also been shown to be closely related to HIF-1 α as well as increased VEGF activity [25]. IL6 signals in cells via classic (membrane-bound) and trans-signalling (soluble) pathways [26,27]. Interleukin 6 receptor α (IL6R α) binds to the gp130 protein receptor to transduce the signal. Membrane-bound IL6R α is found on hepatocytes and different leukocytes [28]. In trans-signalling, soluble IL6 binds to soluble IL6R and the complex binds to cells expressing gp130 [29]. Takenawa *et al* have previously shown the presence of IL6R α on RCC cells [18] and Costes *et al* reported a prognostic value of IL6 and IL6R in primary RCC [30].

Another important signalling system and regulator of tumour angiogenesis, stem cell self-renewal, epithelial cell polarity, cell division, and apoptosis is the Notch signalling pathway [31–34]. Thus, IL6 might trigger a potential autocrine or paracrine Notch-3/

jagged1 (JAG1) loop to boost stem/progenitor self-renewal in the mammary gland [35].

Here, we enrolled patients with mccRCC treated with the VEGFR inhibitor sunitinib in a prospective clinical study, and analysed an expanded panel of candidate predictive biomarkers related to VEGF associated angiogenesis, inflammation, and tumour immune responses.

Materials and methods

Patients and treatment

Forty-six patients with mccRCC were enrolled in an open-label, single-arm phase II study at Haukeland University Hospital, Norway. Between 2007 and 2015, mccRCC patients with radiologically confirmed progressive disease were treated with sunitinib 50 mg/day on schedule 4 weeks on/two weeks off until disease progression, significant toxicity, or consent withdrawal. Study design, inclusion criteria, and clinical response data were reported earlier [4]. In summary, we observed 1 complete response (CR), 7 partial responses (PR), and 18 patients with stable disease (SD) ≥ 6 months. Twelve patients showed progressive disease (PD). Eight patients stopped treatment before week 12 and were recorded as non-evaluable for response rates and progression free survival (PFS). Thus, 38 patients were available for response evaluation. Treatment response was recorded according to RECIST 1.1 and the frequency of OR (CR + PR) was used as primary endpoint. The evaluation of the prognostic value of the biomarkers concerning PFS and OS were secondary endpoints. Clinical information is provided in Table 1.

Ethics

The study followed the ethical principles of the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice. The protocol was approved by the Regional Ethics Committee (REK number 080/07 and REK number 78/05) and the Norwegian Medicines Agency. All participating patients provided signed informed consent before enrolment.

Tissue samples

Tumour tissue was available in 45/46 (97.8%) patients in total. The most recent biopsy, the metastatic lesion ($n = 29$), or the non-resectable primary tumour diagnosed closest to the date of clinical trial inclusion ($n = 12$), was selected for further analysis if

Table 1. Baseline patients characteristics

	Study cohort (<i>n</i> = 46) <i>n</i> (%)
Sex	
Male	29 (63.0)
Female	17 (37.0)
Age, years	
Median	63.1
Range	41.1–84.0
IMDC* risk score	
Good	7 (15.2)
Intermediate	16 (34.8)
Poor	21 (45.7)
Missing	2 (4.3)
WHO** performance status	
0	30 (65.2)
1	16 (34.8)
2	0 (0.0)
Number of disease sites	
1	10 (21.7)
2	11 (23.9)
≥3	25 (54.3)

*International Metastatic Renal Cell Carcinoma Database Consortium.

**World Health Organisation.

several lesions were available. In addition, protein expression of the candidate markers was analysed in primary tumours alone (*n* = 41). All results in this paper refer to the most recent biopsy unless otherwise specified. All metastases and primary ccRCCs were reclassified by an experienced pathologist based on haematoxylin and eosin-stained sections (LB).

Immunohistochemistry (IHC)

Tissue sections (4–5 µm) were stained with primary antibodies for interleukin-6 receptor α (IL6Rα), IL6, JAG1, vascular endothelial growth factor A (VEGF-A), VEGFR2, platelet-derived growth factor receptor β (PDGFRβ), and heat shock protein 27 (HSP27). Slides were deparaffinized in xylene and rehydrated followed by antigen retrieval in a microwave oven. Endogenous peroxidase and alkaline phosphatase were blocked before incubation with the primary antibody followed by incubation with the appropriate visualization kit. Details are provided in supplementary material, Table S1. For negative controls, primary antibodies were omitted or specific blocking peptides for HSP27 and VEGF-A were used. Tissues from different cancer types were used as positive controls. For JAG1, endothelial cells were used as positive internal control.

Evaluation of tissue staining results

All sections were screened at ×40 and ×100 total magnifications to map areas of cancer tissue and

normal tissue. Further, with high power magnification (×200 or ×400), staining intensity and the proportion of positive tumour cells were recorded using a semi-quantitative grading. Staining intensity was defined as absent (0), weak (1), moderate (2), or strong (3). The proportion was rated as ‘no positive tumour cells’ (0), ‘less than 10% positive tumour cells’ (1), ‘10–50% positive tumour cells’ (2), or ‘more than 50% positive tumour cells’ (3). The staining index (SI) is the product of intensity and proportion (range 0–9) [36]. SI was used to quantify cytoplasmic staining of IL6Rα, IL6, JAG1, VEGF-A, VEGFR2, PDGFRβ, and HSP27. Cases were categorized into groups (absent/low versus median/high protein expression) based on the SI distribution for each biomarker under investigation. Thus, cut-points were set to: IL6Rα low (SI = 1–3) versus median/high (SI = 4–9); IL6 absent/low (SI = 0–2) versus median/high (SI = 3–9); JAG1 absent/low (SI = 0–2) versus median/high (SI = 3–9); VEGF-A low (SI = 1–3) versus median/high (SI = 4–9); VEGFR2 absent/low (SI = 0–2) versus median/high (SI = 3–9); PDGFRβ absent/low (SI = 0–1) versus median/high (SI = 2–9); and HSP27 low (SI = 1–3) versus median/high (SI = 4–9). In addition, protein expression in tumour-associated endothelial cells was graded based on staining intensity (0–3) for VEGFR2 and PDGFRβ. The IHC protein expression was evaluated and discussed by two observers blinded with temporary number tags for response data.

Statistical analyses

Comparisons between categorical variables were performed by Fisher’s exact test. In the analyses of the IHC markers, we dichotomized the index score into absent/low versus median/high protein expression and tested the different groups against the frequency of OR in the patients. Logistic regression analysis was used to test the relative importance of predictive factors for sunitinib response. Sample size calculations (alpha 0.05/power 80%) indicated that 20 patients per group based on candidate marker expression were needed to detect a difference between 10 and 50% of patients having an OR to treatment with sunitinib. Thus, 46 patients were enrolled. Kaplan–Meier estimates were constructed for time-to-event endpoints such as PFS and OS, and log rank-test was applied for testing of differences between groups. Log-rank was applied for testing of differences between groups for PFS and OS. All *P* values are two-sided. Statistical investigations were performed using IBM SPSS Statistics version 24.

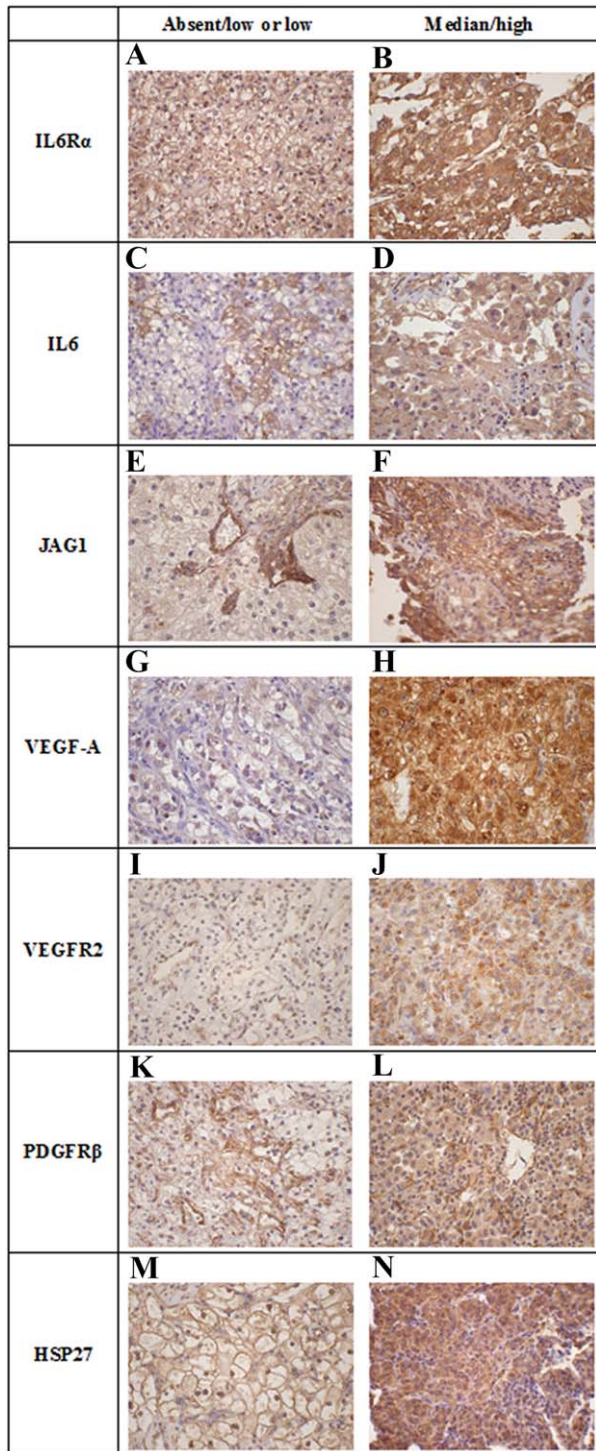


Figure 1. Expression of tumour biomarkers by IHC. Representative microscopic images for IL6R α (A,B), IL6 (C,D), JAG1 (E,F), VEGF-A (G,H), VEGFR2 (I,J), PDGFR β (K,L), and HSP27 (M,N) in tumour tissue. All pictures taken with x400 magnification; A, G, and M represent low tumour expression of the marker applied; C, E, G, I, K, and M represent absent/low tumour expression of the marker applied; and B, D, F, H, J, L, and N represent median/high tumour expression of the marker applied.

Results

Evaluation of IHC

IL6R α

Thirty-eight of 41 (92.7%) cases had significant tumour tissue for quantification of IL6R α . IL6R α was expressed in the cytoplasm and membrane in 38/38 (100%) (median SI = 6) (Figure 1A, B).

Low expression of IL6R α was significantly associated with OR (Fisher's exact test, $p = 0.03$) (Table 2). Sixty-six percent of the patients with response data available showed median/high expression of IL6R α in tumour cells, and only 10% of these patients responded to treatment with sunitinib, whereas 46% of patients with low expression responded (Table 2) (Figure 2). Logistic regression analysis was used to test the relative importance of the candidate predictive factors [International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) risk groups, baseline CRP, baseline European Organization for Research and Treatment of Cancer Quality of Life (EORTC QoL) symptom scale and IL6R α] for OR to sunitinib. Of these, IL6R α was the only significant predictive factor of OR in

Table 2. IHC biomarkers in relation to response

Variable	Best overall tumour response (RECIST ver. 1.1)		
	OR* <i>n</i> (%)	SD** + PD [†] <i>n</i> (%)	<i>P</i> value ^{††}
IL6R α			0.03
SI [†] = 1-3	5(46)	6(54)	
SI = 4-9	2(10)	19(90)	
IL6			0.39
SI = 0-2	5(31)	11(69)	
SI = 3-9	2(13)	13(87)	
JAG1			1.00
SI = 0-2	4(22)	14(78)	
SI = 3-9	3(23)	10(77)	
VEGF-A			1.00
SI = 1-3	2(25)	6(75)	
SI = 4-9	5(20)	20(80)	
VEGFR2			0.66
SI = 0-2	3(27)	8(73)	
SI = 3-9	4(18)	18(82)	
PDGFR β			1.00
SI = 0-1	3(23)	10(77)	
SI = 2-9	4(25)	12(75)	
HSP27			0.38
SI = 1-3	4(33)	8(67)	
SI = 4-9	3(15)	17(85)	

*Objective response (complete + partial response).

**Stable disease.

[†]Progressive disease.

^{††}Fisher's exact test.

^{††}Staining index. Statistically significant comparisons ($p < 0.05$) are shown in bold.

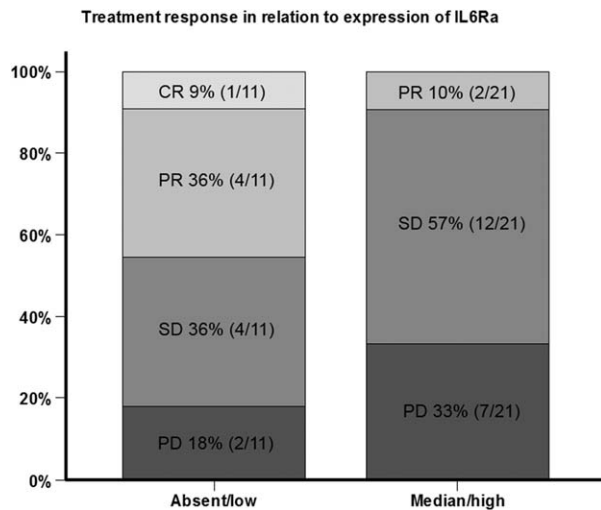


Figure 2. Treatment response in relation to expression of IL6R α . Histogram showing the difference between absent/low and median/high expression of IL6R α in tumour cells according to response. Ten percent of patients with median/high expression experienced an objective response to treatment with sunitinib compared with 46% of patients with absent/low expression.

the final model, with an odds ratio of 7.9 ($p = 0.03$). There was no statistically significant association between IL6R α and PFS or OS (Table 3). Median/

Table 3. Survival analyses according to biomarker expression

Variable	PFS*			OS**		
	Median	95% CI [†]	P value ^{††}	Median	95% CI	P value
IL6R α			0.21			0.22
SI [‡] = 1–3	17.0	12.5–21.4		41.3	0.0–93.0	
SI = 4–9	8.7	5.8–11.6		13.7	11.0–16.5	
IL6			0.04			0.20
SI = 0–2	17.0	8.7–25.2		18.0	0.0–51.9	
SI = 3–9	8.7	6.8–10.6		11.6	8.8–14.4	
JAG1			0.80			0.63
SI = 0–2	12.9	6.3–19.6		13.7	11.2–16.2	
SI = 3–9	16.5	0.0–35.5		19.7	7.6–31.9	
VEGF-A			0.19			0.27
SI = 1–3	5.3	–		48.2	9.9–86.5	
SI = 4–9	12.9	6.4–19.5		13.2	8.7–17.7	
VEGFR2			0.09			0.45
SI = 0–2	17.0	6.5–27.5		15.6	8.8–22.4	
SI = 3–9	9.1	5.9–12.2		12.1	5.9–18.3	
PDGFR β			0.72			0.29
SI = 0–1	10.8	3.7–17.9		13.7	11.0–16.5	
SI = 2–9	14.7	2.8–26.6		25.2	3.0–47.4	
HSP27			0.86			0.62
SI = 1–3	9.1	1.6–16.5		25.2	0.0–53.3	
SI = 4–9	12.9	4.0–21.9		13.9	11.1–16.7	

*Progression free survival.

**Overall survival.

[†]Confidence interval.

^{††}Log rank test.

[‡]Staining index. Statistically significant comparisons ($p < 0.05$) are shown in bold.

Table 4. Analyses of patient characteristics and IHC biomarkers in relation to IL6R α

Variable	Interleukin-6 receptor α		P value**
	SI* = 0–3 <i>n</i> (%)	SI = 4–9 <i>n</i> (%)	
Sex			0.20
Female	6(43)	8(57)	
Male	7(29)	17(71)	
Age			1.00
< median	7(35)	13(65)	
\geq median	6(33)	12(67)	
IMDC [†] risk			0.23
Good	4(67)	2(33)	
Intermediate	2(20)	8(80)	
Poor	5(33)	10(67)	
WHO ^{††} performance status – No. (%)			1.00
0	9(36)	16(64)	
1	4(31)	9(69)	
2	–	–	
Number of disease sites – No. (%)			0.43
1	4(44)	5(56)	
2	3(50)	3(50)	
≥ 3	6(26)	17(74)	
IL6			0.09
SI = 0–2	9(75)	3(25)	
SI = 3–9	11(44)	14(56)	
JAG1			0.08
SI = 0–2	10(77)	3(23)	
SI = 3–9	10(44)	13(56)	
VEGF-A			0.18
SI = 1–3	4(33)	8(67)	
SI = 4–9	3(12)	22(88)	
VEGFR2			1.00
SI = 0–2	5(42)	7(58)	
SI = 3–9	9(36)	16(64)	
PDGFR β			0.30
SI = 0–1	3(27)	8(73)	
SI = 2–9	11(48)	12(52)	
HSP27			0.01
SI = 1–3	8(67)	4(33)	
SI = 4–9	5(20)	20(80)	

*Staining index.

**Fisher's exact test.

[†]International Metastatic Renal Cell Carcinoma Database Consortium.

^{††}World Health Organisation. Statistically significant comparisons ($p < 0.05$) are shown in bold.

high expression of IL6R α was significantly associated with median/high expression of HSP27 (Fisher's exact test, $p = 0.01$) (Table 4). IL6R α was not significantly associated with serum CRP (s-CRP) or lactate dehydrogenase (s-LDH).

IL6

Thirty-eight of 41 (92.7%) cases had sufficient tumour tissue for quantification of IL6. Cytoplasmic IL6 was expressed in 36/38 (94.7%) (median SI = 2) (Figure 1C, D). IL6 was not significantly associated with OR (Table 2). Absent/low expression of IL6 was significantly associated with improved PFS

Table 5. Analyses of patient characteristics and IHC biomarkers in relation to IL6

Variable	Interleukin 6		P value**
	SI* = 0–2 n(%)	SI = 3–9 n(%)	
Sex			0.20
Female	10(67)	5(33)	
Male	10(44)	13(56)	
Age			0.12
< median	8(40)	12(60)	
≥ median	12(67)	6(33)	
IMDC [†] risk			0.37
Good	5(83)	1(17)	
Intermediate	4(46)	6(54)	
Poor	9(60)	6(40)	
WHO ^{††} performance status – No. (%)			1.00
0	13(54)	11(46)	
1	7(50)	7(50)	
2	–	–	
Number of disease sites – No. (%)			0.19
1	7(78)	2(22)	
2	4(57)	3(43)	
≥3	9(41)	13(59)	
IL6R α			0.09
SI = 1–3	9(75)	3(25)	
SI = 4–9	11(44)	14(56)	
JAG1			0.04
SI = 0–2	14(74)	5(26)	
SI = 3–9	6(35)	11(65)	
VEGF-A			0.02
SI = 1–3	8(89)	1(11)	
SI = 4–9	11(39)	17(61)	
VEGFR2			0.05
SI = 0–2	11(73)	4(27)	
SI = 3–9	8(36)	14(64)	
PDGFR β			0.17
SI = 0–1	10(71)	4(29)	
SI = 2–9	9(45)	11(55)	
HSP27			0.18
SI = 1–3	9(69)	4(31)	
SI = 4–9	11(44)	14(56)	

*Staining index.

**Fisher's exact test.

[†]International Metastatic Renal Cell Carcinoma Database Consortium.

^{††}World Health Organisation. Statistically significant comparisons ($p < 0.05$) are shown in bold.

(median 17.0 versus 8.7 months, log rank $p = 0.04$) (Table 3). There was a significant association between absent/low expression of IL6 and absent/low expression of JAG1 (Fisher's exact test, $p = 0.04$), low expression of VEGF-A (Fisher's exact test, $p = 0.02$), and absent/low expression of VEGFR-2 (Fisher's exact test, $p = 0.05$) (Table 5). No statistically significant correlation was present between IL6 and IL6R α (Table 4), s-CRP, or S-LDH.

JAG1

Thirty-eight of 41 (92.7%) cases had significant tumour tissue for quantification of JAG1. JAG1 was

expressed in the cytoplasm and membrane in 28/38 (73.7%) (median SI = 2) (Figure 1E, F). There was no significant association between JAG1 expression and OR, PFS, or OS (Table 2 and 3). Absent/low expression of JAG1 was significantly associated with low expression of VEGF-A (Fisher's exact test, $p = 0.01$), whereas median/high expression of JAG1 tended to be associated with median/high expression of IL6R α (Fisher's exact test, $p = 0.08$) (Table 5).

VEGF-A

Thirty-nine of 41 (95.1%) cases had significant tumour tissue for quantification of VEGF-A. VEGF-A was expressed in the cytoplasm of tumour cells in all patients (median SI = 6) (Figure 1G, H). The expression of VEGF-A was not significantly associated with OR, PFS, or OS (Tables 2 and 3).

VEGFR2

Thirty-nine of 41 (95.1%) cases had significant tumour tissue for quantification of VEGFR2. VEGFR2 was expressed in the cytoplasm and membrane in 31/39 (79.5%) (median SI = 3) (Figure 1I, J). No significant association between VEGFR2 expression and OR was present (Table 2). Cytoplasmic VEGFR2 expression was not significantly associated with OS or PFS (Table 3).

PDGFR β

Thirty-four of 41 (82.9%) cases had significant tumour tissue for quantification of PDGFR β . PDGFR β was expressed in the cytoplasm and membrane in 21/34 (61.8%) (median SI = 2) (Figure 1K, L). PDGFR β expression was not significantly associated with OR, PFS or OS (Tables 2 and 3).

Hsp27

Thirty-nine of 41 (95.1%) cases had significant tumour tissue for quantification of HSP27. HSP27 was expressed in the cytoplasm of tumour cells in all patients (median SI = 6) (Figure 1M, N). HSP27 expression was not significantly associated with OR, OS, or PFS (Tables 2 and 3).

Discussion

Ever since rTKI was introduced as first line treatment for mRCC, an intensive search for predictive markers has been performed to optimize treatment. In a recent paper, we found evidence for a predictive role for CRP in sunitinib treatment of RCC suggesting a potential role for markers of anti-tumour immune responses [4]. Lymphocytic infiltration in RCC has

been shown to be associated with poor survival [37] and IL6 might be used as a surrogate marker of host immunity in patients with RCC [38]. Tissue and serum levels of IL6 are elevated in RCC, and high levels of IL6 have been associated with elevated CRP in RCC patients [18,19]. Dysregulation of the cytokine IL6 and its receptor is involved in the pathogenesis of several diseases, such as autoimmune conditions and cancer [39]. In classic signalling, IL6 binds to the complex of IL6R α and gp130 to induce intra-cellular signals [29,40]. Almost all cells in the body express gp130 [28], but only some have the IL6R α subunit to bind IL6 [39].

Here, we investigated the expression of IL6R α in RCC tumour cells and found that expression of IL6R α may predict response to rTKI treatment. In our study, IL6R α was expressed in all cases, and low expression of IL6R α was significantly associated with OR to sunitinib treatment. Only 10% of patients showing increased expression of IL6R α responded, suggesting that high IL6R α expression might represent an important mechanism of resistance to anti VEGF therapy in ccRCC. The strong association between IL6R α expression and treatment response, as well as the lack of a significant association with PFS and OS, suggest that IL6R α expression adds more predictive than prognostic information in patients with mcrRCC treated with sunitinib. Costes *et al* found a significant association between IL6R expression and OS in patients with primary RCC tumours. In their study, all patients underwent nephrectomy and six out of 38 patients had metastatic disease [30]. Eighteen percent of the 38 patients and 83% of the 6 patients with synchronous metastatic disease showed positive expression of IL6R α . In our cohort of patients with primary inoperable or metastatic disease, 100% of the patients showed positive IL6R α expression.

Regarding the IL6 ligand, absent or low expression was associated with disease outcome in the survival analyses of PFS. The group with low expression of IL6 had almost a doubling of PFS compared to median/high expression. In line with previous reports [18,19], this suggests that IL6 expression has prognostic value independent of the treatment given.

Elevated serum IL6 has been associated with poor survival in RCC [19,30,41]. Tumour cells produce IL6 in response to cellular stress such as hypoxia, and enhanced levels of IL6 are associated with increased tumour cell invasion [23,24]. Kwon *et al* found elevated IL6 to have a stimulating effect on endothelial cells, and this may be a reason for resistance to anti-VEGF therapy [42]. As a response to cellular stress, IL6 activation of the transcription factor STAT3 drives angiogenesis by inducing

expression of VEGF and fibroblast growth factor by tumour cells, and thereby supports vascularization required for tumour growth and metastasis [43,44]. Fu *et al* found that IL6 and IL6R α co-expression might be an independent early-stage immunological prognostic factor for patients with organ-confined ccRCC [45]. Tran *et al* showed a significant increase in PFS in patients treated with another rTKI (pazopanib) versus placebo, when analysing patients with high serum IL6 [46]. Our results are in support of previous reports indicating that high levels of inflammation-associated cytokines are detrimental for the outcome of sunitinib treatment [21].

When correlating IL6R α to the other biomarkers under investigation, we found that median/high expression of IL6R α was significantly associated with median/high expression of HSP27. Both IL6 and HSP27 signalling constitute cellular stress responses and increase the level of VEGF through activation of STAT3 [44,47]. Schuster *et al* found that high HSP27 expression in melanoma metastases predicts response to anti-VEGF treatment [48]. In the present study, we did not find an association between HSP27 expression and treatment response. Blay *et al* previously showed that a higher IL6 level correlated with increased concentration of CRP [49]. In our study, IL6 was not significantly associated with CRP.

JAG1 is one of five Notch ligands. The Notch signalling pathway is a regulator of tumour angiogenesis, stem cell self-renewal, cell fate determination, epithelial cell polarity/adhesion, cell division, and apoptosis [31–34]. In mcrRCC, high JAG1 was associated with poor prognosis [50]. In aggressive breast cancer cells, Sansone *et al* found that IL6 could stimulate Notch-3-dependent upregulation of JAG1 in an autocrine manner in response to hypoxic conditions [35]. In our present cohort, the expression of JAG1 was not related to OR, but absent/low expression of IL6 was significantly associated to absent/low expression of JAG1. These JAG1 results may support a possible interaction of JAG1, Notch, and IL6 [35].

Moreover, absent/low expression of IL6 was shown to be significantly associated with low expression of VEGF-A and absent/low expression of VEGFR2, further supporting an important role of IL6 signalling the regulation of angiogenesis in mRCC.

Along with a stimulating effect on tumour-associated angiogenesis, VEGF-A also plays an important role in the local immune response during wound healing as well as in tumours by inducing accumulation of immature dendritic cells, myeloid-derived suppressor cells, regulatory T cells, and inhibiting the migration of T lymphocytes to the tumour [16]. Whereas VEGF-A was expressed in all

tumours, we found no association between the level of expression and response, in line with other studies [5,6]. VEGF-A signals through VEGFR2 on endothelial cells to activate angiogenesis [51]. Dornbush *et al* found an association between high expression of VEGFR2 and good treatment response [6], and Terakawa *et al* also found high expression of VEGFR2 to be beneficial to sunitinib treatment [52]. In the last paper, the majority of patients were in the good prognostic group whereas in ours, the majority were in the poor prognostic group [4].

PDGF receptors are key regulators of mesenchymal cells of the tumour microenvironment in several malignancies [53]. In cancers, an association between high stromal PDGFR β expression or signalling and poor prognosis is reported [53]. Still, we did not find an association with response in our present data.

In addition to the lack of a control group, our study has some weaknesses. First, the number of patients included is low and thereby the study lacks the statistical power to detect minor differences in response rates between groups based on the biomarkers under investigation. Thus, our findings should be validated in an independent and larger cohort of patients.

Second, the reproducibility of the quantification of protein expression used in this study also needs to be validated in a separate patient cohort. Still, our data suggest that both angiogenesis and tumour immune responses play important roles in anti-VEGF therapy.

Whereas expression levels of the IL6 ligand in tumour cells provided significant prognostic information, reduced expression of its receptor IL6R α was significantly associated with response to sunitinib, thereby suggesting that upregulation of IL6R α might represent an important mechanism of resistance. Expression of IL6R α might be a potential predictive biomarker to guide treatment of patients with mcrRCC.

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Author contributions statement

MP conceived the study, carried out experiments, evaluated the experiments, and analysed data. LB

investigated the material upon experiments. RJE contributed with ideas. LAA contributed in writing the paper. CB contributed in writing the paper. OS conceived the study, evaluated the experiments, and analysed data. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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SUPPLEMENTARY MATERIAL ONLINE

Table S1. Details of antibodies and protocols used for immunohistochemistry