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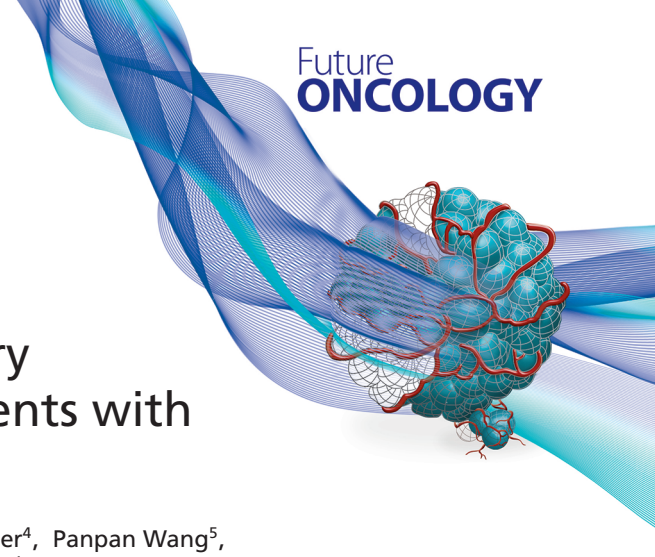

Angiogenic and immunomodulatory biomarkers in axitinib-treated patients with advanced renal cell carcinoma

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Aim: Immunomodulatory mechanisms contributing to angiogenic inhibition in renal tumors are not well characterized. We report associations between efficacy and tumor-associated immune cells and mRNA/miRNA expression in patients from AXIS. **Materials & methods:** Immunohistochemistry (n = 52) and mRNA/miRNA expression analyses (n = 72) were performed on tumor samples. **Results:** In axitinib-treated patients, higher *CXCR4* and *TLR3* expression, respectively, was associated with longer progression-free survival (hazard ratio [95% CI]: 0.3 [0.1–0.8] and 0.4 [0.2–0.9]) and showed interaction with treatment ($p = 0.029$ and $p < 0.001$); lower *CCR7* expression was associated with objective response (odds ratio: 0.1 [95% CI: 0.01–1.0]) and longer overall survival (hazard ratio: 3.9 [95% CI: 1.4–10.3]). **Conclusion:** *CCR7*, *CXCR4* and *TLR3* expression levels may be prognostic/predictive of clinical benefit with axitinib.

Clinical trial identifier: ClinicalTrials.gov NCT00678392.

First draft submitted: 13 March 2020; Accepted for publication: 14 April 2020; Published online: 4 May 2020

Keywords: axitinib • biomarker • renal cell carcinoma • sorafenib • survival • tumor response

Axitinib is a potent and selective tyrosine kinase inhibitor (TKI) of VEGFR 1–3 [1]. VEGFRs are implicated in pathologic angiogenesis, tumor growth and metastatic progression of cancer. Axitinib has been shown to potently inhibit VEGF-mediated endothelial cell proliferation and survival [1].

Axitinib is approved in many countries across the globe for second-line treatment of advanced renal cell carcinoma (RCC) [2]. In the AXIS trial, median progression-free survival (PFS) was significantly longer in axitinib- versus sorafenib-treated patients (hazard ratio [HR]: 0.67, 95% CI: 0.54–0.81; $p < 0.0001$) [3]. Recently, the combinations of axitinib plus avelumab or axitinib plus pembrolizumab were approved by the US FDA and the European Commission for first-line treatment of advanced RCC [4,5].

Significant progress has been made in the identification of prognostic biomarkers in patients with metastatic RCC (mRCC), but no predictive markers of efficacy have been identified for targeted agents such as axitinib in this population [6]. Recent studies focused on evaluating expression levels and signatures of potential genes mRNA and miRNA that might help identify patients with mRCC who are most likely to benefit from antiangiogenic therapy [7,8].

Unsupervised analysis of gene expression data identified four robust clear-cell RCC (ccRCC) subtypes (ccrcc1–ccrcc4) that were associated with different responses to sunitinib [9]. These ccrcc1–4 subtypes were related to previously identified molecular classifications [10,11], which may extend to correlation of response to other VEGFR TKIs and/or immunomodulatory drugs based on molecular typing of the tumors [9]. Other gene expression analyses identified prognostic signatures composed of targets/pathways that regulate angiogenesis and immune response in mRCC [8,12].

Potential miRNA signatures for patient stratification were also identified [13,14]. miR-99b-5p was identified by comprehensive miRNA profiling and revealed a statistically significant correlation with clinical outcome; however, this failed to be confirmed in a subsequent analysis utilizing a more quantitative approach [7].

Using data from the AXIS trial, the objectives of these *post hoc* analyses were to assess potential associations between tumor-infiltrating lymphocytes (TIL; $CD3^+$ cells) and tumor-associated macrophages (TAM; $CD68^+$ cells) and clinical efficacy in axitinib-treated patients with mRCC; and explore mRNA expression levels for genes and potential signatures associated with angiogenesis and tumor immune infiltration and miRNAs that have been previously shown to indicate sensitivity/resistance to VEGFR TKIs in mRCC tumor tissue [7,13,14] and their association with clinical outcomes.

Materials & methods

Patients & study design

Details on AXIS trial design and eligibility criteria were previously reported [3]. In brief, AXIS was a two-arm, randomized, open-label, multicenter Phase III study of axitinib versus sorafenib in patients aged ≥18 years with clear-cell mRCC who progressed after one prior first-line regimen containing sunitinib, bevacizumab plus interferon-alfa, temsirolimus or cytokines (ClinicalTrials.gov identifier: NCT00678392).

Patients were stratified by Eastern Cooperative Oncology Group performance status (ECOG PS) and type of prior therapy and randomized (1:1) to receive axitinib (5 mg twice daily) or sorafenib (400 mg twice daily). The primary end point was PFS and secondary end points included objective response rate and overall survival (OS).

Tumor tissue samples

Archival anonymized, formalin-fixed, paraffin-embedded tumor tissue samples of patients enrolled in the AXIS trial were collected as part of an optional additional research component of the study and those with adequate informed consent were used for biomarker analysis. Only a small subset of tumor samples from the overall study population was available for analysis. Tumor tissue from axitinib-treated patients analyzed for mRNA and miRNA expression were also analyzed by immunohistochemistry (IHC). Analyses were conducted in a blinded fashion with respect to clinical response and demographic information.

Immunohistochemistry

IHC assays for CD3 (mouse clone LN10; Leica Microsystems Inc, IL, USA) and CD68 (mouse clone KP1; Dako, CA, USA) were designed and validated as a laboratory-developed test using Clinical Laboratory Improvement Amendment (CLIA) guidelines. Pathology review of the slides generated from the tumor blocks was conducted according to Good Laboratory Practice in a CLIA and College of American Pathologists-certified facility (Mosaic Laboratories, CA, USA).

Slides were scanned using an Aperio ScanScope® CS system (Aperio, CA, USA) to produce photomicrographs. The percentage of positive cells ([number of positive cells/total number of cells] × 100) and the density of positive cells (number of positive cells/mm^2) was measured.

RNA & miRNA expression

Analyses for mRNA and miRNAs expression levels were performed using the validated HTG EdgeSeq Oncology Biomarker Panel and miRNA Whole Transcriptome Assay (HTG Molecular Diagnostics Inc, AZ, USA). Target genes, miRNA and housekeeping genes evaluated are presented in Supplementary Table 1. Tumor content and tissue necrosis were assessed by a board-certified pathologist and estimated as the number of malignant cells as a percentage of all cells. Acceptance criterion for analysis was set at >70% tumor content and <20% necrosis; macro-dissection was performed if values were outside these parameters. Sequencing was performed on an Illumina NextSeq 500 Sequencer (Illumina Inc, CA, USA). Results were reported if samples passed predefined quality check parameters for each assay. Gene expression data were quantile normalized and $\log_2$ transformed (HTG Molecular Diagnostics).

Statistical analyses

Objective responses were categorized as responders (complete response [CR] and partial response [PR]) and non-responders (stable disease [SD] and progressive disease [PD]). Wilcoxon rank-sum test was applied for continuous expression comparison and Fisher's exact test for association between response categories defined by Response

Table 1. Patient demographics and baseline characteristic, by cohort.

Characteristic	IHC cohort	mRNA cohort			miRNA cohort		
	Axitinib (n = 52)	Axitinib (n = 34)	Sorafenib (n = 33)	p-value†	Axitinib (n = 33)	Sorafenib (n = 32)	p-value†
Age, mean (StD)	58.3 (11.0)	56.8 (11.2)	58.3 (7.8)	>0.999	56.3 (11.0)	58.1 (7.8)	>0.999
Sex, n (%)				0.776			0.775
– Male	38 (73)	25 (74)	26 (79)		24 (73)	25 (78)	
– Female	14 (27)	9 (26)	7 (21)		9 (27)	7 (22)	
Race, n (%)				0.203			0.202
– White	47 (90)	30 (88)	30 (91)		29 (88)	29 (91)	
– Asian	4 (8)	4 (12)	1 (3)		4 (12)	1 (3)	
– Black	0	0	2 (6)		0	2 (6)	
– Other	1 (2)	0	0		0	0	
ECOG PS, n (%)				0.615			0.798
– 0	27 (52)	20 (59)	22 (67)		20 (61)	21 (66)	
– 1	25 (48)	14 (41)	11 (33)		13 (39)	11 (34)	
MSKCC criteria risk group, n (%)				0.053			0.077
– Poor	22 (42)	13 (38)	4 (12)		12 (36)	4 (13)	
– Intermediate	18 (35)	13 (38)	19 (58)		13 (39)	19 (59)	
– Favorable	12 (23)	8 (24)	10 (30)		8 (24)	9 (28)	

† Axitinib-treated vs sorafenib-treated.
ECOG PS: Eastern Cooperative Oncology Group performance status; IHC: Immunohistochemistry; MSKCC: Memorial Sloan Kettering Cancer Center; StD: Standard deviation.

Evaluation Criteria in Solid Tumors and biomarker stratum using the median value as a cutoff point. OS and PFS were compared using the Kaplan–Meier method between treatment arms and between biomarker strata using the median value as a cut-off point. Percent tumor changes from baseline were compared between the biomarker strata with median value as cut-off point using Wilcoxon rank-sum test. Pearson correlation coefficients and p-values were provided for *CD68* mRNA expression versus CD68 immunohistochemistry results (percentage of positive cells and density of positive cells) for axitinib-treated patients. Samples from slides and blocks were analyzed together due to the limited sample size within each treatment arm.

For IHC analysis, a Cox model was applied to PFS in multivariate analysis to adjust for appropriate baseline covariates: prior medications, baseline ECOG PS, time from diagnosis to treatment, liver and bone involvement, number of metastatic sites, corrected calcium and for levels of LDH, neutrophils, hemoglobin and ALP. For significant ($p < 0.05$) results via Kaplan–Meier analysis, receiver operating characteristics (ROC) analysis was used to generate a ROC curve to further assess potential utility as patient selection markers. ROC analysis of PFS was performed at 2, 4, 6 and 8 months.

Due to differences observed in mRNA and miRNA expression between slides and blocks, median values are calculated for slides and blocks separately. For mRNA/miRNA expression analysis and correlations to clinical outcome, the Wilcoxon rank-sum test was stratified by slides and blocks using the van Elteren test and the Fisher's exact test was stratified by slides and blocks using the Cochran–Mantel–Haenszel test. In this exploratory analysis, significant ($p < 0.05$) biomarkers were selected based on the raw two-sided p-value without controlling for false discovery rate.

Results

Patients

Of 723 patients enrolled in AXIS, 52 axitinib-treated patients were evaluable for IHC analysis. Patient demographics and baseline characteristics were similar to those in the entire AXIS population (Table 1) [3]. Mean age was 58.3 (standard deviation: 11.0) years and most patients were male (73.1%), white (90.4%) and all had an ECOG PS of 0 (51.9%) or 1 (48.1%). Overall, 23.1, 34.6 and 42.3% of patients had favorable, intermediate and poor risk, respectively, based on Memorial Sloan Kettering Cancer Center (MSKCC) criteria.

For the mRNA and miRNA analyses, 67 (34 and 33 patients in the axitinib and sorafenib arms, respectively) and 65 (33 and 32 patients in the axitinib and sorafenib arms, respectively) patients were evaluated. The demographics for gender, race and ECOG PS were similar to the IHC analysis cohort (Table 1). No significant differences in the

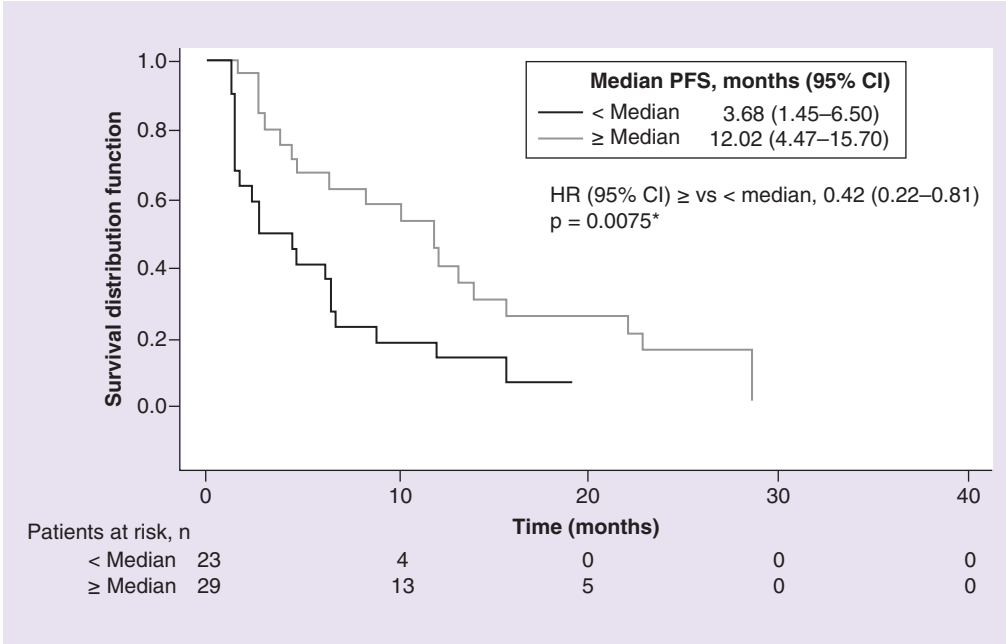

Figure 1. Kaplan–Meier curve of PFS according to median CD68$^+$ cells/mm^2.
*Log rank test. Median = 0.08 cells/mm^2.
HR: Hazard ratio; PFS: Progress-free survival.

Table 2. Multivariate analysis of association of CD68$^+$ cell density and progress-free survival (immunohistochemistry cohort).

Covariate	Two-sided p-value	HR (95% CI)
CD68$^+$ cell density, cells/mm^2, ≥ median vs < median	0.032	0.5 (0.2–0.9)
Neutrophils, > ULN vs ≤ ULN	0.003	8.2 (2.0–33.3)

Final model constructed by backward elimination process with a 5% level of selection.
HR: Hazard ratio; ULN: Upper limit of normal.

demographic and baseline characteristics were observed between the treatment arms in either the mRNA or miRNA analysis sets. However, the MSKCC prognostic group factors approached significance in the mRNA analysis set (p = 0.053), with more patients in the poor-risk group in axitinib-treated patients (38.2%) compared with those in the sorafenib-treated group (12.1%; Table 1).

Tumor-infiltrating immune cells in axitinib-treated patients

The presence and clinical correlation of TILs (CD3$^+$) and TAMs (CD68$^+$) were investigated in archival tumor tissue of axitinib-treated patients by IHC. Median values for cell density were 399.5 cells/mm^2 for CD3$^+$ cells and 0.08 cells/mm^2 for CD68$^+$ cells. There was no significant difference between responders versus nonresponders in cell density of CD3$^+$ (mean: 601.2 vs 531.1 cells/mm^2; p = 0.811) or CD68$^+$ (mean: 0.11 vs 0.10 cells/mm^2; p = 0.425). There was no significant difference in the frequency of response category according to median cut-point stratum (< vs ≥) for density of either CD3$^+$ cells or CD68$^+$ cells (CR + PR: 20.0 vs 23.1%, SD + PD: 80.0 vs 76.9% for CD3$^+$ cells and CD68$^+$ cells, respectively).

A CD68$^+$ ≥median for cell density was associated with longer median PFS compared with <median (12.0 vs 3.7 months; HR: 0.4; log-rank p = 0.008; Figure 1). Prediction of PFS was most accurate for patients who received ≥2 months of axitinib treatment using CD68$^+$ cell density (sensitivity: 83%, specificity: 87%; area under the ROC curve: 0.809). Multivariate analysis of CD68 expression levels in predicting PFS demonstrated that presence of CD68$^+$ cells ≥median (5.21%) and CD68$^+$ cell density >median (0.08 cells/mm^2) significantly predicted longer PFS, after adjusting for important baseline covariates (Table 2). There was a slight trend (p < 0.2), but

Table 3. mRNA association with objective response.

Gene	Treatment		CR + PR, n (%)	SD + PD, n (%)	Odds ratio[†] (95% CI)	p-value[‡]
CCR7	Axitinib	n	7	27	0.11 (0.01–1.00)	0.026
		< Median	6 (37.5)	10 (62.5)		
		≥ Median	1 (5.6)	17 (94.4)		
ITGA4	Axitinib	n	7	27	0.10 (0.01–0.98)	0.026
		< Median	6 (37.5)	10 (62.5)		
		≥ Median	1 (5.6)	17 (94.4)		
VHL	Axitinib	n	7	27	0.10 (0.01–0.98)	0.026
		< Median	6 (37.5)	10 (62.5)		
		≥ Median	1 (5.6)	17 (94.4)		
ESM1	Sorafenib	n	4	27	–	0.044
		< Median	4 (25.0)	12 (75.0)		
		≥ Median	0 (0.0)	15 (100.0)		
GSK3B	Sorafenib	n	4	27	–	0.036
		< Median	4 (26.7)	11 (73.3)		
		≥ Median	0 (0.0)	16 (100.0)		
NOTCH1	Sorafenib	n	4	27	–	0.044
		< Median	4 (25.0)	12 (75.0)		
		≥ Median	0 (0.0)	15 (100.0)		
TLR3	Sorafenib	n	4	27	–	0.023
		< Median	4 (28.6)	10 (71.4)		
		≥ Median	0 (0.0)	17 (100.0)		
VHL	Sorafenib	n	4	27	–	0.044
		< Median	4 (25.0)	12 (75.0)		
		≥ Median	0 (0.0)	15 (100.0)		

Median values are calculated for slides and blocks separately within each treatment. List of mRNA evaluated is provided in Supplementary Table 1; only associations with p-values <0.05 are reported in the table.
[†]An odds ratio <1 is in favor of <median group and an odds ratio >1 is in favor of ≥median group.
[‡]Two-sided unadjusted p-value based on Fisher's exact test.
CR: Complete response; PD: Progressive disease; PR: Partial response; SD: Stable disease.

no significant difference in median OS in patients with $CD68^+$ cell density ≥median versus <median (22.6 vs 17.8 months; HR: 0.6; p = 0.173).

Additionally, a correlation was observed for CD68 protein (% positive cells and cell density) and CD68 mRNA expression (R = 0.477, p = 0.004 and R = 0.399, p = 0.020, respectively) in axitinib-treated patients.

Gene expression analysis of angiogenic & immunomodulatory targets within mRCC

Expression analysis of genes that have been implicated in regulation of angiogenesis and immune modulation in mRCC was performed in archival tumor tissue from axitinib- or sorafenib-treated patients. The median values for each gene/miRNA (reported separately for slides and blocks) with correlation to clinical outcomes are shown in Supplementary Table 2. In axitinib-treated patients, lower expression levels (<median) of *CCR7*, *ITGA4* and *VHL* were associated with objective response (CR + PR, p = 0.026 for all; Table 3). In sorafenib-treated patients, lower expression levels (<median) of *ESM1* (p = 0.044), *GSK3B* (p = 0.036), *NOTCH1* (p = 0.044), *TLR3* (p = 0.023) and *VHL* (p = 0.044) were associated with objective response (Table 3).

Significant associations of mRNA expression levels with PFS and OS within each treatment arm are summarized in Table 4. In particular, in axitinib-treated patients, higher expression levels (≥median) of *CXCR4* were associated with longer PFS compared with <median (9.0 vs 2.8 months; HR: 0.3, 95% CI: 0.1–0.8; p = 0.011). Higher expression levels (≥median) of *TLR3* in axitinib-treated patients and lower expression levels (<median) in sorafenib-treated patients were associated with longer PFS (HR: 0.4, 95% CI: 0.2–0.9; p = 0.023 and HR: 3.9, 95% CI: 1.4–10.7; p = 0.005, respectively; Table 4). Additionally, longer OS (HR: 3.0, 95% CI 1.1–8.0; p = 0.022; Table 4) was observed in sorafenib-treated patients who had lower expression levels (<median) of *TLR3*. Finally, lower

Table 4. mRNA association with progression-free survival and overall survival within each treatment arm.

Gene	Treatment	Median (months)		p-value†	HR (95% CI)
		< Median (95% CI)	≥ Median (95% CI)		
PFS					
– *CXCR4*	Axitinib	2.8 (1.4–6.5)	9.0 (2.8–15.7)	0.011	0.3 (0.1–0.8)
– *PGF*	Axitinib	2.8 (1.4–6.5)	7.7 (2.9–14.1)	0.017	0.4 (0.2–0.9)
– *PRKCA*	Axitinib	9.0 (4.5–14.1)	2.8 (1.4–6.2)	0.048	2.3 (1.0–5.2)
– *TLR3*	Axitinib	4.5 (1.7–6.5)	9.0 (2.7–15.7)	0.023	0.4 (0.2–0.9)
– *TLR3*	Sorafenib	11.9 (3.4–27.5)	4.5 (1.6–6.1)	0.005	3.9 (1.4–10.7)
OS					
– *CCR7*	Axitinib	32.4 (20.0–NR)	13.3 (6.8–18.5)	0.005	3.9 (1.4–10.3)
– *PTPN11*	Axitinib	17.6 (7.0–22.0)	NR (7.0–NR)	0.020	0.4 (0.1–0.9)
– *CCL2*	Sorafenib	34.5 (18.9–34.5)	15.0 (10.9–25.4)	0.019	3.1 (1.2–8.4)
– *MMP-2*	Sorafenib	NR (19.4–NR)	15.7 (10.9–26.8)	0.032	2.8 (1.0–7.4)
– *NF2*	Sorafenib	17.3 (10.7–20.7)	34.5 (15.0–34.5)	0.035	0.4 (0.1–1.0)
– *TLR3*	Sorafenib	34.5 (20.2–34.5)	15.0 (10.7–26.8)	0.022	3.0 (1.1–8.0)

Median values are calculated for slides and blocks separately within each treatment. List of mRNA evaluated is provided in Supplementary Table 1.
†Log-rank two-sided test unadjusted p-values.
HR: Hazard ratio; NR: Not reached.

expression levels (<median) of *CCR7* in axitinib-treated patients was associated with longer OS (HR: 3.9, 95% CI: 1.4–10.3; p = 0.005; Table 4).

In addition, a significant interaction effect with treatment was observed for the expression levels of both *CXCR4* and *TLR3* and PFS (p = 0.029 and p < 0.001, respectively), as well as for the expression levels of *CD163* and PFS (p = 0.036) and OS (p = 0.048) even though the main treatment effects did not reach significance (Figure 2A & B).

Targeted miRNA expression analysis within mRCC

Expression analysis of miRNAs that have been implicated in mRCC and could have prognostic value in response to antiangiogenic therapy was performed in archival tumor tissue from axitinib- or sorafenib-treated patients. In axitinib-treated patients, expression levels (≥median) of miR-192-3p and miR-99B-5p were associated with objective response (p = 0.011 for both). Median PFS was longer in patients with higher expression levels (≥median) of miR-133A-3p versus <median (10.5 vs 2.8 months; HR: 0.4, 95% CI: 0.1–0.9; two-sided p = 0.022). Median PFS was longer in patients with higher expression levels (≥median) of miR-143-5p versus <median (7.7 vs 2.8 months; HR 0.4, 95% CI: 0.2–1.0; two-sided p = 0.037). No miRNA included in these analyses was associated with differences in OS within either treatment arm.

Lower expression levels (<median) of miR-183-5p trended toward a shorter PFS in axitinib-treated patients versus sorafenib-treated patients (4.7 vs 6.5 months; HR: 2.4, 95% CI: 0.9–6.8; two-sided p = 0.088; Figure 2A). Higher expression levels (≥median) of miR-183-5p trended toward a longer PFS in axitinib-treated patients versus sorafenib-treated patients (6.5 vs 2.8 months; HR: 0.6, 95% CI: 0.3–1.2; two-sided p = 0.134); the interaction p-value between treatment arms was significant (p = 0.048). Median OS in patients with higher expression levels (≥median) of miR-221-5p was shorter in axitinib-treated patients versus sorafenib-treated patients (14.4 vs 26.8 months; HR: 2.4, 95% CI: 1.0–5.9; two-sided p = 0.045; Figure 2B); interaction p-value 0.041.

Discussion

The highly angiogenic nature of RCCs has led to multiple approvals of antiangiogenic therapies for patients with advanced RCC. More recent data have shown expression of angiogenic factors and those involved in immune infiltration (e.g., T cells) within these tumors may regulate response and resistance to both antiangiogenic agents and immune checkpoint inhibitors [8]. A role for immune infiltration in RCC tumor progression, coupled with the reduced immune-suppressive properties of axitinib compared with other antiangiogenic agents [15], supported combination of axitinib with immune checkpoint-blocking therapies that have been recently approved for first-line treatment in mRCC [16–18]. Biomarker analyses performed from these studies defined molecular features that differentiate therapy-specific outcomes in first-line advanced RCC [19]. However, the mechanisms by which axitinib

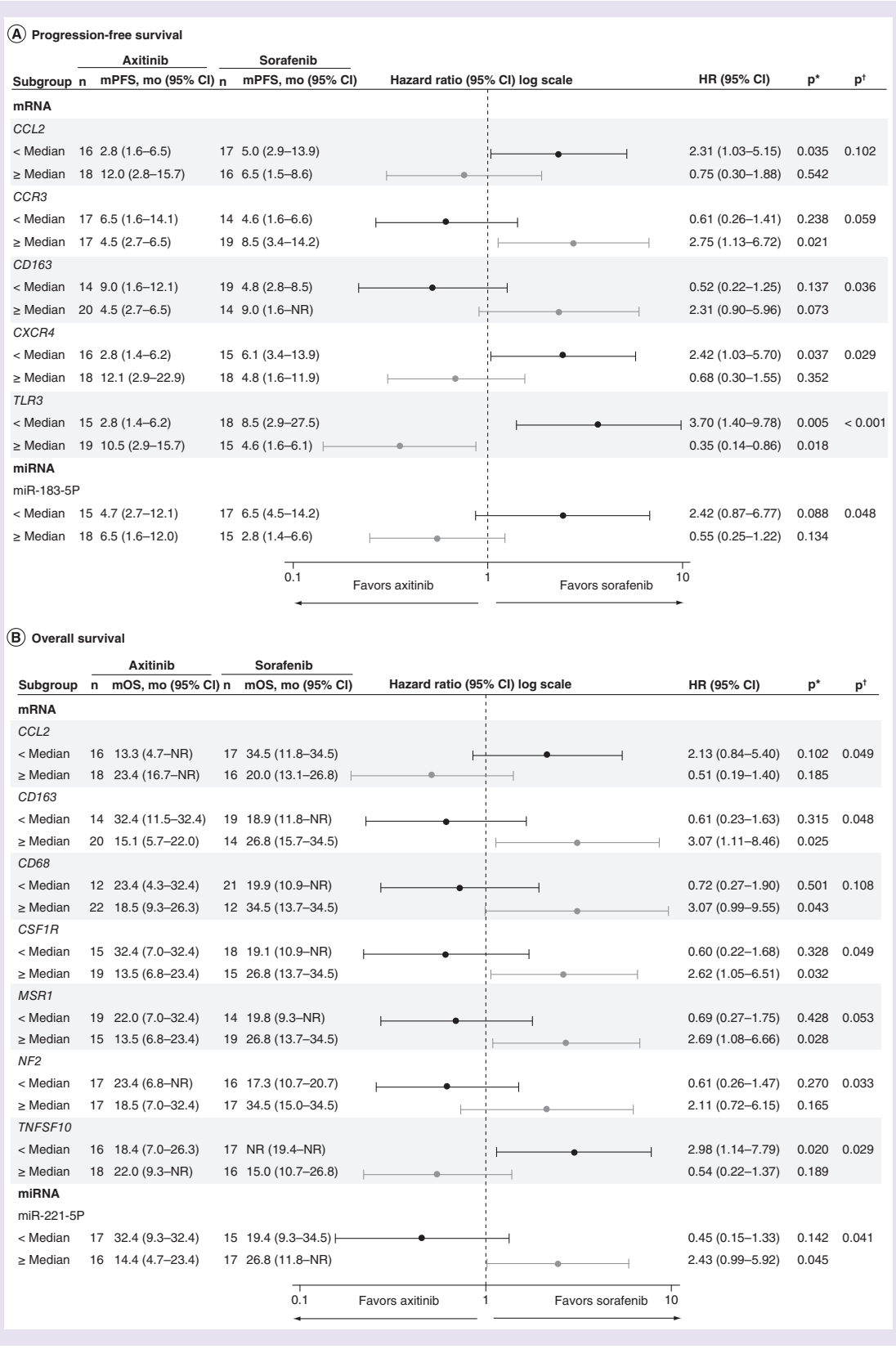

Figure 2. Association of statistically significant mRNA and miRNA with progression-free survival and overall survival between treatment arms.

*Log-rank two-sided test.

†Two-sided unadjusted p-value for overall median cutoff-by-treatment interaction from Cox model, with treatment group and median cutoff status as two main effects.

Median values are calculated for slides and blocks separately.

HR: Hazard ratio; mOS: Median overall survival; mPFS: Median progression-free survival; NR: Not reached.

is supporting potential immune/angiogenic responses remain largely unclear. In this study, tumor immune cell infiltration (CD68 and CD3) and gene/miRNA expression were evaluated in correlation with clinical outcomes in axitinib- or sorafenib-treated patients in the AXIS trial.

Higher levels of $CD68^+$ cells, which are indicative of TAMs, were significantly associated with longer PFS in axitinib-treated patients in this study. One interpretation would be that tumors with high angiogenic status via local TAM-mediated VEGF secretion [20] are sensitized to axitinib therapy. Further supporting this, higher expression levels (≥median) of *CXCR4*, which is induced by HIF-1α within hypoxic conditions, are required for migration of myeloid progenitor (i.e., $CD68^+$) and tumor cells [21] and contribute to neovascularization [22]; these higher expression levels were also associated with longer PFS in these patients. These results might seem inconsistent with previously published results showing that an increase in TAMs contributes to an immunosuppressive and pro-angiogenic environment [23–25] as a mechanism of resistance to antiangiogenic treatments. However, the favorable efficacy observed in this study could indicate potential predictive value of these biomarkers via increased sensitization to anti-VEGFR TKIs.

An alternative hypothesis may be that regimen containing axitinib sensitizes TAMs to a M1 (tumor-killing) phenotype, represented by the pan-macrophage marker CD68 and induces the innate immune system. Higher expression levels of *TLR3*, which can induce a proinflammatory M1 phenotype [26] and stimulate Th1 responses that mediate antitumor activity [27] were found to be associated with longer PFS in this study. Additionally, lower levels of *CD163*, which is an M2 marker, trends with longer OS in axitinib-treated versus sorafenib-treated patients in this study. However, further characterization of the macrophage activation states (M1 vs M2) and their localization within the tumor microenvironment [28], should be explored in future studies of patients with mRCC treated with axitinib.

Interestingly, although our analysis showed correlation between *CD68* mRNA and CD68 protein expression, the mRNA analysis did not demonstrate an association between *CD68* mRNA expression levels and outcomes. However, genes with known prognostic value in mRCC based on protein expression (i.e., *VHL*) showed clinical associations within the analyses, further supporting a connection between RNA and protein expression and RNA platform used. Differences between certain mRNA and IHC protein analyses (i.e., CD68) may be attributed to the small sample size and slightly different sample population, as well as to the fact that mRNA is more prone to degradation during specimen handling and storage.

The IHC analysis of TILs within this study showed no efficacy associations with CD3 expression levels. Markers for tumor-infiltrating immune cells more specific than CD3 (e.g., CD4, CD8, PD-L1) may be more informative of treatment outcomes [8,29–31]. Indeed, a recent study reported that patients with tumors expressing $CD8^+$ cells ≥median and those with CD8 expression ≥median at baseline had longer PFS in the arm treated with combination axitinib/avelumab [19].

Genes that play a dual role within immune and tumor cells, in other words, *CCR7* and *TLR3*, were identified as correlating with clinical outcome. Lower expression (<median) levels of *CCR7* were shown to be associated with better response and longer OS in axitinib-treated patients. This is consistent with the role of *CCR7*, which is expressed by various subset of immune cells and plays a role in cancer cell migration and metastasis formation [32] and similar to findings in another retrospective study that showed high tumoral *CCR7* expression correlated with potential lymphatic involvement and poor prognosis in patients with mRCC treated with TKIs [33]. *TLR3* was associated with longer PFS in both treatment arms. Higher *TLR3* expression levels were associated with longer PFS in axitinib-treated patients, whereas lower *TLR3* expression levels were associated with longer PFS in the sorafenib-treatment arm, which was supported by the comparison between treatment arms. *TLR3* has been shown to induce both tumor and endothelial cell apoptosis while potentially enhancing tumor-infiltrating innate immune cells and T-cell function [34,35]. However, *TLR3* has also been shown to induce tumor cells to undergo a metabolic switch to adapt to hypoxia within the tumor microenvironment [36]. Therefore, potential differences between treatment arms may be attributed to the multi-kinase nature of these TKIs. These results suggest that expression of *TLR3* may have a role in mRCC and potentially be a predictive biomarker for axitinib treatment.

There were limited clinical correlations in our analysis of miRNA expression. Higher expression levels of miR-99B-5p were associated with objective response, which was similar to previous profiling studies of patients with ccRCC treated with antiangiogenic TKIs [7]. The other miRNA targets that were associated/trended with clinical outcomes included miR-133A-3p, miR-143-5p, miR-183-5p and miR-221-5p, which have all been previously described in patients with advanced RCC [13,14]. Interestingly, miR-183 [37], miR-133A [38], miR-143 [39] and miR-221 [40] have been shown to play a role in either innate immune responses and/or immune cell function – suggesting

that perhaps their role in regulating the immune system correlates with responses observed to antiangiogenic TKIs in patients with mRCC.

There are limitations for this analysis, in that the biomarker cohort evaluated is a small, convenience subset of the entire patient population and tumor tissue samples were analyzed as part of a retrospective exploratory study, after completion of the trial. Therefore, the preliminary findings of this study should be further investigated in larger cohorts to determine whether these would be potential biomarkers for patients with mRCC treated with axitinib or VEGFR TKIs. Furthermore, the study design does not allow to distinguish predictive from prognostic potential. This would require samples to be assessed from untreated patients or from patients treated with drugs that have a different mechanism than axitinib.

Conclusion

Higher $CD68^+$ cell density is associated with longer PFS; prediction for PFS was strongest for patients who received ≥2 months of axitinib treatment. There were no efficacy associations with $CD3^+$ levels. Lower (<median) *CCR7* expression levels were associated with better response and OS in axitinib-treated patients. In patients treated with axitinib versus sorafenib, lower *CXCR4* expression levels (<median) were associated with shorter PFS, whereas higher *TLR3* expression levels (≥median) were associated with longer PFS, which suggests that these may be potential biomarkers for RCC treatment selection; however, this requires further evaluation in larger and more contemporaneous studies.

Summary points

- The highly angiogenic nature of renal cell carcinoma (RCC) has led to approval of multiple antiangiogenic therapies for patients with advanced RCC, including the introduction in 2012 of axitinib for second-line treatment of advanced RCC.
- The highly immunogenic nature of RCC was also the foundation for initiation of two Phase III trials of axitinib in combination with the immune checkpoint inhibitors avelumab and pembrolizumab. These combinations (axitinib/avelumab and axitinib/pembrolizumab) have been recently approved for first-line treatment of advanced RCC.
- Biomarker analyses performed from these combination studies defined molecular features that differentiate therapy-specific outcomes in first-line advanced RCC. However, the mechanisms by which axitinib is contributing to potential immune/angiogenic responses in advanced RCC remain largely unclear.
- We investigated associations between tumor-associated immune cells ($CD3^+$ and $CD68^+$) and mRNA/miRNA expression with clinical outcomes in a subset of patients from the axitinib pivotal trial, AXIS.
- Our results showed that higher $CD68^+$ cell density was associated with longer progression-free survival, whereas there were no efficacy associations with $CD3^+$ levels. *CCR7*, *CXCR4* and *TLR3* expression levels were associated with efficacy and may be potential biomarkers for RCC treatment selection; however, this requires further evaluation in larger prospective study.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/fon-2020-0212

Author contributions

Study concepts and design were performed by all authors. Data acquisition was performed by BI Rini, B Escudier and RJ Motzer. Quality control of data and algorithms were performed by DA Murphy and J-F Martini. Data analysis and interpretation were performed by all authors. Statistical analysis was performed by P Wang and S Li. Manuscript preparation, editing and review were performed by all authors.

Acknowledgments

We thank the participating patients and their families, as well as the investigators, sub-investigators, research nurses, study coordinators and operations staff. We would also like to thank AC Donahue for support with the biomarker analyses for this study. Patients treated at Memorial Sloan Kettering Cancer Center were supported in part by Memorial Sloan Kettering Cancer Center Support Grant/Core Grant (P30 CA008748).

Financial & competing interests disclosure

This study was sponsored by Pfizer. DA Murphy, P Wang, S Li, JC Tarazi and J-F Martini are employees of and own stock in Pfizer. JA Williams was an employee of Pfizer when the study was conducted and data were analyzed. BI Rini received a research grant and personal fee from Pfizer. B Escudier served on advisory boards for Bristol-Myers Squibb, Novartis, Pfizer, Eisai, EUSA Pharma, Ipsen, Acceleron, Roche and Exelixis; served as a consultant for Bristol-Myers Squibb, Novartis and Ipsen; and received research funding from Bristol-Myers Squibb and Novartis and honoraria from Pfizer, Novartis, Bristol-Myers Squibb, Roche, Exelixis, Ipsen, Acceleron and Bayer. RJ Motzer served as a consultant for Pfizer, Novartis, Eisai and Exelixis and received research funding from Bristol-Myers Squibb, Pfizer, Roche, Eisai, Exelixis and Novartis. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Medical writing support was provided by V Dror of Engage Scientific Solutions and funded by Pfizer.

Ethical conduct of research

The AXIS trial was conducted in accordance with the Declaration of Helsinki, the International Conference on Harmonisation guidelines on Good Clinical Practice and applicable local regulatory requirements and laws. The protocol, amendments and informed consent forms were approved by an institutional review board or independent ethics committee at each study center. All patients provided written informed consent. Tumor samples were collected from patients who participated in the trial and who provided specific informed consent for the collection of tumor samples for analysis.

Data sharing statement

The authors certify that this manuscript reports original clinical trial data, NCT00678392. Upon request and subject to certain criteria, conditions and exceptions (see https://www.pfizer.com/science/clinical-trials/trial-data-and-results for more information), Pfizer will provide access to individual de-identified participant data from Pfizer-sponsored global interventional clinical studies conducted for medicines, vaccines and medical devices for indications that have been approved in the US and/or EU; or in programs that have been terminated (i.e., development for all indications has been discontinued). Pfizer will also consider requests for the protocol, data dictionary and statistical analysis plan. Data may be requested from Pfizer trials 24 months after study completion. The de-identified participant data will be made available to researchers whose proposals meet the research criteria and other conditions and for which an exception does not apply, via a secure portal. To gain access, data requestors must enter into a data access agreement with Pfizer.

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