A cytokine and angiogenic factor (CAF) analysis in plasma for selection of sorafenib therapy in patients with metastatic renal cell carcinoma


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Background: We investigated cytokines and angiogenic factors (CAFs) in patients with metastatic renal cell carcinoma (mRCC) treated in a randomized phase II clinical trial of sorafenib versus sorafenib + interferon-α (IFN-α) that yielded no differences in progression-free survival (PFS). We aimed to link the CAF profile to PFS and select candidate predictive and prognostic markers for further study.

Methods: The concentrations of 52 plasma CAFs were measured pretreatment (n = 69), day 28, and day 56 using multiplex bead arrays and enzyme-linked immunosorbent assay. We investigated the association between baseline levels of CAFs with PFS and posttreatment changes.

Results: Unsupervised CAF clustering analysis revealed two distinct mRCC patient groups with elevated proangiogenic or proinflammatory mediators. A six-marker baseline CAF signature [osteopontin, vascular endothelial growth factor (VEGF), carbonic anhydrase 9, collagen IV, VEGF receptor-2, and tumor necrosis factor-related apoptosis-inducing ligand] correlated with PFS benefit (hazard ratio 0.20 versus 2.25, signature negative versus positive, respectively; P = 0.0002). While changes in angiogenic factors were frequently attenuated by the sorafenib + IFN combination, most key immunomodulatory mediators increased.

Conclusions: Using CAF profiling, we identified two mRCC patient groups, a candidate plasma signature for predicting PFS benefit, and distinct marker changes occurring with each treatment. This platform may provide valuable insights into renal cell carcinoma biology and the molecular consequences of targeted therapies.

Key words: CAF profiling, interferon, RCC combinations, renal cell carcinoma, sorafenib

Introduction

The advances in understanding of molecular pathways driving renal cell carcinoma (RCC) and the development of new therapies to target those pathways have increased the prospects that effective treatment combinations will be developed. The majority of clear cell RCC demonstrates von Hippel–Lindau gene inactivation, which leads to elevated hypoxia-inducible factor and enhanced transcription of >200 genes including multiple angiogenic mediators such as vascular endothelial growth factor (VEGF) [1–3]. VEGF pathway inhibitors such as sunitinib, sorafenib, bevacizumab, and pazopanib have been shown to prolong progression-free survival (PFS) and even extend overall survival (OS) in this disease [4–10]. However, only a small number of RCC patients achieve a complete response and long-term survival and virtually all experience disease progression.

Multiple investigators have explored the use of angiogenic factors as prognostic and predictive biomarkers in RCC. Higher baseline VEGF levels are associated with worse tumor stage and grade, performance status, and overall prognosis [5, 11–15]. However, studies addressing whether VEGF is a predictive marker for identifying RCC patients likely to benefit from VEGF-targeted therapies have yielded inconsistent results [5, 15, 16]. New biomarkers are needed to guide drug selection, sequence, and dosing and to better classify RCC patients and their patterns of response to therapy.

Linking aspects of the biology of RCC such as angiogenesis and immune modulation with the molecular effects of therapies targeting relevant pathways in RCC patients will inform the development of biologically based markers. The availability of such markers should also accelerate the identification of effective treatment combinations in specific populations of RCC patients. The evaluation of markers in plasma or serum
has become clinically feasible with the advent of multiplexing technologies, which permit simultaneous evaluation of large numbers of biologically relevant proteins [17, 18]. We recently reported a frontline randomized phase II trial of sorafenib versus sorafenib + interferon-α (IFN-α) in metastatic renal cell carcinoma (mRCC) and found no significant differences in PFS between treatment arms [19]. In this follow-up study, we investigated a broad set of cytokines and angiogenic factors (CAFs) in plasma using prospectively defined statistical criteria for analysis to (i) select candidate predictive and prognostic markers for further study, (ii) determine whether combinations of multiple markers could better serve as a predictive index of benefit from therapy, and (iii) establish the set of CAFs that could be used to assess biologic activity of sorafenib versus its combination with IFN in mRCC.

methods

patients

The original phase II clinical trial is described elsewhere [19]. In this trial, patients with pathologically confirmed metastatic clear cell RCC were randomized between frontline sorafenib (Bayer, Wayne, NJ) 400 mg orally twice daily alone or its combination with IFNα2b (Schering, Kenilworth, NJ) 0.5 million units subcutaneously twice daily. Clinical end points investigated included PFS, OS, and duration of response.

sample collection and CAF analysis

Patients provided written Institutional Review Board—approved informed consent to collect blood samples for biomarker analysis. Specimens were obtained at baseline (pretreatment) and on days 28 and 56 (each ≥3 days) of treatment. Plasma and serum were then prepared as previously described and stored at −80°C [18]. For analysis, samples were thawed overnight at 4°C and then centrifuged at 1500 g to remove debris.

Concentrations of 58 CAFs were measured in duplicate in the Blood-based Biomarkers Laboratory at M. D. Anderson Cancer Center. These factors were selected on the basis of their link to established RCC biology, the putative mechanism of action of sorafenib and IFN, and commercial availability. Fifty four CAFs were analyzed in plasma per manufacturers’ instructions with multiplex bead suspension array kits using a Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA), including Human Group I and II cytokine panels (Bio-Rad Laboratories) and two customized panels for measuring matrix metalloproteinase-9, soluble E-selectin, epidermal growth factor (EGF), and transforming growth factor-alpha (LINCOplex; Millipore, Billerica, MA). Plasma concentrations of osteopontin, soluble carbonic anhydrase IX (sCA9), VEGF, soluble vascular endothelial growth factor receptor-2 (sVEGFR-2), and placental growth factor (PIGF) were determined by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). Serum concentrations of collagen IV (ColIV) were also determined by ELISA (Kamiya, Seattle, WA). Six of 58 CAFs (10.3%) were rejected owing to the number of out-of-range samples (see supplemental Methods, available at Annals of Oncology online for details).

For unsupervised hierarchical clustering, the log-transformed concentration of each baseline CAF was standardized by subtracting the sample mean and dividing by the standard deviation. Analysis based on Pearson’s correlation was next conducted to evaluate associations between the patient samples and the CAFs that passed our selection criteria (supplemental Methods, available at Annals of Oncology online).

candidate CAF selection, logistic regression, and signature development

The association between each biomarker expression (high or low versus median) at baseline or on treatment and PFS was evaluated by fitting a multivariate Cox proportional hazards model that included treatment arm, biomarker, and the interaction between these two. We established a biomarker expression ‘index’ containing the expression information from several candidate CAFs and determined whether an interaction exists between such biomarker index and treatment arm. To create a ‘CAF index’ from these candidate markers, we selected CAFs with a median P value for interaction between the markers (testing concentration cut-offs at intervals containing one-sixth of the patients) and treatment arm <0.05. We then selected the corresponding optimal binary split (i.e. that with the smallest P) and assigned a score of +1 for CAF concentrations on the side of the cut-off with PFS favoring sorafenib or 0 for those favoring the combination. Next, we calculated the index for each patient by adding the score for each marker so that the final biomarker index ranged from 0 to 1 in this case 6 (‘Results’ and supplemental Methods, available at Annals of Oncology online). Supplemental Figure S1 (available at Annals of Oncology online) shows the numbers of patients and events in each level of the biomarker index. Linear mixed models were fitted to assess the change in CAFs over time, the difference between treatment arms, and the interaction between time and treatment. All P values were two sided; P < 0.05 was statistically significant. We did not control for multiple analyses because of the exploratory nature of this study. All statistical analyses were completed using SPLUS 8.0 (TIBCO, Palo Alto, CA).

results

patient population

Baseline plasma and serum samples and clinical information were available from 70 (87.5%) of the 80 patients from the clinical trial. One patient in the sorafenib + IFN arm withdrew consent after randomization but before receiving study medication. Therefore, 69 patients (n = 34, sorafenib; n = 35, sorafenib + IFN) were included in the PFS analysis.

Supplemental Table S1 (available at Annals of Oncology online) summarizes patients’ characteristics at baseline. No statistically significant difference was detected in patients’ demographic and clinical characteristics between arms. Days 28 and 56 on-treatment samples were available from 59 (30, sorafenib; 29, sorafenib + IFN) and 57 (28, sorafenib; 29, sorafenib + IFN) patients, respectively.

Disease progressed in 50 of the 69 (72.5%) patients. None of the clinical characteristics in supplemental Table S1 (available at Annals of Oncology online) had a predictive effect on PFS. In this study, the estimated median PFS for the sorafenib arm was 5.8 (95% CI 3.8–9.3 months) versus 7.8 months (95% CI 5.2–13.0 months) for the sorafenib + IFN arm (P = 0.49; Figure 1A).

unsupervised hierarchical clustering by baseline CAF concentration

Table 1 shows the 52 CAFs and their median baseline concentrations. To gain insight into correlations between CAFs in previously untreated metastatic clear-cell RCC, an unsupervised hierarchical clustering analysis was conducted that identified two main groups of patients (n = 38 and 30) (Figure 2). The larger was characterized by relatively higher concentrations of proangiogenic and hypoxia-regulated factors (‘angiogenic group’; 56%), including PIGF, hepatocyte growth factor, platelet-derived growth factor B-B, basic fibroblast
growth factor, stromal cell-derived factor 1 alpha chain, macrophage colony-stimulating factor-1 (M-CSF), and growth-regulated alpha protein (GRO-alpha), plus VEGF, sCA9, and other four markers present in our predictive CAF signature (see below), which clustered close in the same group. The second (‘inflammatory group’; 44%) had notably higher levels of interleukins and other proinflammatory factors [e.g. interleukin 1b (IL-1b), IL-2, IL-4, IL-6, IL-13, IFNα2].
We assessed PFS outcomes in all 69 patients as a group according to pretreatment CAF concentrations above (high) or below (low) the median value for each biomarker. By univariate analysis, baseline levels of 14 CAFs [including low IL-2, IL-5, and monocyte chemotactic protein 1 (MCP-1) and high EGF, IL-12 p40, and M-CSF] were associated with shorter PFS (supplemental Table S2, available at Annals of Oncology online). A multivariate Cox proportional hazards model identified EGF, IL-5, and M-CSF as CAFs with independent prognostic value (supplemental Table S2, available at Annals of Oncology online).

Next, we searched for markers that identified groups of patients who experienced different degrees of benefit from sorafenib versus sorafenib + IFN. The only significant treatment-by-factor interactions for the 52 baseline CAFs were for osteopontin and VEGF (\(P_s\) for interaction 0.004 and 0.01, respectively). In patients with low baseline concentrations of osteopontin (i.e. lower than the median 51.3 ng/ml) or VEGF (19.6 ng/ml), treatment with sorafenib versus sorafenib + IFN resulted in significantly shorter PFS times [median 3.8 versus 11.1 months, respectively, for osteopontin; hazard ratio (HR) 0.36; \(P = 0.02\); and median 7.7 versus 11.1 months, respectively, for VEGF; HR 0.33; \(P = 0.02\) (Table 2)]. In patients with high osteopontin, a trend towards improved PFS was observed in the sorafenib alone arm (Table 2). This suggests that patients with low osteopontin or VEGF being considered for sorafenib treatment could be better treated with sorafenib + IFN, but those with high osteopontin achieve comparable or improved PFS with sorafenib alone.

We next tested a biomarker expression ‘index’ that combined individual CAFs with the goal of identifying groups of patients who experienced different degrees of benefit from the two treatment arms (see ‘Methods’ for details). Six of the 52 CAFs fulfilled our predefined criteria and were included in the index: osteopontin, sCA9, VEGF (ELISA), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), ColIV, and sVEGFR2. Levels greater than the cut-off were associated with shorter PFS in the combination arm for all these except for TRAIL, which showed the opposite effect.

### Table 2. Baseline biomarkers with significant predictive effect on PFS

<table>
<thead>
<tr>
<th>CAF</th>
<th>Baseline concentrationa</th>
<th>Median PFS (months)</th>
<th>Hazard ratio</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteopontin</td>
<td>Low</td>
<td>3.8</td>
<td>0.36</td>
<td>0.02</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>High</td>
<td>7.7</td>
<td>1.94</td>
<td>0.09</td>
</tr>
<tr>
<td>VEGF</td>
<td>Low</td>
<td>7.7</td>
<td>0.33</td>
<td>0.02</td>
</tr>
<tr>
<td>VEGF</td>
<td>High</td>
<td>5.7</td>
<td>1.57</td>
<td>0.26</td>
</tr>
</tbody>
</table>

The predictive effect of treatment arm on PFS, by plasma osteopontin and VEGF concentrations at baseline (\(P_s\) for interaction 0.004 and 0.01, respectively).

*Low’ and ‘high’ refer to below and above the median concentration (51.3 for osteopontin and 19.6 for VEGF), respectively.
PFS, progression-free survival; CAF, cytokine and angiogenic factor; SOR, sorafenib; IFN, interferon; VEGF, vascular endothelial growth factor.

### Predictive biomarker index based on a six-CAF signature at baseline

We next tested a biomarker expression ‘index’ that combined individual CAFs with the goal of identifying groups of patients who experienced different degrees of benefit from the two treatment arms (see ‘Methods’ for details). Six of the 52 CAFs fulfilled our predefined criteria and were included in the index: osteopontin, sCA9, VEGF (ELISA), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), ColIV, and sVEGFR2. Levels greater than the cut-off were associated with shorter PFS in the combination arm for all these except for TRAIL, which showed the opposite effect.
We then calculated the HR for disease progression or death for patients treated with sorafenib + IFN versus sorafenib by using all possible biomarker index values (Table 3 shows results for CAF indexes 3–5). We selected a CAF index value of 4 as our cut-off because the resulting groups were the most balanced \([n = 34 \text{ with } \geq 4 \text{ (‘signature positive’)} \text{ and } n = 34 \text{ with } < 4 \text{ (‘signature negative’)}\) (supplemental Figure S1, available at *Annals of Oncology* online). Significantly, different HR for PFS was observed in the two groups: 2.25 for the positive versus 0.20 for the negative group (Table 3). The interaction effect between ‘signature’ (positive versus negative) and ‘treatment’ (combination versus single agent) was highly significant \((P = 0.0002)\).

These results suggest that mRCC patients candidate for sorafenib treatment who are signature positive obtain more PFS benefit from sorafenib only, whereas those who are signature negative benefit more from sorafenib + IFN. This is shown graphically in Figure 1B.

**changes in CAF concentrations during treatment**

Data on 47 CAFs were available from weeks 4 and 8 of treatment. We assessed individual markers at each time point for changes in concentration from baseline (Figure 3 and supplemental Figure S2, available at *Annals of Oncology* online). Similar to other studies, VEGF robustly increased and sVEGFR-2 decreased in sorafenib-treated patients. While changes in angiogenic factors were often attenuated by the addition of IFN (e.g. VEGF, sVEGFR-2, ColIV, PIGF, E-selectin), immunomodulatory mediators such as IL-6, IL-10, IL-12 p40, IL-18, and M-CSF were noticeably more affected by the combination. The only markers with a significantly different degree of modulation by arm were MCP-1 at 4 weeks \((P = 0.03)\) and IL-18 at 8 weeks \((P < 0.05)\), both increased more with sorafenib + IFN.

**discussion**

In this exploratory study, we conducted a comprehensive profiling analysis of blood samples from patients in our phase II trial of frontline sorafenib versus sorafenib + IFN in metastatic clear-cell RCC [19] to illustrate relevant applications of the CAF platform in randomised clinical trials, including the identification of candidate activity, prognostic, and predictive markers (i.e. those that distinguish patients who received different degrees of benefit from the treatment options). We assessed the concentrations of 52 angiogenic and immunomodulatory cytokines before and during treatment and defined two distinct mRCC groups on the basis of biomarker profile, a candidate signature that helped establish the most appropriate therapy for individual patients, and the sets of marker changes that reflect the biological activity of the drug and combination under study.

Among all CAFs at baseline, only osteopontin and VEGF were predictive of PFS benefit; i.e. patients with high or low levels of the marker had statistically significant differences in PFS between the treatment arms. We further investigated whether an index combining several CAFs would be a better predictor than any factor alone. Using prospectively defined criteria, we found that a six-marker signature comprised of osteopontin, VEGF, sCA9, ColIV, sVEGFR-2, and TRAIL identified a signature negative group of patients who had a PFS benefit from the combination arm \((HR 0.2)\) and another \((signature positive)\) who benefited from sorafenib alone \((HR 2.25; P \text{ for interaction } = 0.0002)\).

To gain insight into the biology and classification of RCC, we conducted a clustering analysis that revealed the most relevant proangiogenic and hypoxia factors are relatively high and aggregate in a majority of mRCC patients (angiogenic group), while a still significant proportion of patients conversely displays higher levels of inflammatory mediators (inflammatory group). Remarkably, all markers in our predictive signature clustered together, behaving de facto as a surrogate for CAFs in the angiogenic group. Given the presence of sorafenib in both treatment arms, it is not surprising that such ‘angiogenic signature’ prevailed for prediction. Patients with low levels of markers in the signature benefited from the addition of IFN to sorafenib, suggesting that those patients may gain more from IFN and less from sorafenib and perhaps from angiogenesis inhibitors in general. Whether our signature is applicable to other tyrosine kinase inhibitors or bevacizumab versus its combination with IFN and whether an alternative ‘inflammatory signature’ exists to predict for benefit from immunomodulatory therapy remains to be established.

Our second most notable finding, for the most part previously unknown, was the broad profile of CAF changes induced by sorafenib and the impact of low-dose IFN on these changes. Sorafenib-induced reciprocal changes in VEGF and sVEGFR-2 concentrations, a class effect consistent with the activity of this drug as a VEGFR tyrosine kinase inhibitor [15]. Several other angiogenic mediators were significantly modulated in sorafenib-treated patients, effects that were blunted \((sVEGFR-2, VEGF, \text{ColIV, E-selectin)}\) or enhanced by the addition of IFN \((MCP-1, IL-18)\). Prominent changes also occurred in several helper T-cell type 2 \((IL-5, IL-6, IL-10, \text{IL-13)}\), type 1 \((IL-12 p40, IFN \gamma)\), and hematopoietic \((M-CSF, \text{GRO-alpha)}\) cytokines. The biologic implications of these distinct likely therapy-induced changes in CAF concentrations are undetermined, but some may affect the

<table>
<thead>
<tr>
<th>CAF index (cut-off)</th>
<th>Signature positive ((P = 0.03))</th>
<th>Signature negative ((P &lt; 0.05))</th>
<th>Interaction (P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOR + IFN vs SOR</td>
<td>1.63 (0.82–3.24)</td>
<td>0.16 (0.05–0.53)</td>
<td>0.0005</td>
</tr>
<tr>
<td>4</td>
<td>2.25 (1.02–4.96)</td>
<td>0.20 (0.08–0.55)</td>
<td>0.0002</td>
</tr>
<tr>
<td>5</td>
<td>10.8 (1.17–99.6)</td>
<td>0.48 (0.25–0.91)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

HR is the hazard ratio for progression-free survival.

*aSelected CAF index level cut-off.*

CAF, cytokine and angiogenic factor; sCA9, soluble carbonic anhydrase IX; VEGF, vascular endothelial growth factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; sVEGFR2, soluble vascular endothelial growth factor receptor-2; ColIV, collagen type IV; CI, confidence interval; IFN, interferon; SOR, sorafenib.
tumor’s behavior and its response to treatment, as suggested by recent preclinical studies [20–23]. These results illustrate the pharmacologic complexity of the systemic response to ‘broad-spectrum’ tyrosine kinase inhibitors. Comparable findings were reported by our group in patients with non-small-cell lung cancer treated with the VEGFR and epidermal growth factor receptor inhibitor vandetanib [18]. Adding low-dose IFN to sorafenib in the current study yielded important differences in CAF modulation, which may help explain not only the benefit obtained by some patients treated with the combination but also the preserved effectiveness observed with lower doses of IFN in clinical trials [24, 25].

Beyond its well-established prognostic value, the use of VEGF for antiangiogenic treatment outcome prediction remains uncertain [5, 15, 16, 26–28]. Similar to the results of the phase III Treatment Approaches in Renal Cancer Global Evaluation Trial study of sorafenib versus placebo where patients with baseline VEGF concentrations in the highest quartile obtained the most PFS benefit from sorafenib [5, 15], our patients with high VEGF benefited more from single-agent sorafenib. Data from other studies in mRCC directly comparing anti-VEGF agents and these with mTOR inhibitors should help clarify this.

These results are exploratory due to the limited number of patient samples and the absence of validation in independent data set, which is undergoing. It is reassuring that the changes we observed during treatment generally coincided with those previously reported for sorafenib and similar drugs [15, 17]. Other investigators have evaluated the value of CAFs in predicting treatment outcome in RCC, but their studies were usually conducted in animal models, single-arm clinical trials, or analyzed smaller number of markers [15, 27, 29–36].

The most effective use of targeted agents in RCC entails a better understanding of the biologic determinants of treatment response. Our results not only suggest that CAF profiling can be applied to identify treatment-specific markers of clinical benefit and resistance but also to provide insight into the mechanisms and toxic effects of these active compounds. While in today’s clinical practice sorafenib may have a lesser role in frontline therapy, and its combination with IFN is unlikely to be further developed, the broad patient classification and candidate angiogenic signature identified in this study may well be relevant to other therapies in RCC and warrant validation in larger and more current datasets.

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references