Biomarker Signatures Correlate with Clinical Outcome in Refractory Metastatic Colorectal Cancer Patients Receiving Bevacizumab and Everolimus

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Running title: Biomarkers change in response to bevacizumab and everolimus

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Abstract

A novel combination of bevacizumab and everolimus was evaluated in refractory colorectal cancer patients in a phase II trial. In this retrospective analysis, plasma samples from 49 patients were tested for over 40 biomarkers at baseline and after one or two cycles of drug administration. Analyte levels at baseline and change on-treatment were correlated with progression-free survival (PFS) and overall survival (OS) using univariate Cox proportional hazard modeling. Multivariable analyses were conducted using Cox modeling. Significant changes in multiple markers were observed following bevacizumab and everolimus treatment. Baseline levels of six markers significantly correlated with PFS and OS, including CRP, Gro-α, IGFBP-1, TF, ICAM-1, and TSP-2 (p<0.05). At C2D1, changes of IGFBP-3, TGFβ-R3, and IGFBP-2 correlated with PFS and OS. Prognostic models were developed for OS and PFS (p=0.0002 and 0.004, respectively). Baseline model for OS consisted of CRP, Gro-α, and TF, while on-treatment model at C2D1 included IGFBP-2, IGFBP-3, and TGFβ-R3. These data demonstrated that multiple biomarkers were significantly modulated in response to bevacizumab and everolimus. Several markers correlated with both PFS and OS. Interestingly, these markers are known to be associated with inflammation and IGF signaling, key modulators of mTOR biology.
Introduction

Colorectal cancer (CRC) is the second leading cause of cancer related death in the United States, and in 2015, the estimated number of new cases of CRC will reach 130,000(1). Three anti-VEGF therapies are currently FDA approved for the treatment of metastatic colorectal cancer: bevacizumab(2), ziv-aflibercept(3), and regorafenib(4). Among them, bevacizumab is the most extensively studied and has demonstrated significant efficacy across a variety of cancers. In combination with chemotherapy, bevacizumab has been shown to improve outcomes in both the 1st and 2nd line settings(2, 5, 6). However, the mechanisms underlying tumor progression on anti-VEGF therapy remain largely unknown and more effective anti-angiogenic strategies are still needed, as are biomarkers to select patients most likely to benefit from these therapies(7).

Mammalian target of rapamycin (mTOR) inhibition represents a theoretically attractive strategy to augment the anti-tumor effects of anti-VEGF therapy. mTOR is a serine/threonine kinase that regulates numerous cellular functions, including nutrient sensing and survival(8). mTOR also regulates angiogenesis and modulates inflammation(9, 10). Importantly, mTOR has been shown to regulate hypoxia inducible factor (HIF-1α)(11, 12), a key player involved in resistance to anti-VEGF therapies. Everolimus is a derivative of the natural mTOR inhibitor rapamycin, with improved solubility, potency, and stability(13).

In an initial phase I clinical trial that combined anti-VEGF and anti-mTOR inhibitors, it was observed that greater anti-angiogenic and anti-tumor effects were observed from the combination treatment than observed with either drug alone(14). Based upon the proposed mechanistic complement, as well as promising phase I data, a phase II
study was conducted(15), testing the combinatorial effect of bevacizumab and everolimus in refractory CRC patients. In this study, we observed that more than 20% of patients had stable disease of more than 6 months, suggesting a potential subset of patients who derived greater benefit from this combination therapy. Notably, most patients enrolled in this trial were resistant to previous bevacizumab-containing therapies, suggesting the addition of everolimus delayed the onset of bevacizumab resistance and improved clinical outcomes(11, 12, 16). How to select the patients with the greatest potential to benefit, and how to better understand the mechanisms of delayed resistance, remain two big challenges in the field.

Our group has developed and applied a novel multiplex ELISA approach that allows for a broad profiling of plasma markers related to angiogenesis and inflammation. This approach has the potential to identify markers that may predict for greater or lesser benefit from anti-VEGF therapies, other anti-angiogenic agents, and combination regimens. Previously, these candidate markers were tested in other clinical settings and specific markers have been identified to have potential prognostic and/or predictive value(17-20).

In this report, we applied the 41-analyte multiplex ELISA approach to this phase II study of bevacizumab and everolimus in colorectal cancer. The intent was to identify candidate predictors of greater or lesser benefit from this regimen. Biomarker levels were determined at baseline and on-treatment, and treatment-related changes were statistically analyzed. Baseline levels and on-treatment changes were correlated with clinical outcomes and prognostic models for OS and PFS were generated.

Materials and Methods
Patient selection, treatment, and outcome

Enrolled in this study were 50 mCRC patients (one patient censored for statistical analysis) who had progressed on, or could not tolerate all of the following standard of care treatments for mCRC: fluoropyrimidines, oxaliplatin, irinotecan, bevacizumab, and cetuximab and/or panitumumab (if wild-type KRAS). This population was highly refractory, having progressed on a median of four prior treatments. It should be noted that 42 patients (84%) had progressed on prior bevacizumab-based therapies. Additional details about the treatment regimen and clinical outcomes for this study have previously been reported(15). Written informed consent was obtained from each patient regarding the use of plasma for this correlative analysis. This study was IRB approved and registered with www.clinicaltrials.gov (study number: NCT00597506). This retrospective analysis conforms to the reporting guidelines established by the REMARK criteria.

Plasma collection, handling, and storage

Blood was collected from each patient by venipuncture into a sodium citrate vacutainer (BD Vacutainer, catalog # 369714), and mixed thoroughly. After mixing, the tubes were centrifuged at 2500 x g for 15 min. The upper layer of plasma was transferred to a fresh tube and centrifuged one more time at 2500 x g for 15 min. The double-spun, platelet-poor plasma was aliquoted, snap frozen, and stored at -80°C until use.

Multiplex and ELISA assays

All biomarkers were measured using the SearchLight multiplex platform (Aushon Biosystems, Inc., Billerica, MA) (Table 1), except for TGF-β R3 (R&D Systems, Inc., Minneapolis, MN), as previously described(18).
Statistical analysis

To evaluate on-treatment changes, L-ratio was calculated using the formula: \( \log_2 \) (post-treatment level/ baseline level) for each analyte at two time points, cycle 2 day 1 (C2D1) and cycle 3 day 1 (C3D1). Signed-rank tests were used to identify significantly modulated markers upon treatment. Significantly modulated markers with a \( p \) value ≤ 0.0001 were graphically illustrated using Waterfall plots demonstrating the change from baseline to C2D1.

Spearman’s rank correlations were calculated for all pairs of analytes at baseline, C2D1 and C3D1. Hierarchical clustering of all markers at baseline and on-treatment was displayed as dendrograms.

Based upon their response criteria, patients were divided into progressive disease (PD) or stable disease/partial response (SD/PR) groups. Biomarker differences between these two patient populations were analyzed and illustrated via Beeswarm plots to show the baseline level variations, as well as the differential modulation of each marker in response to treatment. Baseline biomarker levels, both as continuous values and dichotomized at the median, were associated with clinical outcome using univariate Cox proportional hazards analysis for each analyte for both PFS and OS. On treatment changes, represented by L-ratio 1 and L-ratio 2, were also associated with PFS and OS.

Multivariable analyses were performed using Cox proportional hazards models to generate baseline and on-treatment biomarker signatures. The score selection method was used to control the number of markers in the signature and leave-one-out
cross validation was used to derive a prognostic signature using a SAS macro(21).

Lastly, Kaplan-Meier plots were used to illustrate patients’ survival, and the survival
curves of the low and high risk groups were compared by Wilcoxon test.

Results

Changes in biomarker levels in response to bevacizumab and everolimus

Fifty refractory colorectal cancer patients received a combination of bevacizumab (10
mg/kg/2w) and everolimus (5 or 10 mg daily) in this single-arm, non-randomized
Phase II study(15). To evaluate biomarker responses to treatment, each patient’s
baseline biomarkers profile was used as his/her reference control. Plasma samples
collected at baseline (BL), at C2D1, and at C3D1 were available for biomarker
analysis from 49, 39, and 25 patients, respectively.

In total, 41 biomarkers for each patient were analyzed. Three markers (FGFb, IL-8,
VEGF-C) were excluded from statistical analysis since more than 10% samples fell
below the limit of detection. The median levels, ranges, and fold changes from
baseline for each of the 38 biomarkers are shown in Table 1. Assays were highly
reproducible with CVs generally in the 5-20% range (data not shown). At C2D1,
statistically significant changes were observed in 26 markers (p<0.05), with 12 of
them being highly statistical significant (p<0.0001) (Supplement Table 1). Among
these 12 markers, three were down regulated on treatment, including Ang-2, MCP-1,
and VEGF-R2; while MMP-2, PAI-1 active, PAI-1 total, PIGF, SDF-1, VCAM-1, VEGF-
A, VEGF-D, vWF increased on treatment (Figure 1). Many of the significant changes
observed with these 12 markers at C2D1 persisted at C3D1, with the direction of
change being the same for every marker (Supplement Table 1).
Correlation among biomarkers

To better understand the potential co-regulation of specific biomarkers, Spearman’s rank correlation was used to test pair-wise correlations at baseline and on-treatment. Statistically significant pairs of baseline markers (correlation coefficients ≥0.7, \( p<0.001 \)) included PAI-1 active and PAI-1 total, CRP and IL-6, ICAM-1 and VCAM-1, VCAM-1 and TSP-2, ICAM-1 and CRP, ICAM-1 and TSP-2, PDGF-AA and PDGF-BB. Statistically significant pairs of on-treatment markers at C3D1 included CRP and IL-6, TGF-\( \beta \)1 and PDGF-BB, PDGF-AA and PDGF-BB. All baseline and on-treatment analyte pairs were positively correlated, indicating that biomarkers were either both high (increased) or both low (decreased). Hierarchical clustering maps for all markers were shown in Supplement Figure 1.

Biomarker difference between SD and PD patients

In this clinical study of bevacizumab and everolimus, 23 patients (46%) exhibited stable disease (SD) as their best response, with 13 of these patients (26%) exhibiting SD for greater than 6 months, as specified by the Response Evaluation Criteria in Solid Tumors (RECIST version 1.0). The majority of these SD patients achieved best responses between C2D1 and C3D1. Twenty-one patients (42%) had progress disease (PD) as their best response on treatment with 17 patients (34%) progressed radiographically whereas 4 patients (8%) progressed clinically. This created a balanced distribution of patients that could be categorized into SD and PD groups. After dichotomizing the patients into responder (SD) and non-responder (PD) groups, each marker was tested to evaluate if there were any significant differences between these two groups. First, baseline level of each marker was statistically compared between SD and PD groups, using Wilcoxon rank-sum tests. As shown in Figure 2A,
baseline levels of 10 different markers were significantly lower \((p<0.05)\) in the SD group compared to the PD group, including Ang-2, CRP, D-Dimer, Gro-\(\alpha\), IGFBP-2, IL-6, TGF-\(\beta\)2, TSP-2, VCAM-1, and vWF. Note that the Y-axis scale is log2, indicating that one unit of change reflects a 2-fold difference. After one cycle of treatment, IGFBP-3, TGF\(\beta\)-R3 were observed to be upregulated in SD patients, as compared to very little change observed in the PD patients. Conversely, TSP-1 was down regulated in SD patients, compared to changes observed in PD patients (Figure 2B).

**Univariate correlation of biomarkers with patient outcome**

To test the prognostic and predictive value of the markers, baseline levels and on-treatment change for each marker were associated with PFS and OS, the primary and secondary endpoints of the clinical study, respectively. At baseline, eight markers were significantly associated with PFS \((p<0.05)\): CRP, Gro-\(\alpha\), IGFBP-1, TF, ICAM-1, vWF, TSP-2, and TGF-\(\beta\)1. In all cases except for IGFBP-1, the hazard ratios of the significant markers were >1, indicating that higher levels of the marker correlated with shorter PFS (Table 2). Baseline levels of eight analytes were significantly associated with OS: ICAM-1, CRP, Gro-\(\alpha\), TSP-2, IGFBP-1, TF, MCP-1, and Ang-2 \((p < 0.05)\). As was seen for PFS, higher baseline levels for any given marker was associated with a shorter OS. The lists of markers that correlated with PFS and OS were quite consistent as six of the eight markers were statistically significant for both OS and PFS (CRP, Gro-\(\alpha\), IGFBP-1, TF, ICAM-1, and TSP-2).

Next, the on-treatment change for each marker was associated with PFS and OS. At C2D1, PFS was significantly associated with changes in IGFBP-3, TGF\(\beta\)-R3, and
IGFBP-2 ($p \leq 0.05$), with greater changes in IGFBP-3 and TGFβ-R3 levels resulting in longer PFS, while a greater change in IGFBP-2 levels resulted in shorter PFS times (Table 3). OS was significantly associated with the same three markers observed in PFS (IGFBP-3, TGFβ-R3, IGFBP-2) as well as with three additional markers (MMP-2, MMP-9, VEGF-R2) ($p < 0.05$). MMP-9 was also associated with PFS at the trend level ($p = 0.0736$). At C3D1, only 26 samples were available from 19 SD patients and 7 PD patients. PFS was significantly associated with changes in TSP-1, VEGF-R1, HGF, and IGFBP-1 ($p < 0.05$), as greater changes in TSP-1 levels associated with longer PFS, while greater changes in VEGF-R1, HGF, and IGFBP-1 levels associated with shorter PFS time.

**Multivariable prognostic models**

Using a leave-one-out, cross-validation analysis, multivariable models were developed. High- or low-risk grouping was assigned based on relationship to the median of the combination of biomarkers and coefficients selected in each model, a higher risk corresponding to a higher hazard. The full method of the LOOCV analysis is described in the macro paper (21). At baseline, the model for predicting OS benefit consisted of CRP, Gro-α, and TF. These markers were selected in at least 89.6% of the models. The OS of the high- and low-risk groups were 4.2 and 11.5 months ($p = 0.0002$), respectively, and this difference corresponded to an HR of 2.9.

The on-treatment model at C2D1 for PFS consisted of IGFBP-2, IGFBP-3, and TGFβ-R3. These markers were selected in at least 85% of the models. The PFS of the high- and low-risk groups were 1.9 and 4.6 months ($p = 0.004$), respectively, and this
difference corresponded to an HR of 1.8. Kaplan-Meier plots of these models are shown in Fig 3.

Discussion

When this trial was first initiated, it represented one of the first doublet combinations of a VEGF- and an mTOR-inhibitor in patients with refractory colorectal cancer. Plasma samples were serially collected for each patient, allowing for correlative analyses that explore the changes that occurred on treatment, both in the setting of tumor control and progression. Among the 50 patients enrolled, sample collection was excellent (49/50 patients at baseline) and most assays technically performed well. Three markers (FGFb, IL-8, VEGF-C) were generally read at or below the limit of detection, and since we took a conservative approach to biomarker discovery, these markers were eliminated from analysis. Our analyses were undertaken in an exploratory and hypothesis-generating fashion, and for this reason, $p$ values should be considered descriptive and have not been corrected for multiple parameter testing.

In addition, our study was not randomized and for this reason correlations with clinical outcomes cannot differentiate the prognostic vs. predictive effects of each factor. Lastly, this study did not include a monotherapy group that would be needed to isolate the effects of one drug vs. another.

These caveats notwithstanding, our current analysis identified several key findings. First, a large number of the angiogenic and inflammatory markers that were evaluated were modulated by bevacizumab plus everolimus treatment, suggesting broad biological consequences from this therapy. Of the 38 markers analyzed, 26 were modulated in a statistically significant fashion. Consistent with prior reports by our group and others evaluating anti-VEGF monotherapy, in this study MMP-2, PIGF, VCAM-1, and VEGF-D were increased, while Ang-2, MMP-9, and s-VEGFR2 were
decreased in response to the combination of bevacizumab and everolimus. The magnitudes of these changes appear to be broadly consistent with those previously described for bevacizumab mono-therapy(18, 22), suggesting these changes were driven primarily by VEGF but not mTOR inhibition.

Broad angiogenesis profiling has not been commonly reported with mTOR inhibitors. Several markers were observed to change in response to the combinational treatment, but were not affected in previous analyses of anti-VEGF therapies(18), possibly reflecting mTOR specific effects. These markers included OPN and SDF-1, both of them were related to IGF signaling and inflammation, processes known to be regulated by mTOR(23, 24).

As we have seen in other similar studies, many markers appear to be highly co-regulated, suggesting important cross talk among inflammatory cytokines, growth factors, and matrix derived angiogenic factors. The most significant correlations were noted for CRP and IL-6, ICAM-1 and TSP-2, PAI-1 active and PAI-1 total at baseline, as well as for PDGF-AA and PDGF-BB, CRP and IL-6, TGF-β1 and PDGF-BB at C3D1. These data are consistent with the co-regulation of the IL-6 and TGF-β signaling axes in these patients, a finding well described in preclinical models(25, 26).

From the perspective of clinical utility, in unselected patients, the efficacy of bevacizumab and everolimus was modest in refractory colorectal cancer and does not merit future testing. However, 23 patients (46%) had SD and among them, 13 patients (26%) achieved SD on treatment for a period lasting more than 6 months, suggesting the potential to identify a subset population of patients who may benefit more from this therapy. Despite many biological and technical challenges, multiplex
ELISA approaches have recently identified several strong candidate predictors of benefit from anti-VEGF therapy. For example, in two separate phase III trials in renal cell cancer patients, the inflammatory mediator IL-6 was shown to predict for benefit for pazopanib and for bevacizumab(20, 27). Other candidate clinical predictors for bevacizumab have also been described, including VEGF ligands, HGF, and other inflammatory mediators(28). We and others have also described reproducible patterns of change in multiple markers with anti-VEGF therapy, as well as with other anti-angiogenic therapies(17-20, 29-31).

To explore the relationship between these markers and clinical outcome, patients in this trial were retrospectively dichotomized into PD and SD groups. When biomarkers were compared between these two groups, the baseline levels of most markers were significantly lower in SD patients (Fig 2A), including Ang-2, CRP, IL-6, etc. In response to the co-administration of bevacizumab and everolimus, SD patients tended to have larger increases in IGFBP-3 and TGFβ-R3, as well as greater decreases in TSP-1.

Significant differences in biomarker profiling between PD and SD groups encouraged the approach of using biomarker information to select patients with better clinical benefit. In this trial, baseline level and on-treatment change of each marker were correlated with clinical outcomes (Table 2&3). Several statistically significant candidate markers that could predict for benefit from bevacizumab and everolimus treatment were identified. Six baseline markers were consistently correlated with both PFS and OS: CRP, Gro-α, IGFBP-1, TF, ICAM-1, and TSP-2. Additional markers such as vWF and TGF-β1 were significantly correlated only with PFS; while MCP-1
and Ang-2 were significantly correlated only with OS, but trends were also noted with PFS (MCP-1, \( p=0.076 \); Ang-2, \( p=0.118 \)). Upon treatment, changes in 3 markers were consistently and significantly correlated with both PFS and OS: IGFBP-2, IGFBP-3, and TGF\( \beta \)-R3. IGFBPs and TGF\( \beta \)-R3, as well as VEGF and mTOR, are known to play key roles in immune modulation, suggesting that clinical outcomes may be linked to favorable changes in host immune responses induced by anti-VEGF plus anti-mTOR treatment. These associations were largely consistent across both PFS and OS, suggesting their effects were robust and biologically relevant. Intriguingly, both baseline and on-treatment markers appear to share a common association with inflammation (as exemplified by CRP) and the IGF axis signaling (such as IGFBP-1, 2, 3), suggesting the clinical importance of these pathways.

Many of the current findings appear to be novel. The majority of outcome-related markers identified here have not been previously reported as candidate predictors for anti-VEGF therapy, and their biology may be more directly related to mTOR-regulated pathways. As with VEGF inhibitors, the development of biomarkers to predict benefit from mTOR inhibitors has been challenging. The markers evaluated here have not been extensively profiled in the development of mTOR inhibitors. If confirmed, our current findings may help identify those patients most likely to benefit from combined anti-VEGF plus anti-mTOR therapy and perhaps from anti-mTOR therapy alone. By describing potentially targetable mechanisms of resistance to these agents, these findings may also suggest novel combination regimens.

Lastly, we generated prognostic models for both OS and PFS. A baseline model consisting of CRP, Gro-\( \alpha \), and TF was developed for OS (Figure 3A). Notably, all
three markers were correlated with PFS and OS in univariate analysis, and two of them (CRP and Gro-α) were found to be significantly higher in PD patients compared to SD patients. As discussed above, CRP is a well known mediator of acute phase inflammation, Gro-α has been suggested to play a crucial role in chemotaxis (32); while mTOR is also know to regulate many inflammatory and immune functions(33). The on-treatment changes of IGFBP-2, IGFBP-3 and TGFβ-R3 were selected for PFS model (Figure 3B). Consistently, changes in all these markers were correlated with PFS and OS by univariate analysis. Two of them (IGFBP-3 and TGFβ-R3) underwent larger increases in SD patients. Not surprisingly, sensitivity to mTOR inhibitors may be affected by basal signaling in the IGF axis and by up-regulation of these pathways by feedback loops induced by mTOR inhibition(34).

These biomarker data offer potential insight as to why combining everolimus to bevacizumab increased drug efficacy in previously refractory patients and generates new hypotheses to consider. As discussed, down regulation of OPN and upregulation of SDF-1 appear to be specifically driven by everolimus. Both markers are known to be potent regulators of inflammatory diseases, and notably, OPN is involved in regulating T helper type 1 and Th17 cells (35). Interestingly, Th17 cells produce the cytokine IL-17, whose role in promoting tumor resistance to anti-angiogenic therapy has begun to unravel recently(36). Additionally, multiple myeloid-derived inflammatory mediators have been shown to mediate resistance to anti-VEGF therapy in preclinical models(36-38). Understanding how everolimus regulates OPN in these specific cell populations, and the consequences of intracellular versus soluble OPN(39), is of great interest. Another crucial player is HIF-1, a well-studied transcriptional factor inducing VEGF production and conveying resistance for anti-
tumor drugs(40). Beyond its role in angiogenesis, HIF has been shown to regulate, and be regulated by, many metabolic and inflammatory mediators, including mTOR(11, 41, 42). Everolimus-mediated blockage of mTOR activity may down regulate HIF-1 function, potentially impacting bevacizumab resistance.

In summary, our plasma biomarker analysis in this phase II study identified multiple angiogenic markers uniquely modulated by the combination of bevacizumab and everolimus, exemplified by several factors in the IGF-axis factors and multiple inflammation mediators. Many of these markers, both at baseline and on treatment, were significantly correlated with clinical outcomes, by both univariate analysis and multivariable models. These findings support the increasingly appreciated roles of inflammation in cancer biology and tumor angiogenesis.

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References


Table 1. Levels of biomarkers at baseline and on-treatment. FC, fold change from baseline.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Unit</th>
<th>Median Baseline (n=49)</th>
<th>Median C2D1 (n=39)</th>
<th>Median C3D1 (n=25)</th>
<th>FC Baseline-C2D1</th>
<th>Median C2D1-C3D1</th>
<th>FC C2D1-C3D1</th>
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<td>Ang-2</td>
<td>pg/ml</td>
<td>371.9</td>
<td>146.3-7573.8</td>
<td>289.2</td>
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<td>BMP-9</td>
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<td>0.8-151.4</td>
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<td>2.6-92.8</td>
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<td>CRP</td>
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<td>1.4</td>
<td>4.6</td>
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<tr>
<td>D-dimer</td>
<td>µg/ml</td>
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<td>39.6-154.0</td>
<td>1.13</td>
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<td>17.6</td>
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<td>9.6-863.5</td>
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<td>92.6</td>
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<td>ng/ml</td>
<td>110.1</td>
<td>15.1-273.2</td>
<td>108.5</td>
<td>12.6-360.3</td>
<td>1.9</td>
<td>96.5</td>
</tr>
<tr>
<td>PAI-1 active</td>
<td>ng/ml</td>
<td>3.5</td>
<td>0.003-25.3</td>
<td>11.5</td>
<td>0.01-75.6</td>
<td>11.4</td>
<td>7.2</td>
</tr>
<tr>
<td>PAI-1 total</td>
<td>ng/ml</td>
<td>13.4</td>
<td>3.1-85.4</td>
<td>37.2</td>
<td>3.0-333.0</td>
<td>3.5</td>
<td>28.6</td>
</tr>
<tr>
<td>PDGF-AA</td>
<td>pg/ml</td>
<td>10.9</td>
<td>0.9-542.8</td>
<td>14.2</td>
<td>1.1-527.5</td>
<td>2.0</td>
<td>18.8</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>pg/ml</td>
<td>25.8</td>
<td>0.8-613.4</td>
<td>13.8</td>
<td>1.5-661.0</td>
<td>1.4</td>
<td>19.0</td>
</tr>
<tr>
<td>PEDF</td>
<td>µg/ml</td>
<td>3.6</td>
<td>2.4-5.6</td>
<td>4.1</td>
<td>2.5-5.4</td>
<td>1.1</td>
<td>4.0</td>
</tr>
<tr>
<td>PIGF</td>
<td>pg/ml</td>
<td>7.4</td>
<td>0.9-16.6</td>
<td>12.6</td>
<td>4.9-29.7</td>
<td>2.0</td>
<td>14.6</td>
</tr>
<tr>
<td>P-Selectin</td>
<td>ng/ml</td>
<td>128.9</td>
<td>39.7-576.1</td>
<td>104.5</td>
<td>39.6-440.4</td>
<td>0.9</td>
<td>96.6</td>
</tr>
<tr>
<td>SDF-1</td>
<td>pg/ml</td>
<td>632.9</td>
<td>38.1-1948.8</td>
<td>1083.0</td>
<td>38.1-3166.6</td>
<td>2.0</td>
<td>999.0</td>
</tr>
<tr>
<td>TF</td>
<td>pg/ml</td>
<td>37.8</td>
<td>2.9-510.2</td>
<td>43.4</td>
<td>2.9-105.1</td>
<td>1.3</td>
<td>42.8</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>ng/ml</td>
<td>10.2</td>
<td>4.4-105.3</td>
<td>12.8</td>
<td>5.6-84.2</td>
<td>1.4</td>
<td>11.7</td>
</tr>
<tr>
<td>TGF-β2</td>
<td>ng/ml</td>
<td>28.5</td>
<td>3.0-1182.2</td>
<td>33.5</td>
<td>9.4-1651.8</td>
<td>1.5</td>
<td>29.5</td>
</tr>
<tr>
<td>TGFβ-R3</td>
<td>ng/ml</td>
<td>80.7</td>
<td>39.6-164.5</td>
<td>78.8</td>
<td>48.4-149.7</td>
<td>1.0</td>
<td>79.9</td>
</tr>
<tr>
<td>TSP-1</td>
<td>ng/ml</td>
<td>14.9</td>
<td>2.9-181.7</td>
<td>12.6</td>
<td>2.9-1167.2</td>
<td>7.7</td>
<td>16.1</td>
</tr>
<tr>
<td>TSP-2</td>
<td>ng/ml</td>
<td>35.3</td>
<td>13.1-124.1</td>
<td>35.0</td>
<td>15.0-94.7</td>
<td>1.0</td>
<td>29.5</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>µg/ml</td>
<td>2.1</td>
<td>1.0-7.3</td>
<td>3.9</td>
<td>1.1-9.9</td>
<td>1.8</td>
<td>3.3</td>
</tr>
<tr>
<td>VEGF</td>
<td>pg/ml</td>
<td>38.6</td>
<td>3.0-1098.4</td>
<td>120.5</td>
<td>22.4-709.0</td>
<td>4.2</td>
<td>108.3</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>pg/ml</td>
<td>690.5</td>
<td>173.1-3227.8</td>
<td>956.9</td>
<td>73.9-2737.6</td>
<td>1.5</td>
<td>928.3</td>
</tr>
<tr>
<td>VEGF-R1</td>
<td>ng/ml</td>
<td>67.1</td>
<td>6.0-10004.5</td>
<td>52.3</td>
<td>6.0-1612.5</td>
<td>1.5</td>
<td>35.0</td>
</tr>
<tr>
<td>VEGF-R2</td>
<td>ng/ml</td>
<td>3.7</td>
<td>0.3-18.3</td>
<td>2.2</td>
<td>0.3-8.2</td>
<td>0.7</td>
<td>2.1</td>
</tr>
<tr>
<td>vWF</td>
<td>U/ml</td>
<td>20.1</td>
<td>2.0-217.8</td>
<td>24.9</td>
<td>0.8-159.8</td>
<td>1.4</td>
<td>24.2</td>
</tr>
</tbody>
</table>

Abbreviations: Ang-2, angiopoietin-2; BMP-9, bone morphogenetic protein 9; CRP, C-reactive protein; GRO-α, growth-related oncogene-alpha; HGF, hepatocyte growth factor; ICAM-1, inter-cellular adhesion molecule-1; IGFBP-1, insulin-like growth factor binding protein; IL-6, interleukin-6; MCP-1, monocyte chemotactic protein-1; MMP, matrix metalloproteinase; OPN, osteopontin; PAI-1, plasminogen activator inhibitor-1; PDGF, platelet-derived growth factor; PEDF, pigment epithelium derived factor; PIGF, placenta growth factor; SDF-1, stromal cell derived factor-1; TF, tissue factor; TGF, transforming growth factor; TSP, thrombospondin; VCAM-1, vascular cell adhesion molecule-1; VEGF, vascular endothelial growth factor; sVEGF-R, soluble VEGF receptor; vWF, von Willebrand factor.
Table 2. Correlation of biomarkers baseline levels with clinical outcomes.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>P-value a</th>
<th>,med vs. &gt;med Hazard ratio</th>
<th>95% C. I.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;med vs. &gt;med</td>
<td></td>
</tr>
<tr>
<td>PFS</td>
<td></td>
<td>Hazard ratio</td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>0.0032</td>
<td>2.09</td>
<td>(1.15, 3.83)</td>
</tr>
<tr>
<td>GRO-α</td>
<td>0.0073</td>
<td>2.31</td>
<td>(1.28, 4.18)</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>0.008</td>
<td>0.92</td>
<td>(0.51, 1.67)</td>
</tr>
<tr>
<td>TF</td>
<td>0.0098</td>
<td>1.17</td>
<td>(0.65, 2.08)</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>0.0117</td>
<td>1.66</td>
<td>(0.92, 2.99)</td>
</tr>
<tr>
<td>vWF</td>
<td>0.0335</td>
<td>1.08</td>
<td>(0.6, 1.95)</td>
</tr>
<tr>
<td>TSP-2</td>
<td>0.0455</td>
<td>1.63</td>
<td>(0.91, 2.91)</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>0.0487</td>
<td>1.64</td>
<td>(0.91, 2.93)</td>
</tr>
<tr>
<td>OS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICAM-1</td>
<td>0.0009</td>
<td>2.02</td>
<td>(1.09, 3.74)</td>
</tr>
<tr>
<td>CRP</td>
<td>0.0009</td>
<td>2.2</td>
<td>(1.19, 4.03)</td>
</tr>
<tr>
<td>GRO-α</td>
<td>0.0048</td>
<td>2.5</td>
<td>(1.33, 4.69)</td>
</tr>
<tr>
<td>TSP-2</td>
<td>0.0058</td>
<td>2.9</td>
<td>(1.44, 5.04)</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>0.0126</td>
<td>1.3</td>
<td>(0.7, 2.4)</td>
</tr>
<tr>
<td>TF</td>
<td>0.021</td>
<td>1.47</td>
<td>(0.79, 2.73)</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.0458</td>
<td>1.4</td>
<td>(0.76, 2.58)</td>
</tr>
<tr>
<td>Ang-2</td>
<td>0.0491</td>
<td>1.89</td>
<td>(1.03, 3.48)</td>
</tr>
</tbody>
</table>

aFrom Cox proportional hazard models using continuous analyte values.
Table 3. Correlation of biomarker on-treatment changes with clinical outcomes.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>P-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hazard ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>95% C. I.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PFS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>0.0014</td>
<td>0.36</td>
<td>(0.2, 0.68)</td>
</tr>
<tr>
<td>TGFβR3</td>
<td>0.0084</td>
<td>0.13</td>
<td>(0.03, 0.59)</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>0.0509</td>
<td>1.97</td>
<td>(0.99, 3.9)</td>
</tr>
<tr>
<td><strong>OS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>0.0014</td>
<td>0.30</td>
<td>(0.15, 0.63)</td>
</tr>
<tr>
<td>MMP-9</td>
<td>0.0015</td>
<td>3.18</td>
<td>(1.55, 6.52)</td>
</tr>
<tr>
<td>TGFβ-R3</td>
<td>0.0026</td>
<td>0.12</td>
<td>(0.03, 0.47)</td>
</tr>
<tr>
<td>MMP-2</td>
<td>0.0184</td>
<td>7.62</td>
<td>(1.41, 41.2)</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>0.0247</td>
<td>2.40</td>
<td>(1.11, 5.14)</td>
</tr>
<tr>
<td>VEGF-R2</td>
<td>0.0255</td>
<td>0.61</td>
<td>(0.40, 0.94)</td>
</tr>
<tr>
<td><strong>PFS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSP-1</td>
<td>0.0059</td>
<td>0.77</td>
<td>(0.64, 0.92)</td>
</tr>
<tr>
<td>VEGF-R1</td>
<td>0.0245</td>
<td>1.24</td>
<td>(1.02, 1.5)</td>
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<tr>
<td>HGF</td>
<td>0.038</td>
<td>1.29</td>
<td>(1.01, 1.65)</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>0.0484</td>
<td>1.31</td>
<td>(1.00, 1.72)</td>
</tr>
</tbody>
</table>

<sup>a</sup>From Cox proportional hazard models using the calculated L-ratio values
Figure Legends

Figure 1. Change from baseline to the end of cycle 2 for biomarkers with statistical significance ($p<0.0001$). * denotes censored patients. Gold lines represent patients whose PFS $\geq$ median; black lines represent patients whose PFS < median.

Figure 2. Distinct biomarker features in patients undergoing progressive (PD) vs. stable disease (SD). A. Baseline levels comparison. B. On-treatment changes comparison.

Figure 3. Kaplan-Meier estimates of outcomes using leave-one-out cross validated Cox proportional hazard models. A. Prognostic model for OS using baseline levels consists of CRP, GRO-$\alpha$, and TF. B. Prognostic model for PFS using on-treatment changes consists of IGFBP-2, IGFBP-3, and TGF$\beta$-R3.
Figure 1.
Figure 2

A.

B.
Figure 3

A.

Overall Survival (Probability)

- High Risk
- Low Risk

HR = 2.9 [95% CI: 1.6 - 5.5]

p = 0.0002

Median OS of
- High Risk: 4.2 mon (3.4 - 7.1)
- Low Risk: 11.9 mon (8.8 - 17.2)

Time (Months)

B.

Progression Free Survival (Probability)

- High Risk
- Low Risk

HR = 1.8 [95% CI: 0.96 - 3.5]

p = 0.004

Median PFS of
- High Risk: 1.9 mon (1.7 - 2.4)
- Low Risk: 4.6 mon (2.3 - 7.2)

Time (Months)
Biomarker Signatures Correlate with Clinical Outcome in Refractory Metastatic Colorectal Cancer Patients Receiving Bevacizumab and Everolimus

Yingmiao Liu, Mark D Starr, John C Brady, et al.

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