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Prognostic Value of *ACVRL1* Expression in Metastatic Colorectal Cancer Patients Receiving First-Line Chemotherapy with Bevacizumab: Results from the TRIBE Study

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Abstract

Background—No biomarkers exist to predict benefit from anti-angiogenic therapy in metastatic colorectal cancer (mCRC) patients. *ACVRL1* encodes for ALK1, a member of the transforming growth factor β (TGF β) receptor family, which directs pathologic angiogenesis. We examined intratumoral expression of *ACVRL1* and other angiogenesis pathway related genes to identify molecular markers in TRIBE.

Materials and Methods—Among 503 randomized patients, 228 had sufficient tissue for analysis. Formalin-fixed paraffin-embedded (FFPE) specimens were examined for expression of *VEGF-A*, *VEGF-B*, *VEGF-C*, *VEGFR1*, *VEGFR2*, *ACVRL1*, *EphB4*, and *EGFL7* by RT-PCR. A maximal chi-square approach was used to determine mRNA levels associated with PFS, overall survival (OS), RR, early tumor shrinkage, and depth of response. Recursive partitioning (RP) trees

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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were constructed to identify composite prognostic biomarker profiles. External validation was conducted *in silico* using the Oncomine database.

Results—High *ACVRL1* expression was associated with superior OS in both treatment arms (FOLFOXIRI-bevacizumab: 32.7 vs. 13.5 months, HR 0.38, $P=0.023$; FOLFIRI-bevacizumab: 35.1 vs. 22.0 months, HR 0.36, $P=0.006$), and with prolonged PFS (11.7 vs. 5.9 months, multivariate HR 0.17, $P=0.001$) in patients receiving FOLFOXIRI-bevacizumab, in univariate and multivariate analyses. In RP analysis, *ACVRL1* was the strongest discriminator of RR, PFS, and OS in patients receiving FOLFOXIRI-bevacizumab, and of OS in patients receiving FOLFIRI-bevacizumab. *In silico* validation revealed significant associations between *ACVRL1* expression, disease recurrence, and 1-year survival ($P<0.05$) among all CRC stages.

Conclusion—*ACVRL1* expression may serve as a prognostic biomarker in mCRC patients receiving chemotherapy and bevacizumab and warrants further evaluation in prospective studies.

Keywords

ACVRL1; angiogenesis; bevacizumab; biomarker; metastatic colorectal cancer

INTRODUCTION

Fluoropyrimidine-based regimens combining either oxaliplatin or irinotecan with bevacizumab^{1, 2} are mainstays of metastatic colorectal cancer (mCRC) treatment. Recent efforts to improve upon the standard of care have introduced triple-drug chemotherapy (fluorouracil with leucovorin, oxaliplatin, irinotecan or FOLFOXIRI) and bevacizumab³ as an option for first-line mCRC management. The Triplet plus Bevacizumab (TRIBE) study³ was a randomized, open-label, multicenter phase III trial, comparing bevacizumab with either FOLFOXIRI or FOLFIRI as initial therapy for mCRC. Patients treated with FOLFOXIRI-bevacizumab achieved a superior objective response rate (ORR) (65% v. 53%; $P=0.006$) and progression-free survival (PFS) (12.1 v. 9.7 months, HR 0.75; $P=0.003$), as well as overall survival (OS) (29.8 v. 25.8 months, HR 0.80; $P=0.03$).⁴

Despite these advancements, there are no established biomarkers to select for benefit from bevacizumab based chemotherapy regimens in cancer patients, and correlative studies thus far have yielded conflicting results regarding the clinical utility of angiogenesis biomarkers in CRC. Gene expression of vascular endothelial growth factor (VEGF) ligands and receptors has been associated with clinicopathologic staging and prognosis,^{5, 6} yet other retrospective analyses^{7, 8} and randomized studies^{9–11} have been largely non-confirmatory. Owing to the complexity of tumor vascularity and presence of VEGF-independent angiogenic and drug resistance mechanisms,¹² other genes have been explored as candidate biomarkers of bevacizumab efficacy. For instance, activin receptor like-protein 1 (*ACVRL1*),^{13, 14} epidermal growth factor-like domain 7 (*EGFL7*),^{15, 16} and EphrinB4 (*EphB4*),^{17, 18} have been implicated in malignant angiogenesis, though data regarding their use as biomarkers are sparse and variable. Differences in patient populations, treatment rendered, and measures of gene expression are confounding factors which preclude definitive conclusions about these biomarkers. Evidence from randomized trials should provide strong

rationale for their clinical use and may offer useful hypotheses regarding molecular interactions affecting drug sensitivity and patient outcomes.

Accordingly, we sought to determine the prognostic value of molecular markers reflecting angiogenesis (*VEGF-A*, *VEGF-B*, *VEGF-C*, *VEGFR1*, *VEGFR2*, *ACVRL1*, *EphB4* and *EGFL7*) among mCRC patients receiving first-line chemotherapy and bevacizumab in the TRIBE study. We examined associations between the mRNA expression of individual genes and outcomes and then constructed recursive partitioning trees to identify clinically meaningful gene expression signatures.

MATERIALS AND METHODS

Study Design and Patient Population

The TRIBE³ study accrued 508 patients across 34 Italian centers with un-resectable mCRC who had not received chemotherapy or biologic therapy for their metastatic disease but may have received prior adjuvant chemotherapy. Patients were randomized to receive FOLFOXIRI-bevacizumab (experimental group) or FOLFIRI-bevacizumab (control group) at two-week intervals for twelve cycles, followed by fluorouracil and bevacizumab maintenance therapy until the time of disease progression. The study design, eligibility criteria, treatment details, follow-up and clinical outcomes have been previously reported. Protocols were approved by the respective local ethics committees and conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent for blood and tissue specimen collection for translational studies. Archived formalin-fixed paraffin-embedded (FFPE) tumor tissue was obtained from either the primary tumor or metastasis prior to randomization. Patients with sufficient tissue for examination of at least one molecular marker were included in the present study. This retrospective correlative analysis conforms to the reporting guidelines established by the REMARK criteria.¹⁹

Tumor Tissue Preparation and Gene Expression Analysis

Hematoxylin and eosin (H&E) stained sections of all FFPE tissues were evaluated by a board certified pathologist (G.Z.) for tumor content. FFPE specimens were dissected, and ten-micrometer-thick slides were obtained from areas with the highest tumor concentration and mounted on uncoated glass slides. For histologic diagnosis, three sections representative of the beginning, middle, and end of the tissue were stained with H&E using the standard method. Before microdissection, sections were de-paraffinized in xylene for 10 minutes, hydrated with 100%, 95%, and 70% ethanol, and then washed in H₂O for 30 seconds. Following microdissection of tumor cells, the sections were stained with nuclear fast red (American Master Tech Scientific, Inc., Lodi, CA) for 20 seconds and rinsed in water for 30 seconds. Samples were then dehydrated with 70%, 95%, and 100% ethanol for 30 seconds each, followed by xylene for 10 min. The slides were then completely air-dried. Laser capture microdissection (PALM Microlaser Technologies AG, Bernried, Germany) was carried out in all tumor samples to ensure that only tumor cells were dissected.²⁰ The dissected particles of tissue were transferred to a reaction tube containing 400 µL of RNA buffer for lysis of tumor cells.

After lysis of the tumor cells, RNA and DNA were isolated separately from the specimen. RNA isolation from paraffin-embedded samples was done according to a proprietary procedure defined by Response Genetics, Inc. (RGI; US Patent #6248535). The RNA was then reverse-transcribed to cDNA as described previously.²¹ DNA was either directly extracted or back extracted from the organic phase, both with an RGI patented method (US Patent #6248535).

Quantitation of gene mRNA expression levels of *VEGF-A*, *VEGF-B*, *VEGF-C*, *VEGFR1*, *VEGFR2*, *ACVRL1*, *EphB4*, *EGFL7* and an internal reference (β -actin) cDNA was done using a fluorescence-based real-time detection method (ABI PRISM 7900 TaqMan Sequence Detection System; PerkinElmer Applied Biosystems, Waltham, MA) as previously described.²² Isolated RNA was reverse-transcribed to cDNA, followed by RT-PCR using specific primers and probes. The PCR reaction mixture consisted of 1,200 nmol/L of each primer, a 200 nmol/L probe, 0.4 U of AmpliTaq Gold Polymerase, 200 nmol/L of dATP, dCTP, dGTP, dTTP; 3.5 mmol/L MgCl₂, and 1X TaqMan Buffer A containing a reference dye added to a final volume of 20 mL (PerkinElmer Applied Biosystems, Waltham, MA). Cycling conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 46 cycles at 95°C for 15 seconds and 60°C for 1 minute. For each sample, parallel TaqMan PCR reactions were carried out for each gene of interest and the β -actin reference gene to normalize for input cDNA. Results were obtained as a ratio of the PCR fluorescent signals of each gene of interest relative to the reference gene, β -actin. Primers and probe sequences are summarized in Supplemental Table 1.

KRAS mutation analysis was performed with a Response Genetics Inc. (RGI) designed mutation assay by RT-PCR using specifically designed primers and probes to detect each of the following mutations: Gly12Ala (GGT>GCT) 522; Gly12Asp (GGT>GAT) 521; Gly12Arg (GGT>CGT) 518; Gly12Cys (GGT>TGT) 516; Gly12Ser (GGT>AGT) 517; Gly12Val (GGT>GTT) 520; Gly13Asp (GGC>GAC) 532. After RT-PCR, all collected data was analyzed through an RGI Excel™ template and according to delta-CT values the mutation status was established. *BRAF*V600E mutations were detected by dye terminator sequencing.

Statistical Analysis

Messenger RNA expression levels of *VEGF-A*, *VEGF-B*, *VEGF-C*, *VEGFR1*, *VEGFR2*, *ACVRL1*, *EphB4*, and *EGFL7* were summarized and analyzed by Wilcoxon two-sample tests or Kruskal-Wallis tests to detect differences based on gender, primary tumor site (left vs. right), *KRAS* and *BRAF* mutation status.

The maximal chi-square approach was used to identify which mRNA levels may delineate subsets of patients more likely to benefit from FOLFIRI-bevacizumab in terms of PFS. Based upon these analyses, the following optimal cutoff points were determined (relative expression level to the internal reference gene, β -actin): *VEGF-A* (5.715), *VEGF-B* (3.295), *VEGF-C* (0.135), *VEGFR1* (0.245), *VEGFR2* (0.52), *ACVRL1* (0.40), *EphB4* (0.435), and *EGFL7* (0.435).

A classification and regression tree (CART) method, based on recursive partitioning, was used to explore gene expression variables for identifying homogenous subgroups for tumor response, PFS, and OS.²³ The recursive partitioning analysis is a non-parametric statistical method for modeling a response variable and multiple predictors. The recursive partitioning analysis includes two essential processes: tree-growing and tree pruning. The splitting rule of recursive partitioning is based on the Gini diversity index (1 minus the sum of squared probabilities over all levels of response) or deviance whenever appropriate. Our recursive partitioning analysis included all patients with tumor tissue specimen who were evaluable for tumor response (N = 228).

Genes which showed significant associations with outcomes in both treatment groups were then evaluated in the Oncomine microarray data compendium, as an external validation. Differential expression of *ACVRL1* in relevant studies was compiled and annotated for significance using the statistical measures provided by the Oncomine platform (Thermo Fisher, Ann Arbor, MI).

All values are reported as medians and ranges or cut-off values, with a significance *P* value cutoff 0.05. *P*-values for the associations between gene expression levels and each clinical outcome were adjusted for multiple testing using permutation-based false discovery rate with confidence intervals (FDR CIs) (Millstein and Volfson 2013). Analyses were performed using Statistical Analysis Software (SAS) version 9.4 (SAS Institute Inc., Cary, NC, USA), functions *rpart* and *fdrci* of R package (R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>).

RESULTS

Patient Characteristics and Baseline Intratumoral Gene Expression

Of the 508 patients enrolled in TRIBE, 228 (45%) had tumor tissue sufficient for gene expression analysis (Supplemental Figure 1). Patient and tumor characteristics were similar between the FOLFOXIRI-bevacizumab and FOLFIRI-bevacizumab subgroups in the present study and to the overall trial population (Table 1). The median follow-up time for patients in this correlative analysis was 49.9 months. Patients receiving FOLFOXIRI-bevacizumab had superior RR (68.5% vs. 58.8%) and PFS (median PFS: 11.7 vs 10.8 months), with similar OS (median OS: 32.7 vs 33.0 months) (Table 1).

Baseline tumor mRNA expression was determined using RT-PCR for the following genes: *VEGF-A*, *VEGF-B*, *VEGF-C*, *VEGFR1*, *VEGFR2*, *ACVRL1*, *EphB4*, and *EGFL7*. Expression of these genes did not differ significantly between patients in the experimental and control groups (Supplemental Table 2). In subgroup analysis, men had significantly higher *VEGF-B* expression than women (*P* = 0.02). Patients with *KRAS* wildtype tumors had increased *EphB4* expression relative to those with *KRAS* mutant tumors (*P* = 0.04), and patients whose tumors harbored V600E *BRAF* mutations had significantly increased *ACVRL1* expression compared to patients with *BRAF* wildtype tumors (*P* = 0.03). When *RAS* and *BRAF* mutation status were both taken into account, *RAS/BRAF* wildtype tumors had significantly higher *VEGF-A* (*P* = 0.026) and *VEGF-B* (*P* = 0.028) expression, relative to those with either *RAS* or *BRAF* mutations (Supplemental Table 3). There were no

significant differences in baseline gene expression based on primary tumor location (Supplemental Table 4).

Associations between Gene Expression and Progression-Free and Overall Survival

Increased *ACVRL1* was associated with superior PFS (11.7 v. 5.9 months; multivariate HR 0.17, 95% CI 0.06 to 0.49; $P=0.001$) and OS (32.7 v. 13.5 months; HR 0.38, 95% CI 0.17 to 0.88; $P=0.023$) among patients receiving FOLFOXIRI-bevacizumab (Table 2, 3; Figure 1). Within the FOLFIRI-bevacizumab cohort, increased *ACVRL1* was associated with prolonged OS (35.1 v. 22.0 months; HR 0.36, 95% CI 0.17 to 0.74; $P=0.006$), but was not associated with PFS (Table 2, 3; Figure 1).

Elevated *EphB4* levels were associated with inferior PFS in patients receiving FOLFIRI-bevacizumab (9.3 v. 10.8 months; HR 2.46, 95% CI 1.09 to 5.53; $P=0.029$) and FOLFOXIRI-bevacizumab (10.8 v. 12.2 months; HR 2.01, 95% CI 1.03 to 3.95; $P=0.042$) in multivariate analysis, but was not associated with OS in either treatment arm.

Increased *VEGF-C* expression was associated with lack of benefit from FOLFIRI-bevacizumab with regards to PFS (8.6 v. 11.3 months; HR 2.19, 95% CI 1.10 to 4.37; $P=0.026$) in multivariate analysis, but not OS. *VEGF-C* was not associated with PFS or OS in patients receiving FOLFOXIRI-bevacizumab.

VEGFR1 expression had opposing prognostic associations in both treatment groups. In patients randomized to FOLFIRI-bevacizumab, high *VEGFR1* expression was associated with inferior PFS (9.3 v. 20.0 months; HR 4.89, 95% CI 1.24 to 19.27; $P=0.023$). In contrast, in patients receiving FOLFOXIRI-bevacizumab, increased *VEGFR1* was associated with a trend towards improved PFS (11.3 v. 8.9 months; HR 0.23, 95% CI 0.05 to 1.20; $P=0.082$), with a similar trend for OS (33.6 v. 18.8 months; HR 0.51, 95% CI 0.19 to 1.36; $P=0.18$).

In univariate analysis, increased *EGFL7* expression was associated with inferior OS in patients receiving FOLFIRI-bevacizumab (26.3 v. 33.5 months; HR 1.82, 95% CI 1.07 to 3.11; $P=0.020$), but this effect was not seen in multivariable analysis or in patients treated with FOLFOXIRI-bevacizumab.

Lastly, increased *VEGFR2* expression was associated with shortened PFS (9.3 v. 11.6 months; HR 1.97, 95% CI 1.03 to 3.77; $P=0.041$) and OS (32.1 v. 33.5 months; HR 1.88, 95% CI 1.02 to 3.46; $P=0.042$) in patients treated with FOLFIRI-bevacizumab. This trend was not evident in the FOLFOXIRI-bevacizumab group.

Associations between Gene Expression and Overall Response Rate, Early Tumor Shrinkage, and Depth of Response

Among patients receiving FOLFIRI-bevacizumab, decreased *EphB4* (71% v. 23%, $P=0.001$) and *VEGFR2* (77% v. 41%, $P=0.002$) expression were each associated with significantly higher RR compared to those with low expression of either marker. There were no significant relationships between the other genes and RR in either treatment group (Supplemental Table 5).

Spearman correlations were then determined between gene expression levels and early tumor shrinkage (ETS) as well as depth of response (DoR). ETS was defined as the percentage of tumor shrinkage at 8 weeks compared to baseline, and DoR was defined as the percentage of tumor shrinkage at the nadir, in the absence of new lesions or progression of non-target lesions.

We have previously shown that patients enrolled in TRIBE with an ETS $\geq 20\%$ achieved a superior PFS and OS²⁴. Within the FOLFOXIRI-bevacizumab group, low *VEGF-A* expression was associated with ETS ($P = 0.017$). In the FOLFIRI-bevacizumab group, lower *EphB4* ($r^2 = -0.28$, $P = 0.0082$) or *VEGF-C* expression ($r^2 = -0.22$, $P = 0.034$) levels demonstrated modest correlations with DoR. Lastly, using $\geq 20\%$ as a cutoff to dichotomize ETS, low *VEGFR2* ($P = 0.023$) and *EphB4* ($P = 0.049$) expression were both significantly associated with ETS among patients treated with FOLFIRI-bevacizumab (Supplemental Figure 2).

Recursive Partitioning Analysis of Candidate Molecular Markers

ACVRL1 expression was the strongest positive discriminant of OS in both treatment groups, and *VEGF-A* was also a common discerning biomarker between the treatment arms (Supplemental Figure 3). With respect to PFS, *VEGF-C* expression was the main determinant among patients receiving FOLFIRI-bevacizumab, whereas *ACVRL1* was more prognostic in the FOLFOXIRI-bevacizumab group (Supplemental Figure 4). Among the candidate markers, *EphB4* expression provided the best separation between responders and non-responders in the FOLFIRI-bevacizumab group, with low *EphB4* expression associated with increased tumor response (Supplemental Figure 5). In the FOLFOXIRI-bevacizumab group, *ACVRL1* was the primary discriminant for RR. Expression of *VEGF-B* was associated with RR in both treatment groups.

External In Silico Validation of *ACVRL1* Expression

We queried the Oncomine microarray compendium with progressively broad search filters. We found four relevant studies which tested intratumoral *ACVRL1* expression in patients with CRC: TCGA Colorectal,²⁵ Smith Colorectal,²⁶ Smith Colorectal 2,²⁷ and Jorissen Colorectal 3.²⁸ The TCGA dataset only included 19 patients with mCRC who had documented follow-up time. In addition, *ACVRL1* expression was determined using a different platform (Illumina) compared to the other three studies (which employed Human Genome U133 Plus 2.0 Array). Therefore, this dataset was not used for further analysis. Across the remaining three studies which included patients with all stages of CRC, increased *ACVRL1* expression was significantly associated with improved recurrence free survival as well as OS (Table 4). The two studies conducted by Smith et al. included patients with stage IV disease with documented follow-up. Among these patients ($n=56$), there remained a trend between increased *ACVRL1* expression and improved 1-year survival; however, this did not reach statistical significance ($P = 0.28$).

DISCUSSION

To date, no biomarkers exist to select mCRC patients likely to benefit from antiangiogenic based chemotherapy regimens. We examined the baseline intratumoral mRNA expression of genes involved in angiogenesis pathways to identify molecular surrogates of clinical outcomes. Our correlative analysis of the TRIBE study is the first to reveal a potential prognostic role for *ACVRL1* expression in mCRC patients receiving cytotoxic chemotherapy with bevacizumab.

Insights into the development and regulation of tumor vasculature have revealed *VEGF*-independent signaling pathways which partly mediate bevacizumab resistance.¹² One such pathway involves ALK1 (encoded by the *ACVRL1* gene), a member of the transforming growth factor β (TGF β) receptor family, that directs physiologic and pathologic angiogenesis.²⁹ Along with TGF β , ALK1 binds bone morphogenic protein 9 (BMP9) to phosphorylate Smads 1, 5, and 8 and activate transcription of proteins regulating vascular development and remodeling,^{29, 30} along with cellular proliferation.³¹ ALK1 also binds VEGFR3 to promote malignant lymphangiogenesis.^{29, 32} Clinically, tumor *ACVRL1* expression has been associated with survival in head and neck cancer patients,³³ but has not been explored in CRC patients. In our analysis, high *ACVRL1* expression was associated with both PFS and OS, lending evidence for its use as a prognostic marker in cancer patients treated with bevacizumab based chemotherapy.

Another mechanism of VEGF-independent angiogenesis is mediated by EphB4, an aberrantly expressed receptor tyrosine kinase,^{17, 34} with both tumor suppressor and oncogenic roles in CRC development.^{35, 36} EphB4 and its solitary ligand, EphB2,^{37, 38} regulate vasculogenesis through bidirectional signaling,^{39, 40} resulting in the activation of multiple downstream effectors³⁶ (e.g. insulin-like growth factor, fibroblast growth factor, platelet derived growth factor, ephrenergins, amphiregulin) and pathways (e.g. EGFR/Ras/MEK/ERK,³⁵ Wnt- β -catenin,³⁷ PI3K/Akt/mTOR⁴⁰). In a retrospective study¹⁸ of mCRC patients receiving chemotherapy and bevacizumab, *EphB4* expression was inversely associated with tumor response and survival. Similarly, our data showed low *EphB4* expression to be associated with superior PFS in both treatment groups, and of tumor response among patients receiving FOLFIRI-bevacizumab.

In our univariable and recursive partitioning analyses, we identified *EGFL7* as a negative prognosticator with regards to PFS and OS. This is in line with prior investigations associating *EGFL7* overexpression with lymph node metastasis and inferior survival among colon cancer patients.¹⁵ *EGFL7* codes for an extracellular matrix associated protein restricted to endothelial cells and whose expression is normally upregulated during angiogenesis and vascular injury.^{41–44} *In vitro* studies have revealed that *EGFL7* blunts immunestimulatory cytokines (e.g. interferon-gamma) and adhesion molecule production, allowing for escape from the anti-tumor immune response.^{45, 46} In addition, *EGFL7* has been shown to promote tumor angiogenesis partly by activating Notch signaling^{45, 47} and VEGFR3 expression.^{42, 45} In human cell lines, knockdown of *EGFL7* suppresses endothelial cell proliferation and blood vessel formation.⁴² In mice models, *EGFL7* suppression has

been found to enhance the efficacy of anti-VEGF therapeutics.¹⁶ Altogether, our data further supports *EGFL7* as a prognostic marker as well as a potential therapeutic target.

While gene expression levels of different *VEGF* and *VEGFR* isoforms have been correlated with CRC tumor recurrence⁴⁸ and survival,^{6, 49, 50} the evidence has been mixed,^{7, 8} and such discrepancies may be attributed to heterogeneous gene expression within primary tumors⁵¹ and between metastatic sites.^{52, 53} In our study, low *VEGF-C* expression was associated with improved PFS in patients receiving FOLFIRI-bevacizumab. This is consistent with prior studies showing an inverse relationship between *VEGF-C* levels and survival in CRC patients.^{51, 54} Mechanistically, cell line studies have revealed that *VEGF-C*, a ligand for the VEGFR2 and VEGFR3 receptors, promotes malignant angiogenesis and lymphangiogenesis,⁵⁵ as well as proliferation, invasion, and metastasis.⁵⁶ Our study also showed opposing relationships between *VEGFR1* expression and survival in both treatment groups. The significance of these findings is unclear, and given the small number of patients with tissue sufficient for *VEGFR1* analysis, these results should be interpreted with caution.

Notably, *VEGF-A*, the therapeutic target of bevacizumab, did not emerge as a significant biomarker of tumor response or survival in either treatment group of our study. Although our recursive partitioning analysis identified *VEGF-A* as a potential marker for OS, the strength of this association is limited given the crossing of the corresponding Kaplan-Meier curves (data not shown). This is consistent with findings from the AVF2107 trial, which randomized patients to treatment with 5-fluorouracil and irinotecan with or without bevacizumab and also failed to demonstrate predictive or prognostic associations for *VEGF-A* expression.⁹ Conversely, the *VEGF-A* splice variant, VEGF_{165b}, may be a more appropriate marker for bevacizumab efficacy as supported by a correlative analysis of the ECOG 3200 trial.¹¹

It is noteworthy that *VEGF-C* emerged as a marker of DoR and PFS, though it was not significantly associated with RECIST-defined overall response rate. In line with findings from the PRIME,⁵⁷ CRYSTAL⁵⁸ and OPUS⁵⁸ trials, a *post-hoc* analysis of the TRIBE study²⁴ demonstrated significant associations between ETS, DoR and survival. Our current study now provides a potential molecular surrogate to characterize such associations. Mindful of the limitations of subgroup analysis, these findings merit further investigation and suggest that refining efficacy outcomes to account for the onset and extent of response is necessary in patients receiving targeted therapies.

Certain limitations of our study should be acknowledged. Given the relatively small sample size of our retrospective analysis, larger biomarker-embedded prospective trials are required to validate our findings. Importantly, the lack of a control group not receiving bevacizumab in the TRIBE study limits our ability to draw any conclusions about the predictive value of these biomarkers, which is more clinically relevant. Similarly, the specificity of the prognostic association of *ACVRL1* expression to anti-angiogenic therapy is limited as all patients received both chemotherapy and bevacizumab. Nonetheless, these findings are clinically meaningful as there is no role for anti-angiogenic monotherapy in the management of mCRC. Intratumoral variability in gene expression, including potential discrepancies between primary and metastatic tissue may have also affected the results of the current

study. Moreover, tumor-stromal differences in gene expression, and treatment-induced changes over time are factors which warrant exploration.

In summary, our study provides evidence for *ACVRL1* as a potential biomarker in mCRC patients receiving first-line chemotherapy and bevacizumab. As angiogenic mechanisms are complex, composite molecular profiles, rather than one particular marker, are likely necessary to adequately inform clinical decisions. Future translational investigations should delineate interactions between angiogenesis pathways which may affect the utility of these biomarkers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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CLINICAL PRACTICE POINTS

- Bevacizumab improves survival in mCRC patients treated with chemotherapy, yet not all patients benefit from anti-angiogenic therapy.
- No validated biomarkers exist to predict benefit from anti-angiogenic based chemotherapy regimens.
- *ACVRL1* encodes for ALK1, a member of the TGF β receptor family, which directs pathologic angiogenesis.
- We examined intratumoral expression of markers of angiogenesis (*VEGF-A*, *VEGF-B*, *VEGF-C*, *VEGFR1*, *VEGFR2*, *ACVRL1*, *EphB4* and *EGFL7*) in mCRC enrolled in the TRIBE study.
- We found that high *ACVRL1* expression was associated with superior OS in both treatment arms.
- In recursive partitioning analysis, *ACVRL1* was the strongest discriminator of RR, PFS, and OS in patients receiving FOLFOXIRI-bevacizumab, and of OS in patients receiving FOLFIRI-bevacizumab.
- *In silico* validation revealed significant associations between *ACVRL1* expression, disease recurrence, and 1-year survival ($P<0.05$) among all CRC stages.
- *ACVRL1* expression may serve as a prognostic biomarker in mCRC patients receiving bevacizumab based chemotherapy and warrants further evaluation in prospective studies.
- These results may guide the development of biomarker-embedded trials and the selection of colorectal cancer patients likely to benefit from anti-angiogenic regimens.

Figure 1.
Kaplan-Meier Cumulative Survival Curves Stratified by Gene Expression Levels and Treatment Arm
A. OS by *ACVRL1* Expression in Patients Receiving FOLFIRI-Bevacizumab
B. OS by *ACVRL1* Expression in Patients Receiving FOLFOXIRI-Bevacizumab

Table 1

Patient and Tumor Characteristics

Characteristic	FOLFIRI-Bevacizumab		FOLFOXIRI-Bevacizumab		P
	TRIBE Population(N = 253)	Study Subset(N = 115)	TRIBE Population(N = 250)	Study Subset(N = 113)	
Age, years					0.29
Median (range)	60 (29–75)	60 (29–74)	61 (29–75)	59 (33–75)	
Sex					0.57
Male	153 (60%)	66 (57%)	148 (59%)	69 (61%)	
Female	100 (40%)	49 (43%)	102 (41%)	44 (39%)	
Primary Tumor Site					0.59
Right	61 (26%)	37 (33%)	87 (36%)	40 (37%)	
Left	175 (74%)	76 (67%)	152 (64%)	69 (63%)	
Both	1 (0.4%)	0 (0%)	0 (0%)	0 (0%)	
Resection of Primary Tumor					0.35
Yes	165 (65%)	98 (85%)	173 (69%)	101 (89%)	
Number of Metastatic Sites					0.34
0–1	105 (42%)	43 (37%)	108 (43%)	53 (47%)	
2	103 (41%)	48 (42%)	102 (41%)	41 (36%)	
3–5	45 (18%)	24 (21%)	40 (16%)	19 (17%)	
Time to Metastases					0.93
Synchronous	204 (81%)	89 (77%)	196 (78%)	88 (78%)	
Metachronous	49 (19%)	26 (23%)	54 (22%)	25 (22%)	
Liver Limited Metastases					0.098
Yes	77 (30%)	31 (27%)	87 (35%)	42 (37%)	
KRAS Mutation Status					0.43
Wildtype	99 (39%)	51 (44%)	94 (38%)	48 (42%)	
Mutant	95 (38%)	60 (52%)	103 (41%)	64 (57%)	
Unknown	59 (23%)	4 (3%)	53 (21%)	1 (1%)	
BRAF Mutation Status					0.67
Wildtype	182 (72%)	104 (90%)	181 (72%)	100 (88%)	
Mutant	12 (5%)	8 (7%)	16 (6%)	11 (10%)	

Characteristic	FOLFIRI-Bevacizumab		FOLFOXIRI-Bevacizumab		P
	TRIBE Population(N = 253)	Study Subset(N = 115)	TRIBE Population(N = 250)	Study Subset(N = 113)	
Unknown	59 (23%)	3 (3%)	53 (21%)	2 (2%)	0.80
RAS/BRAF Mutation Status					
Wildtype	45 (18%)	24 (21%)	48 (19%)	25 (22%)	
RAS Mutant	118 (47%)	73 (63%)	116 (46%)	72 (64%)	
BRAF Mutant	12 (5%)	8 (7%)	16 (6%)	11 (10%)	
Unknown	78 (31%)	10 (9%)	70 (28%)	5 (4%)	0.21
Adjuvant Chemotherapy*					
Yes	32 (13%)	20 (17%)	32 (13%)	13 (12%)	0.41
ECOG Performance Status					
0	210 (83%)	101 (88%)	224 (90%)	103 (91%)	
1–2	42 (17%)	14 (13%)	26 (10%)	10 (9%)	0.89
High LDH**					
No	99 (39%)	46 (40%)	84 (34%)	43 (38%)	
Yes	122 (48%)	55 (48%)	129 (52%)	54 (48%)	
Unknown	32 (13%)	14 (12%)	37 (15%)	16 (14%)	
Response Rate (RR)	53.1%	58.8%	65.1%	68.5%	0.13
Median PFS, months (95% CI)	9.7(9.3–10.9)	10.8(9.4–11.6)	12.1(10.9–13.2)	11.7(10.2–13.4)	0.21
Median OS, months(95% CI)	25.8(22.7–30.8)	33.0(24.8–39.6)	31.0(26.9–35.1)	32.7(26.7–36.8)	0.77

* Adjuvant chemotherapy received 12 months within study enrollment

** LDH greater than 300 IU/L.

Association between Intratumoral Gene Expression Levels and Progression-Free Survival

Table 2

Gene	N	FOLFIRI+BEV			FOLFOXIRI+BEV		
		Median PFS (months, 95% CI)	Univariate HR (95% CI) [†]	Multivariate HR (95% CI) [‡]	Median PFS (months, 95% CI)	Univariate HR (95% CI) [†]	Multivariate HR (95% CI) [‡]
VEGF-A							
5.715	40	11.7 (8.6, 18.2)	1 (reference)	1 (reference)	11.2 (9.3, 12.2)	1 (reference)	1 (reference)
>5.715	43	9.4 (8.4, 11.6)	1.63 (0.93, 2.84)	1.64 (0.82, 3.30)	11.4 (9.3, 13.5)	1.15 (0.69, 1.94)	1.31 (0.75, 2.28)
<i>P</i> value*			0.42	0.16		0.58	0.35
VEGF-B							
3.295	29	11.7 (8.8, 20.0)	1 (reference)	1 (reference)	10.4 (7.9, 12.2)	1 (reference)	1 (reference)
>3.295	50	9.4 (7.8, 11.3)	1.84 (0.98, 3.46)	1.67 (0.82, 3.43)	11.9 (9.5, 14.0)	0.92 (0.53, 1.59)	0.89 (0.47, 1.69)
<i>P</i> value*			0.24	0.16		0.75	0.72
VEGF-C							
0.135	69	11.3 (9.7, 16.1)	1 (reference)	1 (reference)	10.7 (9.0, 13.7)	1 (reference)	1 (reference)
>0.135	22	8.6 (6.7, 9.7)	2.15 (1.14, 4.07)	2.19 (1.10, 4.37)	10.8 (9.3, 33.6)	0.62 (0.34, 1.15)	0.59 (0.30, 1.18)
<i>P</i> value*			0.057			0.12	0.14
VEGFR1							
0.245	11	20.0 (9.5, 30.8)	1 (reference)	1 (reference)	8.9 (2.2, 16.9)	1 (reference)	1 (reference)
>0.245	28	9.3 (6.2, 11.9)	3.34 (1.24, 9.03)	4.89 (1.24, 19.27)	11.3 (9.5, 22.0)	0.51 (0.20, 1.28)	0.23 (0.05, 1.20)
<i>P</i> value*						0.11	0.082
VEGFR2							
0.52	35	11.6 (9.5, 16.6)	1 (reference)	1 (reference)	9.9 (7.4, 12.1)	1 (reference)	1 (reference)
>0.52	37	9.3 (7.8, 13.4)	1.40 (0.80, 2.48)	1.97 (1.03, 3.77)	11.3 (9.5, 12.8)	0.91 (0.51, 1.63)	0.81 (0.43, 1.52)
<i>P</i> value*			0.83			0.74	0.51
ACVRL1							
0.40	10	8.8 (1.9, 11.1)	1 (reference)	1 (reference)	5.9 (0.9, 12.0)	1 (reference)	1 (reference)
>0.40	95	10.8 (9.3, 12.7)	0.50 (0.24, 1.02)	0.51 (0.25, 1.06)	11.7 (10.3, 15.0)	0.30 (0.11, 0.79)	0.17 (0.06, 0.49)
<i>P</i> value*			0.38	0.071			

Gene	N	FOLFIRI+BEV			FOLFOXIRI+BEV		
		Median PFS (months, 95% CI)	Univariate HR (95% CI) [†]	Multivariate HR (95% CI) [‡]	Median PFS (months, 95% CI)	Univariate HR (95% CI) [†]	Multivariate HR (95% CI) [‡]
EphA4							
0.435	75	10.8 (9.4, 11.5)	1 (reference)	1 (reference)	12.2 (9.9, 15.6)	1 (reference)	1 (reference)
>0.435	13	9.3 (3.5, 12.7)	1.94 (0.89, 4.21)	2.46 (1.09, 5.53)	10.8 (7.7, 12.0)	1.64 (0.87, 3.11)	2.01 (1.03, 3.95)
<i>P</i> value*			0.27			0.089	
EGFL7							
0.435	82	11.1 (8.8, 12.7)	1 (reference)	1 (reference)	10.8 (9.3, 15.7)	1 (reference)	1 (reference)
>0.435	21	9.7 (6.2, 13.1)	1.43 (0.79, 2.61)	0.92 (0.47, 1.77)	11.7 (9.5, 13.7)	1.14 (0.66, 1.97)	1.12 (0.61, 2.05)
<i>P</i> value*			0.68	0.79		0.62	0.71

* Based on adjusted *P*value for FOLFIRI Arm and log-rank test for FOLFOXIRI + BEV arm in the univariable analysis ([†]).

[‡]*P*value was based on Wald test in the multivariable Cox regression model ([‡]) adjusted for age, gender, BRAF mutation status, ECOG, number of metastatic sites, and time to metastases.

Table 3

Association between Intratumoral Gene Expression Levels and Overall Survival

Gene	N	FOLFIRI+BEV			FOLFOXIRI+BEV		
		Median OS (months, 95% CI)	Univariate HR (95% CI)	Multivariate HR (95% CI) ‡	Median OS (months, 95% CI)	Univariate HR (95% CI)	Multivariate HR (95% CI) ‡
VEGF-A							
5.715	40	32.3 (21.9, 50.3)	1 (Reference)	1 (Reference)	30.2 (19.0, 36.1)	1 (Reference)	1 (Reference)
>5.715	43	32.7 (20.6, 43.6)	1.19 (0.71, 1.99)	1.00 (0.52, 1.89)	38.2 (28.2, 44.3)	0.70 (0.42, 1.17)	0.70 (0.40, 1.22)
<i>P</i> value*			0.50	0.99		0.16	0.21
VEGF-B							
3.295	29	32.1 (20.5, 49.1)	1 (Reference)	1 (Reference)	26.7 (18.2, 42.0)	1 (Reference)	1 (Reference)
>3.295	50	33.1 (20.8, 43.6)	1.06 (0.61, 1.82)	0.89 (0.48, 1.62)	35.0 (26.2, 42.7)	0.69 (0.41, 1.18)	0.79 (0.46, 1.38)
<i>P</i> value*			0.84	0.69		0.17	0.42
VEGF-C							
0.135	69	37.8 (25.2, 48.7)	1 (Reference)	1 (Reference)	28.5 (19.6, 35.0)	1 (Reference)	1 (Reference)
>0.135	22	32.6 (14.7, 39.8)	1.45 (0.83, 2.54)	1.60 (0.86, 2.97)	38.2 (24.1, 70.2)	0.69 (0.39, 1.20)	0.68 (0.37, 1.22)
<i>P</i> value*			0.18	0.13		0.19	0.19
VEGFR1							
0.245	11	55.6 (18.8, 55.6)	1 (Reference)	1 (Reference)	18.8 (9.0, 40.9)	1 (Reference)	1 (Reference)
>0.245	28	23.8 (14.1, 32.7)	3.72 (1.35, 10.24)	3.15 (0.91, 10.88)	33.6 (24.1, 44.3)	0.43 (0.19, 0.99)	0.51 (0.19, 1.36)
<i>P</i> value*				0.070			0.18
VEGFR2							
0.52	35	33.5 (22.0, 49.1)	1 (Reference)	1 (Reference)	27.3 (19.0, 44.3)	1 (Reference)	1 (Reference)
>0.52	37	32.1 (20.3, 37.8)	1.28 (0.75, 2.18)	1.88 (1.02, 3.46)	32.7 (19.8, 41.4)	1.04 (0.59, 1.83)	0.93 (0.50, 1.71)
<i>P</i> value*			0.35			0.89	0.81
ACVRL1							
0.40	10	22.0 (5.7, 25.6)	1 (Reference)	1 (Reference)	13.5 (1.1, 34.3)	1 (Reference)	1 (Reference)
>0.40	95	35.1 (26.3, 43.6)	0.38 (0.19, 0.77)	0.36 (0.17, 0.74)	32.7 (27.3, 41.4)	0.39 (0.18, 0.86)	0.38 (0.17, 0.88)
<i>P</i> value*							

Gene	N	FOLFIRI+BEV			FOLFOXIRI+BEV		
		Median OS (months, 95% CI)	Univariate HR (95% CI)	Multivariate HR (95% CI) ‡	Median OS (months, 95% CI)	Univariate HR (95% CI)	Multivariate HR (95% CI) ‡
EphA4							
0.435	75	35.1 (26.1, 45.0)	1 (Reference)	1 (Reference)	34.3 (28.1, 43.1)	1 (Reference)	1 (Reference)
>0.435	13	32.1 (10.2, 44.4)	1.51 (0.78, 2.93)	1.66 (0.83, 3.34)	28.2 (16.3, 42.0)	1.52 (0.88, 2.63)	1.38 (0.77, 2.47)
<i>P</i> value *			0.20	0.15		0.12	0.28
EGFL7							
0.435	82	33.5 (23.9, 45.0)	1 (Reference)	1 (Reference)	30.8 (20.4, 40.9)	1 (Reference)	1 (Reference)
>0.435	21	26.3 (14.1, 39.6)	1.82 (1.07, 3.11)	1.35 (0.77, 2.35)	33.6 (26.2, 56.8)	0.82 (0.48, 1.39)	0.94 (0.54, 1.63)
<i>P</i> value *				0.29		0.46	0.82

* Based on log-rank test in the univariable analysis (†).

‡ *P*value was based on Wald test in the multivariable Cox regression model (‡) adjusted for age, gender, BRAF mutation status, ECOG, number of metastatic sites, and timing to metastases.

Table 4

ACVRL1 In Silico

Analysis

Study Sample	Ref.	Endpoint	Gene Reporter	N1 (alive or no event)	N2 (dead or event)	Fold Change	P value
Smith Colorectal	[27]	1-year recurrence	226950_at	130	8	-1.349	
		3-year recurrence	226950_at	77	31	-1.147	
		5-year recurrence	226950_at	38	34	-1.227	
		1-year survival	226950_at	153	21	-1.191	
		3-year survival	210838_s_at	103	51	-1.095	
		5-year survival	226950_at	52	67	-1.178	
Smith Colorectal 2	[28]	3-year recurrence	210838_s_at	30	17	-1.201	
Jorissen Colorectal 3	[29]	3-year recurrence	226950_at	71	19	-1.381	
		5-year recurrence	226950_at	30	56	-1.463	
Smith + Smith 2 (Stage IV)	[27, 28]	1-year survival	226950_at	36	20	-1.123	0.28