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Plasma VEGF-A Short Isoforms as a Predictive Biomarker for Bevacizumab Efficacy in First-Line Treatment of Metastatic Colorectal Cancer: A Phase II Study (WJOG7612GTR)

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Abstract

This prospective study was designed to determine whether plasma vascular endothelial growth factor-A short isoforms (pVEGF-Asi) could predict responsiveness to bevacizumab (BV) and to investigate additional circulating biomarkers in metastatic colorectal cancer (mCRC) patients receiving modified FOLFOX6/XELOX in combination with BV (mFOLFOX6/XELOX + BV). Plasma samples obtained before treatment initiation were collected from 100 mCRC patients treated with first-line mFOLFOX6/XELOX + BV. Levels of 11 angiogenesis-related circulating proteins, including pVEGF-Asi, together with 22 cancer-related gene mutations identified in circulating tumor DNA, were evaluated. For the primary endpoint, the hazard ratio (HR) for progression-free survival (PFS), estimated using a Cox proportional hazards model, was prespecified to be <1.15 when comparing patients classified as having high versus low pVEGF-Asi levels based on the median value. The median pVEGF-Asi concentration was 37 pg/ml (range 6.5–262). Comparison of high and low pVEGF-Asi groups yielded an HR for PFS of 1.3 [95% confidence interval (CI) 0.8–2.1; log-rank, P = 0.25], exceeding the predefined criterion of 1.15. Multivariate analysis identified significant associations between PFS and plasma intercellular adhesion molecule-1 (pICAM-1) (≥190.0 versus <190.0 ng/ml; HR 2.1; 95% CI 1.3–3.5), RAS mutation status (mutant versus wild type; HR 2.5; 95% CI 1.5–4.3), and FBXW7 mutation status (mutant versus wild type; HR 2.8; 95% CI 1.2–6.8). Overall survival was significantly correlated with pICAM-1 (HR 2.0; 95% CI 1.1–3.7) and RAS mutations (HR 2.6; 95% CI 1.5–4.6). BV administration did not mitigate the unfavorable PFS associated with elevated pVEGF-Asi levels, indicating that pVEGF-Asi is unlikely to serve as an effective predictive biomarker for mFOLFOX6/XELOX + BV treatment. Additional studies are required to clarify the clinical relevance of circulating ICAM-1 levels and mutations in RAS and FBXW7.

Keywords: Metastatic colorectal cancer, Circulating biomarkers, pVEGF-A short isoforms, pICAM-1 level, RAS and FBXW7 mutation

Introduction

Angiogenesis and tumor progression are critically regulated by the vascular endothelial growth factor (VEGF) signaling pathway [1]. Elevated VEGF expression and increased circulating VEGF concentrations have been linked to disease advancement, metastatic spread, and unfavorable prognosis in gastrointestinal malignancies, including colorectal cancer (CRC) [2–5]. Bevacizumab (BV) is a monoclonal antibody with anti-angiogenic activity that neutralizes VEGF-A and suppresses downstream VEGF signaling [6]. Clinically, BV is routinely administered in combination with cytotoxic chemotherapy regimens, such as fluorouracil (FU) and leucovorin (LV) combined with oxaliplatin (FOLFOX or XELOX) or irinotecan (FOLFIRI), as standard first- and/or second-line treatment for metastatic CRC (mCRC) [7]. Despite its

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widespread use, no validated biomarker is currently available to predict BV treatment efficacy.

Given that VEGF-A is the direct molecular target of BV, circulating VEGF-A levels have been proposed as potential predictors of therapeutic benefit. VEGF-A comprises multiple isoforms generated by alternative RNA splicing. Shorter isoforms, including VEGF-A110 and VEGF-A121, lack basic amino acid domains required for extracellular matrix binding and therefore circulate freely, whereas longer isoforms bind to heparin and heparan sulfate proteoglycans within the extracellular matrix [8–10]. These biological properties suggest that plasma VEGF-A short isoform concentrations (pVEGF-Asi) may reflect tumor-derived VEGF-A secretion. Measurement of pVEGF-Asi using the immunological multiparametric chip technique (IMPACT) has been reported to predict BV benefit in advanced gastric cancer [11] and pancreatic cancer [12], but not in mCRC, non-small-cell lung cancer, or renal cell carcinoma [13]. Variability among studies may be related to differences in plasma collection procedures [ethylenediamine tetraacetic acid (EDTA) versus citrate], with EDTA-based sampling being recommended [5, 13, 14]. However, the predictive value of pVEGF-Asi measured in EDTA-collected plasma for BV efficacy in mCRC remains unresolved.

Analysis of circulating DNA has emerged as a non-invasive strategy for detecting tumor-associated genetic alterations and for estimating prognosis and treatment response in various malignancies. Increased levels of total circulating cell-free DNA (cfDNA) have been associated with poor outcomes in mCRC patients prior to oxaliplatin-based chemotherapy [15]. In addition, circulating tumor DNA (ctDNA) has demonstrated clinical relevance in mCRC, with KRAS and NRAS (RAS) mutation status [16, 17] and ERBB2 amplification detected in ctDNA being associated with responses to anti-epidermal growth factor receptor (EGFR) antibodies and anti-human epidermal growth factor receptor-2 therapies, respectively [18].

Based on these observations, we conducted a single-arm phase II study to evaluate associations between candidate biomarkers, including pVEGF-Asi and ctDNA, and clinical outcomes in mCRC patients treated with modified FOLFOX6 plus BV (mFOLFOX6 + BV) or XELOX plus BV (XELOX + BV) as first-line therapy.

MTC staging.

Materials and Methods

Patient selection

Eligibility was limited to individuals diagnosed by histology with unresectable metastatic colorectal cancer (mCRC) who had not previously undergone systemic chemotherapy. Allowed exceptions included adjuvant fluoropyrimidine monotherapy completed more than 180 days earlier or adjuvant regimens containing oxaliplatin finished more than 1 year prior to disease recurrence. Candidates were additionally required to have an Eastern Cooperative Oncology Group performance status (PS) of 0–1. All participants provided written informed consent. Ethical approval for the protocol was obtained from the institutional review boards of all participating centers. The study was conducted in accordance with the Declaration of Helsinki and was registered in the University Hospital Medical Information Network (UMIN) Clinical Trials Registry under the identifier UMIN000012442.

Study design

This investigation was performed as a multicenter, non-randomized, phase II clinical trial coordinated by the West Japan Oncology Group in Japan. Patients were treated with either mFOLFOX6 plus BV or XELOX plus BV, based on the treating physician's judgment. The primary outcome measure was progression-free survival (PFS), evaluated by comparing patients stratified into high and low pVEGF-Asi groups using the median pVEGF-Asi value as the cutoff. PFS was defined as the interval from study enrollment to documented tumor progression or death from any cause. Secondary outcome measures included overall survival (OS), overall response rate (ORR), and safety, with additional exploratory analyses assessing associations between plasma biomarkers and these endpoints.

Treatments

Participants received either BV at a dose of 5 mg/kg followed by mFOLFOX6, consisting of oxaliplatin (85 mg/m² administered intravenously), l-leucovorin (200 mg intravenously), and fluorouracil (FU) given as a 400 mg/m² intravenous bolus followed by a 2400 mg/m² continuous infusion over 46 h every 2 weeks, or BV at 7.5 mg/kg followed by XELOX, comprising oxaliplatin (130 mg/m² intravenously on day 1) and oral capecitabine (1000 mg/m² twice daily from the evening of day 1 through the morning of day 15, totaling 28 doses) administered every 3 weeks. Treatment cycles

continued until radiographic disease progression, unacceptable toxicity, or patient withdrawal.

Evaluation

Baseline imaging studies using computed tomography or magnetic resonance imaging were performed within 28 days before enrollment and were repeated at 8-week intervals until disease progression was confirmed. Tumor response was determined by the investigators in accordance with RECIST version 1.1. Clinical assessments, including symptom evaluation, physical examination, and laboratory testing, were conducted at least on day 1 of each treatment cycle and additionally as clinically indicated. Adverse events were categorized and graded using the Common Terminology Criteria for Adverse Events, version 4.0.

Sample collection and processing

Tumor samples preserved as formalin-fixed, paraffin-embedded (FFPE) blocks and obtained at initial diagnosis were retrospectively retrieved. Venous blood (14 ml) was drawn into EDTA-containing collection tubes immediately before the first and second treatment cycles, as well as at protocol completion. All blood samples were processed within 1 h of collection by centrifugation at 1200 × g for 10 min. Genomic DNA was extracted from FFPE tumor tissue using the Allprep DNA/RNA FFPE Kit (Qiagen, Valencia, CA) in accordance with standard procedures. Plasma-derived circulating tumor DNA (ctDNA) was purified using the cobas® cfDNA Sample Preparation Kit (Roche Diagnostics Ltd., Penzberg, Germany). The yield and purity of isolated nucleic acids were confirmed using the PicoGreen dsDNA Reagent (Thermo Scientific, Wilmington, DE).

Somatic mutation analysis

Amplicon-based next-generation sequencing was applied to both tumor DNA and ctDNA utilizing the Ion AmpliSeq™ Colon and Lung Cancer Research Panel (version 2; Thermo Fisher Scientific K.K., Tokyo, Japan), which covers 22 genes implicated in cancer: KRAS, EGFR, BRAF, PIK3CA, AKT1, ERBB2, PTEN, NRAS, STK11, MAP2K1, ALK, DDR2, CTNNB1, MET, TP53, SMAD4, FBXW7, FGFR3, NOTCH1, ERBB4, FGFR1, and FGFR2. Library preparation involved multiplex PCR amplification of up to 10 ng of tissue DNA or ctDNA using the Ion AmpliSeq Library Kit 2.0 (Life Technologies, Carlsbad, CA), following the manufacturer's workflow. After purification, libraries were combined and sequenced on the Ion Torrent Proton platform using the Ion PI Hi-Q Chef Kit and Ion PI Chip Kit v3 (Life Technologies). Sequence data were processed with Torrent Suite software version 5.10 (Life Technologies). Alignment was performed against the hg19 human reference genome, and variant detection was carried out using Variant Caller version 5.10, as previously described [19].

Plasma proteins analysis

Quantification of plasma biomarkers—including fibroblast growth factor-2 (pFGF-2), Fms-related tyrosine kinase 1 (pFLT1), FLT4 (pFLT4), intercellular adhesion molecule-1 (pICAM-1), interleukin 8 (pIL-8), kinase insert domain receptor (pKDR), platelet-derived growth factor-C (pPDGF-C), placental growth factor (pPGF), selectin E (pSELE), VEGF-A (pVEGF-A), and VEGF-C (pVEGF-C)—was performed using the IMPACT-2 platform, a proprietary multiplex enzyme-linked immunosorbent assay developed by Roche Diagnostics Ltd. This analytical system preferentially detects short VEGF-A isoforms (VEGF-A110 and VEGF-A121) with greater sensitivity than longer isoforms (VEGF-A165 and VEGF-A189).

Statistical analysis

Patients were stratified into high- or low-expression groups for each plasma biomarker using the corresponding median concentration as the cutoff. The working hypothesis assumed that patients with elevated pVEGF-Asi levels would derive greater benefit from bevacizumab (BV), thereby neutralizing their otherwise inferior prognosis in the absence of BV; under this assumption, the expected hazard ratio (HR) for progression-free survival (PFS) between groups was 1.0. Conversely, the null hypothesis postulated that outcomes would remain poorer among patients with high pVEGF-Asi levels despite BV-containing chemotherapy (HR ≥1.15). To reject the null hypothesis with a two-sided α error of 5% and 80% statistical power, approximately 90 PFS events were required. Allowing for potential cases in which biomarker assessment was not feasible, total enrollment was set at 100 patients.

For exploratory univariate analyses evaluating relationships between circulating biomarkers and either PFS or overall survival (OS), a P value ≤0.01 was considered indicative of clinical relevance in order to account for multiple testing. Somatic mutations detected

in fewer than five patients were excluded from statistical evaluation due to insufficient sample size.

Both univariate and multivariate analyses were conducted using Cox proportional hazards regression models. Associations between mutational status and baseline patient characteristics were examined using the chi-square (χ^2) test. Survival outcomes were estimated using Kaplan–Meier methodology, with intergroup comparisons performed via the log-rank test. Statistical computations were carried out using JMP software (version 14.0; SAS Institute, Cary, NC) and GraphPad Prism (version 8; GraphPad Software Inc., La Jolla, CA).

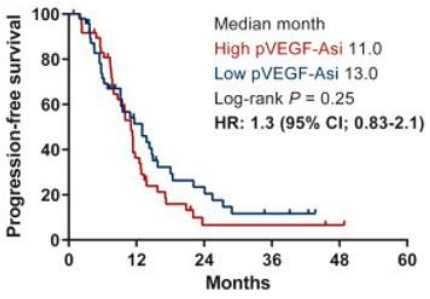
Results and Discussion

Patient characteristics and treatment outcomes

From January 2014 through April 2015, a total of 102 patients were registered across 23 participating centers, and eligibility was confirmed for 100 individuals who were subsequently analyzed. Among the eligible cohort, 52 patients received mFOLFOX6 + BV, while 48 were treated with XELOX + BV. For the entire study population, median progression-free survival (PFS) was 11.4 months [95% confidence interval (CI) 9.5–13.0 months], the overall response rate (ORR) was 65.9% (95% CI 54.6%–76.0%), and median overall survival (OS) was 33.7 months (95% CI 28.7–38.1 months). Comparisons between treatment regimens showed numerically longer PFS (HR 0.66; 95% CI 0.41–1.04; P = 0.075) and OS (HR 0.71; 95% CI 0.43–1.16; P = 0.175) in patients receiving mFOLFOX6 + BV compared with XELOX + BV, although these differences did not reach statistical significance.

Association of pVEGF-Asi level with PFS, ORR, and OS

Plasma samples obtained before treatment initiation were available from all 100 eligible patients; however, pVEGF-Asi quantification failed in three cases. Using the median plasma concentration of pVEGF-Asi (37 pg/ml; range 6.5–262.0 pg/ml) as the cutoff value, patients were categorized into a high-level group (n = 49) and a low-level group (n = 48). Baseline clinical characteristics were largely balanced between groups, with the exception of performance status (PS), as a PS of 1 was more frequently observed among patients with high pVEGF-Asi levels (35%) than among those with low levels (17%) **(Table 1)**. The ORR did not differ significantly between the low and high pVEGF-Asi groups (65% versus 69%; P = 0.69). Analysis of PFS demonstrated that patients with elevated pVEGF-Asi had a hazard ratio of 1.3 relative to those with lower levels (95% CI 0.8–2.1; median PFS 11 versus 13 months; P = 0.25), indicating that the predefined null hypothesis of an HR ≥1.15 could not be excluded **(Figure 1a)**. In contrast, OS was significantly reduced in the high pVEGF-Asi group, with a hazard ratio of 1.7 (95% CI 1.1–2.9; median OS 26 versus 38 months; P = 0.029**) (Figure 1b)**. Given the established prognostic relevance of BRAF mutations, additional analyses were performed after excluding patients with BRAF-mutant tumors (n = 7 based on tissue DNA and n = 4 based on baseline ctDNA).



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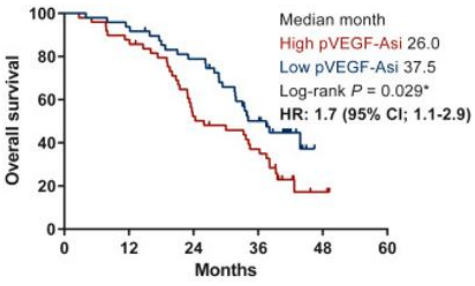

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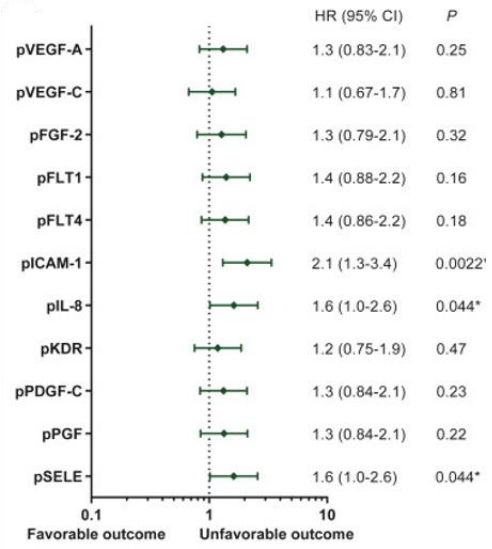

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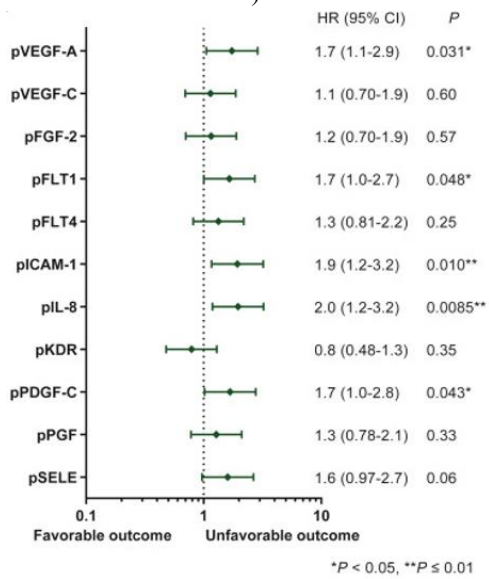


Figure 1. Associations between plasma angiogenesis-related proteins and clinical outcomes.

Table 1. Baseline characteristics stratified by pVEGF-Asi status

Characteristic		**Pre-treatment plasma VEGF-A level**	
	p-value	High (n=49)	Low (n=48)
		n %	n %
Gender			
Male	0.69	24; 49.0	26; 54.2
Female		25; 51.0	22; 45.8
Age (years)	65.0 (44–78)	62.5 (36–78)	Median (range)
Chemotherapy regimen			
mFOLFOX6 + bevacizumab	0.42	23; 46.9	27; 56.3
XELOX + bevacizumab		26; 53.1	21; 43.8
ECOG performance status			
0	0.063	32; 65.3	40; 83.3
1		17; 34.7	8; 16.7
Primary tumor location			
Colon	0.54	31; 63.3	27; 56.3
Rectum		18; 36.7	21; 43.8
Tumor sidedness			
Right-sided	1.0	15; 30.6	14; 29.2
Left-sided		34; 69.4	34; 70.8
Histology			
Papillary or tubular	1.0	42; 85.7	43; 89.6
Poorly differentiated, mucinous, or signet-ring cell		6; 12.2	5; 10.4
Unknown		1; 2.0	0; 0
RAS mutation status (ctDNA)			
Mutant	0.29	15; 30.6	20; 41.7
Wild-type		34; 69.4	28; 58.3
BRAF mutation status (ctDNA)			

Mutant	0.36	1; 2.0	3; 6.3
Wild-type		48; 98.0	45; 93.8

BV, bevacizumab; mFOLFOX6, modified FOLFOX6; PS, performance status; pVEGF-Asi, plasma vascular endothelial growth factor-A short isoforms; left, descending colon, sigmoid colon, rectosigmoid colon, and rectum; right, cecum, ascending colon, and transverse colon; muc, mucinous adenocarcinoma; pap, papillary; por, poorly differentiated adenocarcinoma; tub, tubular adenocarcinoma; sig, signet-ring cell carcinoma.

Other circulating biomarkers of plasma protein

Forest plots illustrating hazard ratios for PFS and OS according to high versus low expression levels are shown in **Figures 1a and 1d**, respectively. Clinically meaningful associations were identified for plasma intercellular adhesion molecule-1 (pICAM-1) with PFS (HR 2.1; 95% CI 1.3–3.4; P = 0.002) and OS (HR 1.9; 95% CI 1.2–3.2; P = 0.010), as well as for plasma interleukin 8 (pIL-8) with OS (HR 2.0; 95% CI 1.2–3.2; P = 0.009). ORRs in patients with low versus high biomarker levels were 79% and 59% for pICAM-1 (P = 0.05) and 71% and 64% for pIL-8 (P = 0.51), respectively. Changes in circulating protein levels measured before the first and second chemotherapy cycles and at treatment completion were also analyzed in relation to treatment response. No statistically significant associations were identified between biomarker dynamics and response categories (complete or partial response versus stable or progressive disease) for any of the 11 assessed proteins.

Correlation of somatic mutations with clinical outcomes

Somatic mutation profiling was conducted using 99 FFPE tumor specimens and 100 baseline plasma ctDNA samples. Successful sequencing was achieved in 80 FFPE samples and all ctDNA samples. Thirteen distinct genomic alterations detected across the cohort are summarized in **Figure 2**. Differences in mutation frequencies between archival tumor tissue and pre-treatment ctDNA were observed for several genes, including TP53 (61% versus 47%), KRAS (53% versus 35%), FBXW7 (14% versus 8%), PIK3CA (13% versus 5%), and BRAF (9% versus 4%).

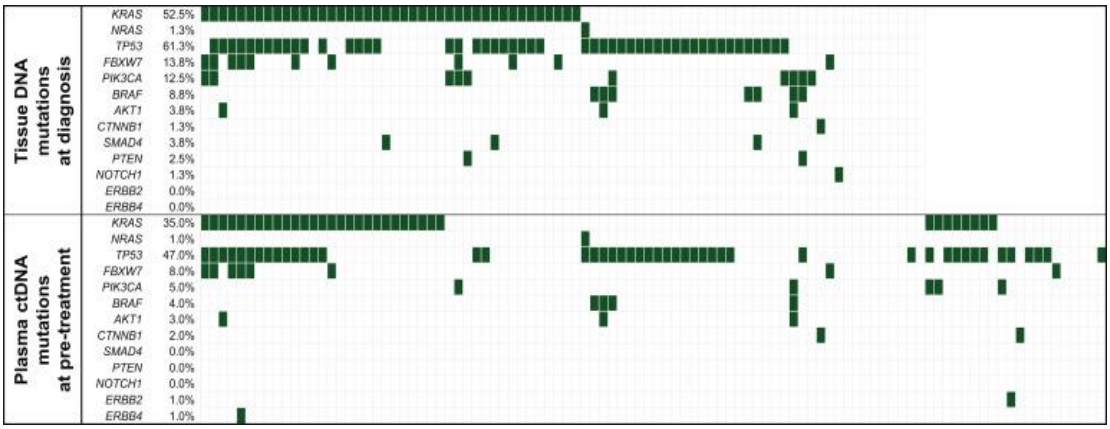
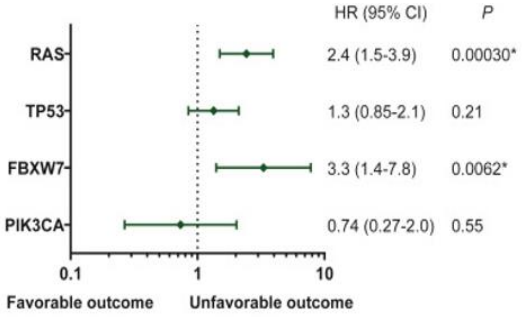


Figure 2. Somatic non-synonymous mutations were identified in tumor tissue collected at diagnosis and plasma collected prior to treatment. Deep sequencing using an amplicon-based targeted panel covering 22 genes was applied. Thirteen genes harboring non-synonymous mutations in either tissue or plasma are displayed. Green denotes the presence of a non-synonymous mutation, and each column corresponds to an individual patient. Sequencing was successfully performed in 80 FFPE tissue samples and in all 100 ctDNA samples. ctDNA, circulating tumor DNA.

For patients whose tumor tissue DNA showed mutant RAS (KRAS or NRAS), median PFS was 9.5 months, compared with 13.0 months in wild-type patients (HR 1.5; 95% CI 0.9–2.4; P = 0.10). Corresponding median OS was 32 months for mutant RAS versus not reached for wild type (HR 1.9; 95% CI 1.1–3.6; P = 0.03). Forest plots comparing PFS and OS for selected ctDNA mutations with higher prevalence are presented in **Figures 3a and 3b**. Among ctDNA samples, RAS-mutant patients (n = 36) exhibited reduced PFS (HR 2.4; 95% CI 1.5–3.9; P = 0.0003) and OS (HR 2.2; 95% CI 1.3–3.6; P = 0.0032) relative to wild-type RAS patients

(n = 64). Patients with FBXW7 mutations in ctDNA (n = 8) also demonstrated shorter PFS (HR 3.3; 95% CI 1.4–7.8; P = 0.0062). Objective response rates in wild-type versus mutant ctDNA groups were 72.3% versus 60.6% for RAS (P = 0.33) and 69.4% versus 50.0% for FBXW7 (P = 0.43).



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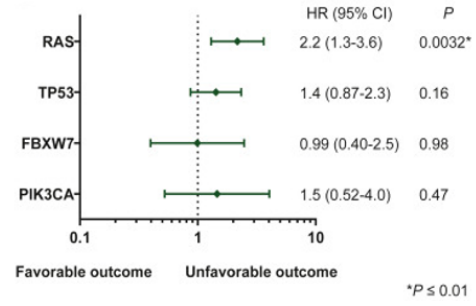

b)

Figure 3. Forest plots illustrating ctDNA mutation status. Hazard ratios (HR), 95% confidence intervals (CI), and significance values were calculated using univariate Cox regression analyses for PFS (a) and OS (b). CI, confidence interval; ctDNA, circulating tumor DNA; HR, hazard ratio; OS, overall survival; PFS, progression-free survival. ∗P ≤ 0.01.

Multivariate analysis of circulating biomarkers

Variables showing significance (P ≤ 0.01) in univariate testing—namely, pICAM-1, FBXW7 ctDNA mutation, and RAS ctDNA mutation for PFS, and pICAM-1, pIL-8, and RAS ctDNA mutation for OS—were incorporated into multivariate models. Previously reported prognostic variables, including tumor location and histological subtype, were also included. The results are summarized in **Tables 2 and 3.**

For PFS, statistically independent factors included ctDNA RAS mutation (HR 2.5; 95% CI 1.5–4.3; P = 0.00060), ctDNA FBXW7 mutation (HR 2.8; 95% CI 1.2–6.8; P = 0.021), and elevated pICAM-1 (HR 2.1; 95% CI 1.3–3.5; P = 0.0027). Regarding OS, ctDNA RAS mutation (HR 2.6; 95% CI 1.5–4.6; P = 0.0010) and pICAM-1 levels (HR 2.0; 95% CI 1.1–3.7; P = 0.025) remained independent predictors.

Table 2. Multivariate analysis for circulating biomarker levels and established prognostic factors (PFS)

Variable	Multivariate Analysis			Univariate Analysis		
	HR	P-value	95% CI	HR	P-value	95% CI
Plasma ICAM-1 level High vs. Low	2.1	0.0027∗∗	1.3–3.5	2.1	0.0022∗	1.3–3.4
RAS status (ctDNA) Mutant vs. Wild-type	2.5	0.00060∗∗	1.5–4.3	2.4	0.00030∗	1.5–3.9
FBXW7 status Mutant vs. Wild-type	2.8	0.021∗∗	1.2–6.8	3.3	0.0062∗	1.4–7.8
Tumor sidedness Right-sided vs. Left-sided	0.67	0.14	0.40–1.1	0.79	0.35	0.48–1.3
Histological type Poorly differentiated / mucinous / signet-ring cell vs. Papillary / tubular	1.1	0.82	0.45–2.7	0.60	0.24	0.26–1.4

HR, hazard ratio
MT, mutant type
pap, papillary
pICAM-1, plasma intercellular adhesion molecule-1
right, cecum, ascending colon, and transverse colon
tub, tubular adenocarcinoma
CI, confidence interval
left, descending colon, sigmoid colon, rectosigmoid colon, and rectum
muc, mucinous adenocarcinoma
PFS, progression-free survival
por, poorly differentiated adenocarcinoma

sig, signet-ring cell carcinoma
WT, wild type
∗P ≤ 0.01 in univariate analysis (chi-square test).
∗∗P < 0.05 in multivariate analysis (chi-square test).

Table 3. Multivariate analysis for circulating biomarker levels and established prognostic factors (OS)

Variable	Multivariate Analysis			Univariate Analysis		
	HR	P-value	95% CI	HR	P-value	95% CI
Plasma ICAM-1 level High vs. Low	2.0	0.025∗∗	1.1–3.7	1.9	0.010∗	1.2–3.2
Plasma IL-8 level High vs. Low	1.4	0.24	0.80–2.5	2.0	0.0085∗	1.2–3.2
RAS status (ctDNA) Mutant vs. Wild-type	2.6	0.0010∗∗	1.5–4.6	2.2	0.0032∗	1.3–3.6
Tumor sidedness Right-sided vs. Left-sided	1.3	0.41	0.73–2.2	1.4	0.19	0.84–2.4
Histological type Poorly differentiated / mucinous / signet-ring cell vs. Papillary / tubular	2.4	0.066	0.94–5.9	1.2	0.61	0.56–2.7

WT, wild type.
sig, signet-ring cell carcinoma;
right, cecum, ascending colon, and transverse colon;
pIL-8, plasma interleukin 8;
pap, papillary;
MT, mutant type;
HR, hazard ratio;
CI, confidence interval;
muc, mucinous adenocarcinoma;
OS, overall survival;
pICAM-1, plasma intercellular adhesion molecule-1;
por, poorly differentiated adenocarcinoma;
left, descending colon, sigmoid colon, rectosigmoid colon, and rectum;
tub, tubular adenocarcinoma;
∗P ≤ 0.01 in univariate analysis (chi-square test).
∗∗P < 0.05 in multivariate analysis (chi-square test).

In this study, plasma VEGF-A short isoforms (pVEGF-Asi) did not predict the clinical benefit of bevacizumab (BV), whereas elevated plasma intercellular adhesion molecule-1 (pICAM-1) levels and RAS mutations in circulating tumor DNA (ctDNA) emerged as independent prognostic indicators for patients with metastatic colorectal cancer (mCRC) receiving modified FOLFOX6 or XELOX (mFOLFOX6/XELOX) plus BV.

We measured 11 angiogenesis-related plasma proteins using the validated IMPACT-2 panel, which has been widely applied in numerous international trials for biomarker analysis, especially for pVEGF-Asi [11–13]. Previous evidence suggested that higher VEGF-A levels are linked to worse outcomes in patients undergoing chemotherapy without anti-angiogenic therapy [11–13]. On this basis, we hypothesized that BV could improve outcomes in patients with elevated pVEGF-Asi, potentially offsetting its negative prognostic effect, and leading to similar progression-free survival (PFS) between patients with high versus low pVEGF-Asi (anticipated HR = 1.0, threshold HR = 1.15).

For context, in first-line treatment of mCRC with irinotecan plus bolus 5-FU/LV (IFL) without BV, the PFS hazard ratio comparing high versus low pVEGF-A groups was reported as 1.28 [14]; accordingly, we set our threshold HR at 1.15, approximately half of this reported value. In contrast, when BV was combined with IFL, prior work indicated HRs of 0.52 for patients with high pVEGF-A and 0.64 for those with low pVEGF-A [13], which translates to a PFS HR of roughly 1.04 when comparing low versus high VEGF-A under BV-containing IFL therapy. Based on this, we considered an expected HR of 1.0 for high pVEGF-Asi patients receiving mFOLFOX6/XELOX + BV to be reasonable. Considering that pVEGF-A had previously failed to act as a predictive marker in IFL + BV therapy [13], our initial assumption may have been optimistic.

In our cohort, the observed HR of 1.3 for PFS is close to the previously reported 1.28 for high versus low pVEGF-A in patients treated without BV. Assuming that the HRs for PFS and overall survival (OS) for mFOLFOX6/XELOX alone mirror those of IFL alone,

the addition of BV appears to have a similar impact on patients with high and low pVEGF-Asi (0.98× for PFS, 1.01× for OS). These findings indicate that BV provides comparable benefit irrespective of baseline pVEGF-Asi, reinforcing that pVEGF-Asi is unlikely to serve as a predictive biomarker for mFOLFOX6/XELOX + BV efficacy.

However, the clinical utility of plasma VEGF-A as a biomarker remains controversial. Some studies report no significant difference in outcomes according to VEGF-A levels in mCRC patients treated with FOLFIRI + BV [16] or aflibercept [20]. By contrast, a significant difference in time to progression was observed when an optimal VEGF-A threshold was applied in patients receiving FOLFIRI + aflibercept [21].

In our analysis, elevated pVEGF-Asi levels measured in EDTA-collected plasma were associated with a poorer prognosis in mCRC patients receiving mFOLFOX6/XELOX + BV; however, potential confounding variables could not be entirely excluded. The role of VEGF-A as an independent prognostic factor remains debated, despite its frequent identification as a prognostic marker in mCRC. Additionally, while performance status (PS) is known to influence outcomes negatively, we observed a higher proportion of patients with PS 1 in the high pVEGF-Asi group. To disentangle these effects, a multivariate analysis was performed to evaluate the relationship between pVEGF-Asi and established prognostic factors for overall survival (OS), both including and excluding PS. This analysis revealed that PS was the sole independent prognostic factor for OS (HR 1.9; 95% CI 1.1–3.3; P = 0.034), suggesting that PS and pVEGF-Asi likely act as confounders in this patient population.

Plasma ICAM-1 (pICAM-1), a member of the immunoglobulin superfamily of adhesion molecules, is expressed on multiple cell types, including leukocytes, endothelial cells, fibroblasts, epithelial cells, and tumor cells [22], and is implicated in angiogenesis [23, 24]. In this study, pICAM-1 levels were significantly associated with both PFS and OS in patients receiving mFOLFOX6/XELOX + BV. Previous reports have demonstrated elevated soluble ICAM-1 in sera of patients with gastrointestinal malignancies, including colorectal cancer [25, 26], with higher levels correlating with advanced tumor stage and worse outcomes, though most studies focused on resectable CRC [25]. For metastatic disease, only one prior study has linked pICAM-1 to PFS and OS in patients treated with BV + everolimus [27]. Our findings reinforce that pICAM-1 functions as a prognostic biomarker in mCRC.

We also found that both PFS and OS were significantly shorter in patients harboring RAS mutations detectable in ctDNA compared with wild-type ctDNA, whereas RAS mutations in tumor tissue DNA did not significantly affect PFS. Except for a single patient lacking tumor tissue data, all patients with RAS-mutated ctDNA also exhibited RAS mutations in tumor tissue; however, several patients with RAS-mutated tumor tissue did not have detectable mutations in ctDNA. This discrepancy can be attributed to two factors: technical limitations, as the detection method used (~0.3%–1% sensitivity) may not identify low-frequency alleles, and biological factors, since sufficient ctDNA may not have been shed from tumor sites into the circulation. Concordance between tumor tissue and ctDNA RAS mutation status was highest in patients with liver-only metastasis (85.7%), compared with peritoneal (33.3%) or lung-only (14.3%) metastases. Differences in metastatic location, consistent with previous reports, may therefore explain the variation in ctDNA detection [28, 29]. Patients with RAS-mutated ctDNA had a median of two metastatic sites versus one in patients with RAS mutations detected only in tumor tissue. Moreover, patients with a RAS mutant allele fraction ≥5% in ctDNA exhibited shorter PFS and OS than those with fractions <5%, corroborating earlier findings [30]. These data suggest that RAS mutations detectable in ctDNA reflect a higher tumor burden, contributing to worse outcomes.

Patients with FBXW7 mutations in ctDNA experienced significantly reduced PFS, and FBXW7-mutated ctDNA emerged as an independent predictor of poor PFS, although no association with OS was observed. FBXW7, a tumor suppressor gene, encodes a protein containing an F-box and seven WD40 repeats [31]. FBXW7 mutations occur in approximately 6%–10% of CRC cases [32–34], with loss of function driving oncogenesis and tumor progression [33, 35]. Previous studies have linked FBXW7 missense mutations to adverse prognosis [36], although treatment-specific effects remain unclear. Other reports have suggested associations of FBXW7 mutations with resistance to chemotherapy, including BV [37] and cetuximab [38, 39], although sample sizes were limited. Additionally, FBXW7 deficiency [40] and high cryptochrome 2 expression, negatively regulated by FBXW7 [41], have been implicated in oxaliplatin resistance in CRC cell lines and patients receiving oxaliplatin-containing regimens in the adjuvant setting.

Conclusion

This study presents several limitations that restrict the strength of our conclusions. Cut-offs for all plasma protein biomarkers were uniformly defined using the median value of each protein, but it is uncertain if these thresholds were optimal. Notably, prior studies have reported that VEGF-A cut-offs derived from receiver operating characteristic (ROC) analysis could influence efficacy outcomes in mCRC patients receiving anti-angiogenic agents combined with chemotherapy [21]. Additionally, our definition of statistical criteria for assessing pVEGF-Asi as a potential predictive biomarker for mFOLFOX6/XELOX + BV was based on extrapolation from studies using the IFL regimen, due to the single-arm design of our trial. Whether the relationship between pVEGF-Asi and the additive effect of BV depends on the chemotherapy backbone (IFL versus mFOLFOX6/XELOX) remains unresolved.

FBXW7 mutations detected in ctDNA were independently associated with shorter PFS, although no significant effect was observed for OS between low and high levels. This suggests that FBXW7 mutation may predict responsiveness to mFOLFOX6/XELOX + BV, but the contribution of each drug to this effect cannot be disentangled due to the single-arm nature of the study and the lack of post-treatment efficacy data. Regarding BRAF mutations in ctDNA, although univariate analysis suggested poorer ORR and OS (data not shown), the small number of patients with these mutations limited the ability to perform robust statistical comparisons, preventing a firm conclusion about their clinical relevance.

In conclusion, pVEGF-Asi did not demonstrate predictive utility for the efficacy of mFOLFOX6/XELOX + BV. However, exploratory analyses indicated that higher pICAM-1 levels and the presence of circulating RAS mutations could serve as prognostic indicators in mCRC patients treated with this regimen. Further research is needed to confirm the clinical significance of circulating ICAM-1, mutant RAS, and FBXW7 in this context.

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