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ORIGINAL RESEARCH

A phase II study to explore biomarkers for the use of mFOLFOX6/XELOX plus bevacizumab as a first-line chemotherapy in patients with metastatic colorectal cancer (WJOG7612GTR)

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Background: The purpose of this prospective study was to assess the ability of plasma vascular endothelial growth factor-A short isoforms (pVEGF-Asi) to predict bevacizumab (BV) efficacy and to explore other circulating biomarkers in metastatic colorectal cancer (mCRC) patients treated with modified FOLFOX6/XELOX plus BV (mFOLFOX6/XELOX + BV).

Patients and methods: Pre-treatment plasma samples were collected from 100 mCRC patients receiving first-line chemotherapy with mFOLFOX6/XELOX + BV. The plasma levels of 11 angiogenesis-associated molecules, including pVEGF-Asi and 22 cancer-associated gene mutations in circulating tumor DNA, were analyzed. For the primary endpoint, we assumed that the hazard ratio (HR) for progression-free survival (PFS) calculated using a Cox proportional hazards model was <1.15, comparing patients with a high versus those with a low pVEGF-Asi level divided according to the median pVEGF-Asi value.

Results: The median value of pVEGF-Asi was 37 (range 6.5-262) pg/ml. The HR for PFS between the high and low pVEGF-Asi patient groups was 1.3 [95% confidence interval (CI) 0.8-2.1; log rank, $P = 0.25$], which was larger than the predefined threshold of 1.15. The multivariate analysis demonstrated that PFS was significantly associated with plasma intercellular adhesion molecule-1 (pICAM-1) (≥190.0 versus <190.0 ng/ml; HR 2.1; 95% CI 1.3-3.5), *RAS* (mutant versus wild; HR 2.5; 95% CI 1.5-4.3), and *FBXW7* (mutant versus wild; HR 2.8; 95% CI 1.2-6.8), whereas overall survival was significantly associated with pICAM-1 (HR 2.0; 95% CI 1.1-3.7) and *RAS* (HR 2.6; 95% CI 1.5-4.6).

Conclusions: The addition of BV was unable to compensate for the poor PFS associated with a high pVEGF-Asi level, suggesting that pVEGF-Asi is unlikely to be a good predictive biomarker of the efficacy of mFOLFOX6/XELOX + BV therapy. The clinical significance of circulating ICAM-1, mutant *RAS*, and mutant *FBXW7* levels should be studied further.

Key words: metastatic colorectal cancer, circulating biomarkers, pVEGF-A short isoforms, pICAM-1 level, *RAS* and *FBXW7* mutation

INTRODUCTION

The vascular endothelial growth factor (VEGF) pathway plays an important role in angiogenesis and tumor growth.[1] The overexpression of VEGF and increased circulating VEGF levels are reportedly associated with tumor progression, metastasis, and a poor prognosis in patients with gastrointestinal tumors, including colorectal cancer (CRC).[2-5] Bevacizumab

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(BV) is an anti-angiogenic monoclonal antibody that binds to VEGF-A and inhibits the activation of the VEGF signaling pathway.[6] BV is commonly used in combination with cytotoxic agents such as fluorouracil (FU) and leucovorin (LV) with either oxaliplatin (FOLFOX or XELOX) or irinotecan (FOLFIRI) as a standard first- and/or second-line chemotherapy for metastatic CRC (mCRC).[7] BV has long been included in standard therapy, but no predictive biomarker of its efficacy is available in clinical practice. The circulating level of VEGF-A, which is a target of BV, has been expected to be a predictive biomarker of the efficacy of BV-containing chemotherapy. VEGF-A has several isoforms as a result of alternative RNA splicing, and the shorter isoforms, such as VEGF-A110 and VEGF-A121, are freely diffusible because of the absence of basic amino acid residues that bind to the extracellular matrix; the longer isoforms, meanwhile, bind to heparin and heparan sulfate proteoglycans in the extracellular matrix.[8-10] These features suggest that the circulating levels of plasma VEGF-A short isoforms (pVEGF-Asi) are likely to be associated with the amount of VEGF-A secreted by tumor cells. The level of pVEGF-Asi, such as VEGF-A110 and VEGF-A121, measured using the immunological multiparametric chip technique (IMPACT) was reported to have potential as a predictor of the clinical benefits of BV in patients with advanced gastric[11] and pancreatic cancer,[12] but not in patients with mCRC, non-small-cell lung cancer, or renal cell carcinoma.[13] This discrepancy might depend on the method used to collect plasma samples [ethylenediamine tetraacetic acid (EDTA) or citrate]; the use of EDTA when collecting plasma samples has been recommended.[5,13,14] However, whether the pVEGF-Asi level measured in plasma samples collected using EDTA can predict the efficacy of BV in patients with mCRC remains unclear.

Circulating DNA can be used to detect gene alterations and has recently attracted attention as a non-invasive tool for predicting patient prognosis and treatment efficacy for various cancers. The total circulating cell-free DNA (cfDNA) level has been reported to predict a poor outcome in patients with mCRC before oxaliplatin-based chemotherapy.[15] Furthermore, the clinical utility of circulating tumor DNA (ctDNA) has also been reported for mCRC. The *KRAS* and *NRAS* (*RAS*) mutation status[16,17] and the detection of *ERBB2* amplification[18] in ctDNA are reportedly associated with the effects of anti-epidermal growth factor receptor (EGFR) antibodies and anti-human epidermal growth factor receptor-2 antibodies, respectively.

Here, we conducted a single-arm phase II study to investigate the associations between potential biomarkers, such as pVEGF-Asi and ctDNA, and treatment outcomes in mCRC patients treated with modified FOLFOX6 plus BV (mFOLFOX6 + BV) or XELOX plus BV (XELOX + BV) as a first-line chemotherapy.

PATIENTS AND METHODS

Patient selection

Patients with histologically confirmed unresectable mCRC and no prior chemotherapy (except for adjuvant chemotherapy with fluoropyrimidine alone completed >180 days previously or adjuvant chemotherapy containing oxaliplatin completed >1 year before relapse) and an Eastern Cooperative Oncology Group performance status (PS) of 0-1 were eligible (detailed information regarding the eligibility criteria is included in the Supplementary Appendix, available at https://doi.org/10.1016/j.esmoop.2022.100592). Written informed consent was obtained from all the patients. The study protocol was approved by the ethics committees of all the participating centers. This trial was conducted in compliance with the Declaration of Helsinki and was registered in the University Hospital Medical Information Network (UMIN) Clinical Trials Registry, number UMIN000012442.

Study design

This study was a multicenter, single-arm, phase II trial conducted by the West Japan Oncology Group in Japan. Patients received either mFOLFOX6 + BV or XELOX + BV, according to each investigator's selection. The primary endpoint was progression-free survival (PFS) as compared between patients with a high and those with a low pVEGF-Asi level divided according to the median pVEGF-Asi value. PFS was defined as the time from the date of enrollment to the date of the confirmation of progressive disease or death from any cause. Secondary endpoints were overall survival (OS), overall response rate (ORR), and safety; the relationships between plasma biomarkers and these clinical outcomes were also explored.

Treatments

Patients received either BV (5 mg/kg) followed by mFOLFOX6 [oxaliplatin, 85 mg/m^2 intravenous infusion; l-LV, 200 mg intravenous infusion; FU, 400 mg/m^2 bolus intravenously and 2400 mg/m^2 continuous infusion over 46 h) every 2 weeks or BV (7.5 mg/kg) followed by XELOX [oxaliplatin, 130 mg/m^2 intravenously on day 1; and capecitabine, 1000 mg/m^2 orally twice daily from the evening of day 1 to the morning of day 15 (28 doses)] every 3 weeks. The study treatment was repeated until disease progression, unacceptable toxicity, or patient refusal. Detailed information regarding dose modifications is included in the Supplementary Appendix, available at https://doi.org/10.1016/j.esmoop.2022.100592.

Evaluation

Radiological tumor assessments (computed tomography or magnetic resonance imaging) were carried out within 28 days before enrollment and were repeated every 8 weeks until disease progression. Responses were assessed by each investigator according to RECIST (version 1.1). Interviews regarding each patient's symptom and examinations of each patient's condition and laboratory data were repeated on the first day of each cycle, at a minimum, and as indicated anytime thereafter. Adverse events were graded according to the Common Terminology Criteria for Adverse Events, version 4.0.

Sample collection and processing

Archived formalin-fixed, paraffin-embedded (FFPE) tissue specimens obtained at the time of diagnosis were collected. Blood samples (14 ml) were collected into tubes containing EDTA just before the first and second cycle and at the end of the protocol treatment; the samples were then centrifuged at 1200 × *g* for 10 min within 1 h after collection. Tumor tissue DNA in FFPE was isolated using the Allprep DNA/RNA FFPE Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Plasma ctDNA was isolated using the cobas® cfDNA Sample Preparation Kit (Roche Diagnostics Ltd., Penzberg, Germany) according to the manufacturer's instructions. The quality and quantity of the nucleic acid were verified using PicoGreen dsDNA Reagent (all from Thermo Scientific, Wilmington, DE).

Somatic mutation analysis

Using tissue DNA and ctDNA, amplicon-targeted sequencing was carried out with the Ion AmpliSeq™ Colon and Lung Cancer Research Panel (ver. 2; Thermo Fisher Scientific K.K., Tokyo, Japan), which targets 22 cancer-associated genes: *KRAS, EGFR, BRAF, PIK3CA, AKT1, ERBB2, PTEN, NRAS, STK11, MAP2K1, ALK, DDR2, CTNNB1, MET, TP53, SMAD4, FBXW7, FGFR3, NOTCH1, ERBB4, FGFR1,* and *FGFR2*. For library preparation, tissue DNA or ctDNA (up to 10 ng) was subjected to multiplex PCR amplification using the Ion AmpliSeq Library Kit 2.0 (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. Purified libraries were pooled and then sequenced using an Ion Torrent Proton instrument, the Ion PI Hi-Q Chef Kit, and the Ion PI Chip Kit v3 (all from Life Technologies). DNA sequencing data were accessed through the Torrent Suite version 5.10 program (Life Technologies). Reads were aligned with the hg19 human reference genome, and potential mutations were identified using Variant Caller version 5.10, as previously described.[19]

Plasma protein analysis

The plasma levels of 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) were measured using IMPACT-2 (Roche proprietary multiplex enzyme-linked immunosorbent assay platform) at Roche Diagnostics Ltd. This assay has a greater sensitivity for shorter isoforms of pVEGF-A (VEGF-A110, VEGF-A121) than for longer isoforms (VEGF-A165, VEGF-A189).

Statistical analysis

The median value of each plasma marker was used as a cut-off to categorize the patients into low and high groups. We expected that the hazard ratio (HR) for PFS compared between patients with high and low levels of pVEGF-Asi would be 1.0, based on the hypothesis that BV is likely to be more effective for patients with high pVEGF-Asi levels than for those with low pVEGF-Asi levels, compensating for their poor prognosis without BV, and our null hypothesis was that the prognosis of patients with high pVEGF-Asi levels would be worse than those with low pVEGF-Asi levels (HR $\geq$ 1.15) even with BV plus chemotherapy. About 90 events were required to reject the null hypothesis of HR $\geq$1.15 with a two-sided α error of 5% and a power of 80%. Considering that these biomarkers might not be assessable in some cases, the planned enrollment was set at 100 patients.

In an exploratory univariate analysis of the associations between PFS or OS and circulating biomarkers, we regarded factors with a *P* value $\leq$0.01 as being clinically relevant, considering the multiplicity of comparison. Mutant genes observed in fewer than five patients were regarded as being inapplicable for statistical analysis, since statistical comparisons would be inadequate because of the small number.

The Cox proportional hazards regression model was used to carry out univariate and multivariate analyses. The relations between the mutation status and patient characteristics were evaluated using the chi-square (χ^2) test. Kaplan—Meier curves were used to estimate survival, and the log-rank test was used to compare times to events between groups. JMP (version 14.0, SAS Institute, Cary, NC) and GraphPad Prism software (version 8, GraphPad Software Inc., La Jolla, CA) were used for the statistical analysis.

RESULTS

Patient characteristics and treatment outcomes

Out of 102 patients enrolled at 23 institutes between January 2014 and April 2015, the eligibility of 100 patients was confirmed. The baseline characteristics are summarized in Supplementary Table S1, available at https://doi.org/10.1016/j.esmoop.2022.100592. Fifty-two and 48 patients received mFOLFOX6 + BV and XELOX + BV, respectively. Overall, the median PFS was 11.4 months [95% confidence interval (CI) 9.5-13.0 months], the ORR was 65.9% (95% CI 54.6% to 76.0%), and the median OS was 33.7 months (95% CI 28.7-38.1 months). Patients treated with mFOLFOX6 + BV had a slightly better PFS (HR 0.66; 95% CI 0.41-1.04; $P = 0.075$) and OS (HR 0.71; 95% CI 0.43-1.16; $P = 0.175$) than those treated with XELOX + BV.

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

Pre-treatment plasma samples were obtained from all 100 eligible patients. The pVEGF-Asi level could not be measured in three samples. The median pVEGF-Asi concentration, which was used as a cut-off, was 37 pg/ml (range 6.5-262.0 pg/ml), and patients were divided into high ($n = 49$) and low ($n = 48$) groups. The patient characteristics of the high and low pVEGF-Asi groups were similar except for the PS (Table 1). The proportion of patients with a PS of 1 was higher in the high pVEGF-Asi group (35%) than in the low pVEGF-Asi group (17%). No significant

Table 1. Baseline characteristics according to pVEGF-Asi status

Variable		Pre-treatment pVEGF-Asi level				
		Low ($n = 48$)		High ($n = 49$)		*P*
		n	%	*n*	%	
Sex	Male	26	54.2	24	49.0	0.69
	Female	22	45.8	25	51.0	
Age in years	Median (range)	62.5 (36-78)		65.0 (44-78)		
Regimen	mFOLFOX6 + BV	27	56.3	23	46.9	0.42
	XELOX + BV	21	43.8	26	53.1	
PS	0	40	83.3	32	65.3	0.063
	1	8	16.7	17	34.7	
Primary site	Colon	27	56.3	31	63.3	0.54
	Rectum	21	43.8	18	36.7	
Tumor location	Right	14	29.2	15	30.6	1.0
	Left	34	70.8	34	69.4	
Histological type	pap/tub	43	69.6	42	85.7	1.0
	por/muc/sig	5	10.4	6	12.2	
	Unknown	0	0	1	2.0	
RAS (ctDNA)	Positive	20	41.7	15	30.6	0.29
	Negative	28	58.3	34	69.4	
BRAF (ctDNA)	Positive	3	6.3	1	2.0	0.36
	Negative	45	93.8	48	98.0	

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

difference was observed in the ORR between the low (65%) and high (69%) pVEGF-Asi groups ($P = 0.69$). Compared with the low pVEGF-Asi group, the high pVEGF-Asi group had an HR of 1.3 (95% CI 0.8-2.1; median PFS, 11 versus 13 months; $P = 0.25$) for PFS, indicating that the null hypothesis of an HR for PFS $\geq$1.15 could not be rejected (Figure 1A). Patients in the high pVEGF-Asi group had a significantly shorter OS than those in the low pVEGF-Asi group, with an HR of 1.7 (95% CI 1.1-2.9; median OS, 26 versus 38 months; $P = 0.029$) (Figure 1B). Given that the *BRAF* mutation is a major prognostic factor, we investigated the PFS and OS between the low and high pVEGF-Asi groups after excluding patients with *BRAF* mutation ($n = 7$ for tissue sample and $n = 4$ for ctDNA sample before treatment initiation). However, the results were similar to those for analyses that included patients with *BRAF* mutation (Supplementary Figure S1, available at https://doi.org/10.1016/j.esmoop.2022.100592).

Other circulating biomarkers of plasma protein

The median levels of circulating plasma molecules are summarized in Supplementary Table S2, available at https://doi.org/10.1016/j.esmoop.2022.100592, and forest plots comparing high and low groups of each protein for PFS and OS are shown in Figure 1C and D, respectively. We regarded the association of pICAM-1 with PFS (high versus low: HR 2.1; 95% CI 1.3-3.4; $P = 0.002$) and OS (high versus low: HR 1.9; 95% CI 1.2-3.2; $P = 0.010$) and that of pIL-8 with OS (high versus low: HR 2.0; 95% CI 1.2-3.2; $P = 0.009$) as being clinically relevant. In the low and high groups, the ORRs were 79% and 59% for pICAM-1 ($P = 0.05$) and 71% and 64% for IL-8 ($P = 0.51$), respectively. Furthermore, we investigated the associations between the treatment response and the changes in the levels of all circulating plasma molecules before the first and second cycle of chemotherapy as well as at the end of the treatment. No significant differences were found between complete response/partial response and stable disease/progressive disease for all 11 molecules (Supplementary Figure S2, available at https://doi.org/10.1016/j.esmoop.2022.100592).

Correlation of somatic mutations with clinical outcomes

Ninety-nine FFPE tissue samples and 100 plasma ctDNA samples were collected for the somatic mutation assay. The FFPE tissue and ctDNA analyses were successful for 80 and 100 samples, respectively. Thirteen detected gene alterations are shown in Figure 2. Some differences in the respective mutation rates between pre-treatment ctDNA and archival tissue DNA were observed for *TP53* (47% and 61%), *KRAS* (35% and 53%), *FBXW7* (8% and 14%), *PIK3CA* (5% and 13%), and *BRAF* (4% and 9%).

The median PFS of patients with mutant-type versus wild-type *RAS* (*KRAS* and *NRAS*) in tumor tissue DNA was 9.5 versus 13.0 months (HR 1.5; 95% CI 0.9-2.4; $P = 0.10$), while the median OS was 32 months versus not reached (HR 1.9; 95% CI 1.1-3.6; $P = 0.03$). The forest plots for PFS and OS comparing mutant-type and wild-type patients for some ctDNA alterations with relatively high proportions are shown in Figure 3A and B, respectively. Patients with *RAS* mutant type in ctDNA ($n = 36$) had a shorter PFS (HR 2.4; 95% CI 1.5-3.9; $P = 0.0003$) and OS (HR 2.2; 95% CI 1.3-3.6; $P = 0.0032$) than those with *RAS* wild type ($n = 64$).

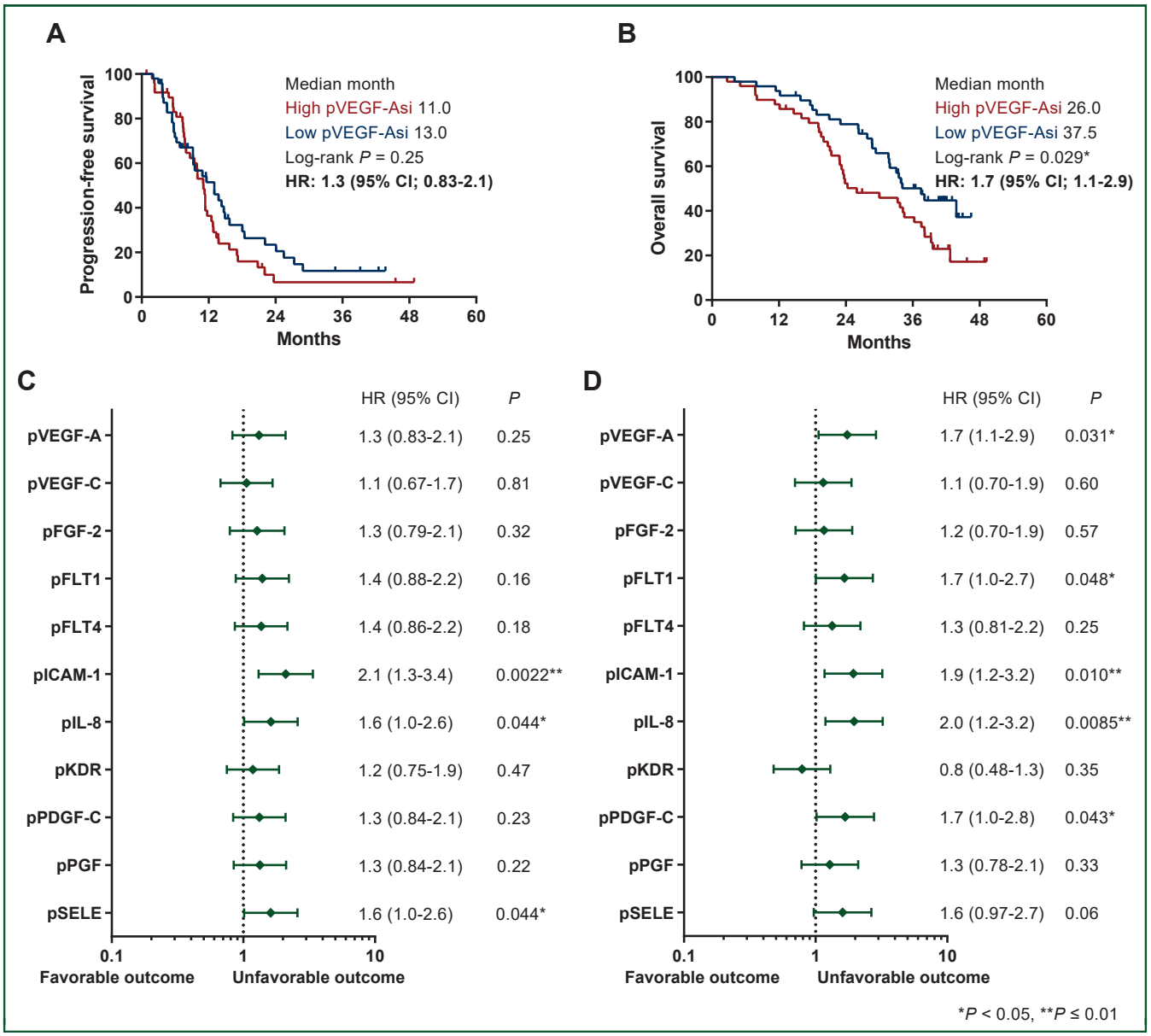

Figure 1. Correlations between the angiogenesis-related protein levels in the plasma and clinical outcome.
(A, B) Correlations between the plasma VEGF-A short isoform level (pVEGF-Asi) and PFS and OS. (A) PFS of patients with a high pVEGF-Asi level versus a low pVEGF-Asi level; no significant difference in PFS was observed between the high and low pVEGF-Asi groups (HR 1.3; 95% CI 0.83-2.1; $P = 0.25$). (B) OS of patients with a high pVEGF-Asi level versus a low pVEGF-Asi level; a significantly shorter OS was observed for the high pVEGF-Asi group (HR 1.7; 95% CI 1.1-2.9; $P = 0.029$*). *$P < 0.05$. (C, D) Forest plot of angiogenesis-related proteins assessed by IMPACT-2. The HR, 95% CI, and statistical significance for each of 11 proteins were determined for PFS (C) and OS (D) using univariate Cox regression analyses. *$P < 0.05$, **$P \leq 0.01$.
CI, confidence interval; HR, hazard ratio; OS, overall survival; pFGF-2, plasma fibroblast growth factor-2; pFLT1, plasma Fms-related tyrosine kinase 1; PFS, progression-free survival; pICAM-1, plasma intercellular adhesion molecule-1; pIL-8, plasma interleukin 8; pKDR, plasma kinase insert domain receptor; pPDGF-C, plasma platelet-derived growth factor-C; pPGF, plasma placental growth factor; pSELE, plasma selectin E; pVEGF-Asi, plasma vascular endothelial growth factor-A short isoforms.

Patients with the *FBXW7* mutation in ctDNA ($n = 8$) also had a shorter PFS (HR 3.3; 95% CI 1.4-7.8; $P = 0.0062$). In the wild- and mutant-type ctDNA groups, the ORRs were 72.3% and 60.6% for *RAS* ($P = 0.33$) and 69.4% and 50.0% for *FBXW7* ($P = 0.43$), respectively.

Multivariate analysis for circulating biomarkers

Based on the univariate analyses, the pICAM-1 level, *FBXW7* ctDNA mutation, and *RAS* ctDNA mutation for PFS and the pICAM-1 level, pIL-8 level, and *RAS* ctDNA mutation for OS were considered as candidate variables ($P \leq 0.01$) and were included in the multivariate analyses. The multivariate analyses were therefore carried out using these variables and known variables previously reported as prognostic factors in medical literature, such as tumor location and histological type. The results of the multivariate analyses are shown in Tables 2 and 3. The *RAS* and *FBXW7* mutation status and the pICAM-1 level remained as statistically significant independent factors for PFS (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 pICAM-1 level: HR 2.1; 95% CI 1.3-3.5; $P = 0.0027$), while the *RAS* mutation and pICAM-1 level were independent factors for OS (ctDNA *RAS*

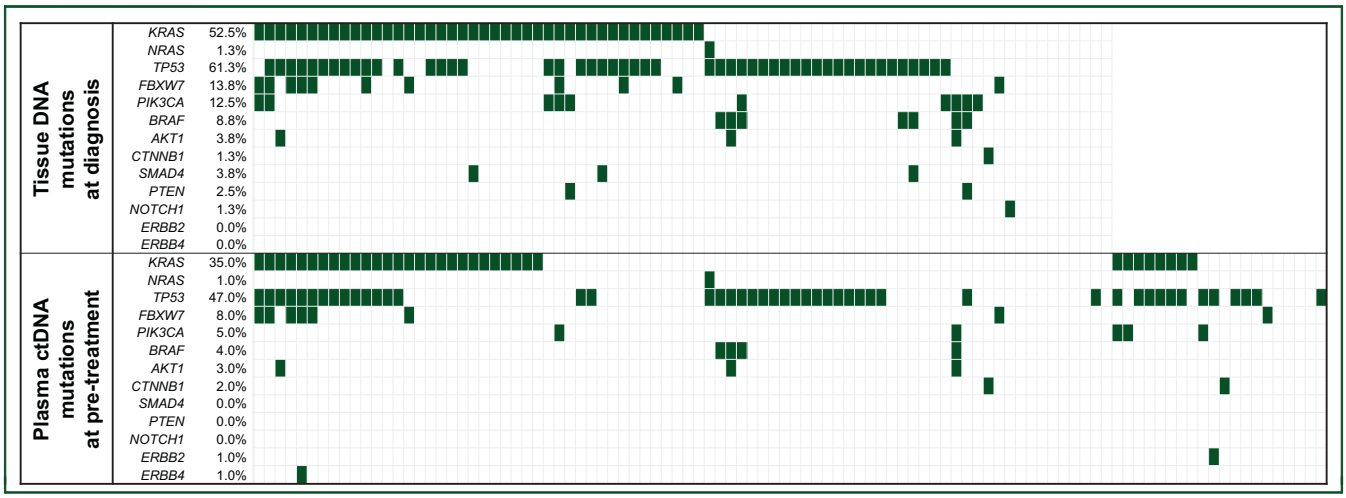

Figure 2. Mutations detected in tumor tissue samples obtained at diagnosis and in plasma samples obtained before treatment. Somatic non-synonymous mutations were detected using amplicon-based targeted deep sequencing for 22 genes. Thirteen genes for which somatic non-synonymous mutations were detected either in tissue or plasma samples are demonstrated. Green indicates a non-synonymous mutation. The columns represent patients. The FFPE tissue and ctDNA analyzes were successful for 80 and 100 samples, respectively.
ctDNA, circulating tumor DNA.

mutation: HR 2.6; 95% CI 1.5-4.6; $P = 0.0010$; pICAM-1 level: HR 2.0; 95% CI 1.1-3.7; $P = 0.025$).

DISCUSSION

Our study showed that the pVEGF-Asi level was not a predictive biomarker for the efficacy of BV and that high levels of pICAM-1 and *RAS* mutation in ctDNA were independent prognostic factors in mCRC patients treated with mFOLFOX6 or XELOX (mFOLFOX6/XELOX) + BV.

We used the IMPACT-2 panel to measure 11 angiogenesis-associated factors in plasma. This panel has been validated and adopted for biomarker analysis, especially for pVEGF-Asi, in numerous large-scale global clinical trials for evaluating the efficacy of BV-containing regimens.[11-13] Based on these previous reports[11-13] that a high pVEGF-A level was associated with a poor prognosis in patients receiving chemotherapy without anti-angiogenic agents such as BV, we expected that BV would be more effective for patients with high pVEGF-Asi levels than for those with low pVEGF-Asi levels and would compensate for the deteriorative influence of pVEGF-Asi, resulting in similar PFS periods (expected HR of 1.0 and threshold HR of 1.15) between patients with high and those with low pVEGF-Asi levels. The reported HR for PFS comparing high and low pVEGF-A groups was 1.28 when BV was not used in combination with irinotecan plus bolus 5-FU/LV (IFL) as a first-line treatment for mCRC[14]; our threshold HR was determined to be 1.15, corresponding to about half of the previously reported HR of 1.28. On the other hand, a previous study showed that the additional effect of BV on IFL resulted in an HR of 0.52 for a high pVEGF-A group and an HR of 0.64 for a low pVEGF-A group in a first-line treatment setting for mCRC.[13] Therefore, the HR for PFS comparing low and high pVEGF-A groups was estimated to be 1.04 when BV was combined with IFL (Supplementary Figure S3, available at https://doi.org/10.1016/j.esmoop.2022.

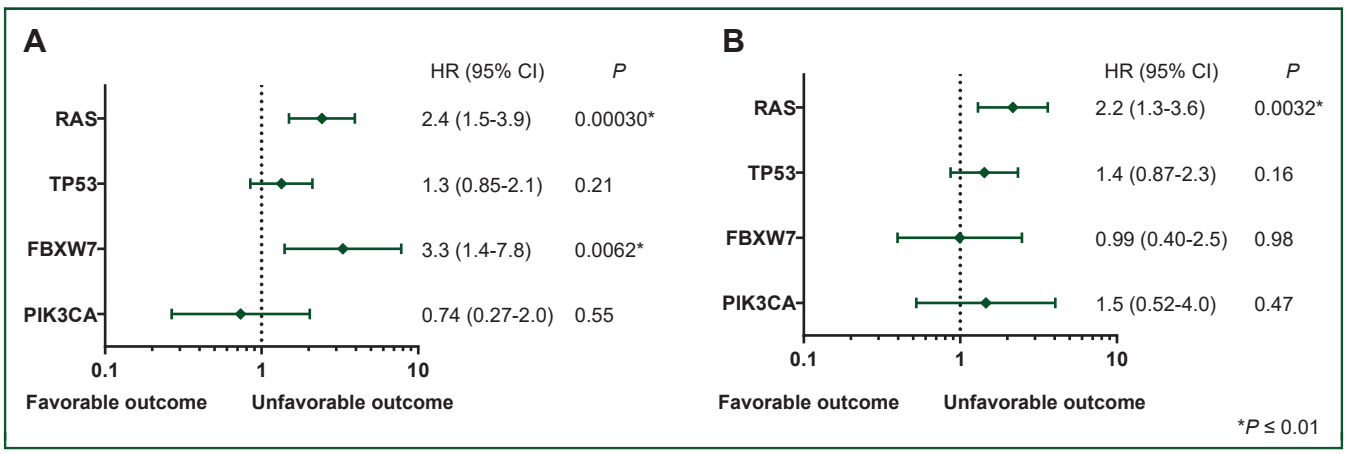

Figure 3. Forest plot of ctDNA mutation status. The HR, 95% CI, and statistical significance for each of the gene mutations in ctDNA were determined for PFS (A) and OS (B) using univariate Cox regression analyses.
CI, confidence interval; ctDNA, circulating tumor DNA; HR, hazard ratio; OS, overall survival; PFS, progression-free survival. *$P \leq 0.01$.

Table 2. Multivariate analysis for circulating biomarker levels and other well-known prognostic factors (PFS)

PFS			Univariate			Multivariate	
Variable		HR	95% CI	*P*	HR	95% CI	*P*
pICAM-1	High versus low	2.1	1.3-3.4	0.0022*	2.1	1.3-3.5	0.0027**
RAS	MT versus WT	2.4	1.5-3.9	0.00030*	2.5	1.5-4.3	0.00060**
FBXW7	MT versus WT	3.3	1.4-7.8	0.0062*	2.8	1.2-6.8	0.021**
Tumor location	Right versus left	0.79	0.48-1.3	0.35	0.67	0.40-1.1	0.14
Histological type	por/muc/sig versus pap/tub	0.60	0.26-1.4	0.24	1.1	0.45-2.7	0.82

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

100592). Expecting a similarly higher efficacy of BV combined with mFOLFOX6/XELOX for high pVEGF-Asi patients, as observed with IFL, our expected HR of 1.0 was considered to be reasonable and feasible. Given that pVEGF-A was not recognized as a predictive marker for IFL + BV in a previous report,[13] our statistical hypothesis might have been generous. Furthermore, our obtained HR of 1.3 was very close to that of 1.28 for a comparison of low and high pVEGF-A patients treated without BV. Assuming that the HRs for PFS and OS comparing low and high pVEGF-Asi patients treated with mFOLFOX6/XELOX alone were the same as the HRs for PFS and OS comparing low and high pVEGF-A patients treated with IFL alone, the results of this study suggest that the effects of the addition of BV to the mFOLFOX6/XELOX regimen on the HRs for PFS and OS were similar among patients with low or high pVEGF-Asi levels (0.98 times for PFS and 1.01 times for OS). Thus, the benefits gained from the addition of BV might be similar in the high and low pVEGF-Asi groups. Consequently, pVEGF-Asi is unlikely to be a predictive biomarker of the efficacy of mFOLFOX6/XELOX + BV. However, the significance of plasma VEGF-A as a biomarker is controversial, even in other regimens that combine an anti-angiogenic inhibitor and a cytotoxic agent. In some studies, no differences in outcomes were observed according to VEGF-A levels in mCRC patients receiving FOLFIRI + BV[16] or aflibercept.[20] On the other hand, a significant difference in time to progression was observed between patients divided according to the optimal cut-off in mCRC patients treated with FOLFIRI + aflibercept.[21]

In this study, we showed that the pVEGF-Asi level in EDTA-collected plasma might predict a poor prognosis in mCRC patients treated with mFOLFOX6/XELOX + BV, although the possible effects of confounding factors could not be completely ruled out. Whether VEGF-A is an independent prognostic factor has been controversial, although VEGF-A has been frequently reported as a prognostic factor in mCRC patients. In addition, although PS is associated with a poor prognosis, our results showed that the proportion of patients with a PS of 1 was higher in the high pVEGF-Asi group. So, we conducted a multivariate analysis to clarify the interactions for OS between pVEGF-Asi and well-known prognostic factors (with and without PS) (Supplementary Table S3, available at https://doi.org/10.1016/j.esmoop.2022.100592). As a result, PS was the only independent prognostic factor for OS (HR 1.9; 95% CI 1.1-3.3; $P = 0.034$). These results suggest that PS and the pVEGF-Asi level were likely to be confounding factors in patients with mCRC treated with mFOLFOX6/XELOX + BV.

pICAM-1 is a member of the immunoglobulin superfamily adhesion molecule, which is expressed on various cells including white blood cells, endothelial cells, fibroblasts, and epithelial and tumor cells.[22] It is also known to play important roles in angiogenesis.[23,24] pICAM-1 was significantly related to the PFS and OS in patients treated with mFOLFOX6/XELOX + BV in the present study. Soluble ICAM-1 is reportedly elevated in the sera of gastrointestinal cancer patients, including those with CRC,[25,26] and high serum levels of soluble ICAM-1 were correlated with tumor stage and a poor prognosis in CRC,[25] although most studies examined patients with resectable CRC. For mCRC, only one article has reported a significant association of pICAM-1 with PFS and OS in patients treated with BV +

Table 3. Multivariate analysis for circulating biomarker levels and other well-known prognostic factors (OS)

OS			Univariate			Multivariate	
Variable		HR	95% CI	*P*	HR	95% CI	*P*
pICAM-1	High versus low	1.9	1.2-3.2	0.010*	2.0	1.1-3.7	0.025**
pIL-8	High versus low	2.0	1.2-3.2	0.0085*	1.4	0.80-2.5	0.24
RAS	MT versus WT	2.2	1.3-3.6	0.0032*	2.6	1.5-4.6	0.0010**
Tumor location	Right versus left	1.4	0.84-2.4	0.19	1.3	0.73-2.2	0.41
Histological type	por/muc/sig versus pap/tub	1.2	0.56-2.7	0.61	2.4	0.94-5.9	0.066

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

everolimus.[27] Our results clearly demonstrated that pICAM-1 is a prognostic marker in patients with mCRC.

In this study, we also demonstrated that both the PFS and the OS of patients with *RAS*-mutated ctDNA were significantly shorter than those of patients with wild-type *RAS* in ctDNA, whereas the PFS of patients with *RAS*-mutated tumor tissues was not significantly shorter. Furthermore, with the exception of one patient whose tumor sample was unavailable, all the patients with *RAS*-mutated ctDNA were also positive for the *RAS* mutation in their tumor tissues, while *RAS*-mutated ctDNA was not detected in some patients with *RAS*-mutated tumor tissues. These results reflect two factors: one is a technical issue, as the sensitivity of the detection method (∼0.3%-1%) used in our assay might be insufficient; the other is a biological issue, as a sufficient amount of ctDNA might not have been released from the tumor tissue into the blood. The concordance rate of the *RAS* mutation status between tumor tissue and ctDNA was higher in patients with liver metastasis alone (85.7%) than in patients with peritoneal (33.3%) or lung (14.3%) metastasis alone. Thus, the discrepancy in the *RAS* mutation status might be caused by differences in the metastatic site, as reported in other studies.[28,29] The median number of metastatic sites was 2 in patients with *RAS*-mutated ctDNA and 1 in patients with *RAS* mutation not detected in ctDNA but detected in tumor tissue. Furthermore, the PFS and OS in patients with a *RAS* mutant allele fraction $\geq$5% were shorter than those in patients with a fraction $<$5% in ctDNA, supporting a previous report[30] (Supplementary Figure S4, available at https://doi.org/10.1016/j.esmoop.2022.100592). These data suggest that *RAS* mutation detectable in ctDNA reflects a large tumor burden and was therefore associated with a worse prognosis.

Patients with *FBXW7*-mutated ctDNA had a significantly shorter PFS, and *FBXW7*-mutated ctDNA was an independent factor associated with a poor PFS, but not with a poor OS, in this study. *FBXW7* is a tumor-suppressor gene that encodes the F-box protein family member and seven tandem WD40 repeats.[31] The *FBXW7* mutation is reportedly observed in 6%-10% of CRC cases,[32-34] and the loss of FBXW7 function leads to oncogenesis and the progression of cancers, including CRC.[33,35] In a previous study, *FBXW7* missense mutations showed a negative prognostic association,[36] although the difference between the treatment regimens was unclear. Other studies have shown that *FBXW7* mutation was associated with resistance to chemotherapy, including BV[37] and cetuximab,[38,39] although the sample sizes of these studies were very small. FBXW7 deficiency[40] and a high expression of cryptochrome 2,[41] which is negatively regulated by FBXW7, are reportedly associated with resistance to oxaliplatin in CRC cell lines and CRC patients treated with oxaliplatin-containing regimens in adjuvant settings.

The present study had some limitations that prevent a definitive conclusion. We uniformly defined the cut-off for all plasma protein factors according to the median value of each plasma protein level; whether these cut-offs were optimal remains uncertain. As described above, significant differences in efficacy were observed according to a VEGF-A cut-off determined by a receiver operating characteristic analysis in mCRC patients treated with an anti-angiogenic agent combined with chemotherapy.[21] Furthermore, we defined the statistical criteria for regarding pVEGF-Asi as a potential predictive biomarker of the efficacy of the mFOLFOX6/XELOX + BV regimen based on an extrapolation of data from a previous study examining the IFL regimen, since our study had a single-arm design; however, whether the relationship between pVEGF-Asi and the additive efficacy of BV is affected by the chemotherapy regimen (IFL or mFOLFOX/XELOX) remains unclear. In addition, *FBXW7* mutation in ctDNA was shown to be an independent predictor of a poor PFS, but the effects of low and high levels were not significantly different in terms of OS. This result suggests that the *FBWX7* status might be a predictor of mFOLFOX6/XELOX + BV efficacy, but which drug is affecting this result remains unclear because of the single-arm design of this study and the difficulty in interpreting the discrepancy between the results for PFS and OS, since post-treatment efficacy data were not collected. Moreover, we cannot discuss the clinical significance of the *BRAF* status in ctDNA, although patients with *BRAF* mutation in ctDNA had poor ORR and OS in our univariate analysis (data not shown), because our study was relatively small in size and the number of patients with *BRAF* mutation was inadequate for statistical comparison.

In conclusion, the clinical relevance of pVEGF-Asi as a predictor of the efficacy of mFOLFOX6/XELOX + BV treatment was not demonstrated. Our exploratory biomarker analyses revealed that the pICAM-1 level and circulating *RAS* mutation status might be prognostic biomarkers in mCRC patients treated with mFOLFOX6/XELOX + BV. The clinical significance of circulating ICAM-1, mutant *RAS*, and mutant *FBXW7* should be studied further.

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