Original Article

Biomarker analysis of the phase II JO25567 study comparing erlotinib with or without bevacizumab in first-line advanced *EGFR*$^{+}$ non-small-cell lung cancer

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Background: Epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs), such as erlotinib, are standard-of-care for patients with *EGFR* mutation-positive non-small-cell lung cancer (NSCLC), but most patients progress within 1 year. Previously, we demonstrated that erlotinib plus bevacizumab (EB) improved progression-free survival (PFS) in patients with *EGFR*-positive non-squamous NSCLC in the randomized JO25567 study. To understand this effect, we conducted comprehensive exploratory biomarker analyses.

Methods: Using blood and tissue specimens from patients enrolled in the JO25567 study, angiogenesis-related serum factors, plasma vascular endothelial growth factor-A (pVEGFA), angiogenesis-related gene polymorphisms, and messenger RNAs (mRNAs) in tumor tissue were analyzed. Interactions between potential predictors and treatment effect on PFS were analyzed in a Cox model. Continuous variable predictors were evaluated by multivariate fractional polynomial interaction methodology and subpopulation treatment effect pattern plotting (STEPP).

Results: Overall, 152 patients treated with EB or erlotinib alone (E) were included in the analysis. Among 26 factors analyzed in 134 baseline serum samples, high follistatin and low leptin were identified as potential biomarkers for worse and better outcomes with EB, with interaction P values of 0.0168 and 0.0049, respectively. Serum concentrations of 12 angiogenic factors were significantly higher in patients with high follistatin. Low pVEGFA levels related to better outcomes with EB, interaction P=0.033. *VEGF-A165a* was

the only predictive tissue mRNA, showing a similar trend to pVEGFA. No valid results were obtained in 13 polymorphisms of eight genes.

Conclusions: EB treatment showed better treatment outcomes in patients with low pVEGFA and serum leptin, and limited efficacy in patients with high serum follistatin.

Keywords: Follistatin; plasma vascular endothelial growth factor-A (pVEGFA); leptin; *VEGF-A165a*

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Introduction

Lung cancer is a leading cause of cancer-related death worldwide, and a significant proportion of patients with non-small-cell lung cancer (NSCLC) have activating mutations in the epidermal growth factor receptor (*EGFR*) gene (1,2). First-generation EGFR-tyrosine kinase inhibitors (TKIs), such as erlotinib, were the standard of care for patients with activating *EGFR* mutation-positive NSCLC (3-5). The third-generation EGFR-TKI, osimertinib has demonstrated improved progression-free survival (PFS) and overall survival (OS) compared with the first-generation EGFR-TKIs, erlotinib and gefitinib (6,7). However, most patients experience disease progression during their treatment course with EGFR-TKIs. New strategies may overcome development of resistance.

Highlight box

Key findings

- In this exploratory biomarker analyses of the phase II JO25567 study, EB showed favorable treatment outcomes in patients with low pVEGFA and serum leptin, and limited efficacy in patients with high serum follistatin.

What is known and what is new?

- The JO25567 study found that EB significantly prolonged median PFS compared with E, without unexpected adverse effects; HR 0.54 (95% CI: 0.36–0.79).
- A consistent predictive biomarker for bevacizumab treatment has not yet been validated across any cancer type. This study is thought to be advantageous for biomarker analyses for bevacizumab due to the relatively homogeneous patient population selected.

What is the implication, and what should change now?

- We expect our results will provide a basis for validation studies and contribute toward understanding the therapeutic positioning of the combination therapy among various solutions to improve EGFR-TKI outcomes for patients with *EGFR*-positive NSCLC.

Bevacizumab is a humanized monoclonal antibody against vascular endothelial growth factor-A (VEGF-A), and has been shown to provide additional efficacy when used in combination with chemotherapy in multiple tumor types (8,9). Bevacizumab inhibits VEGF-induced neovascularization needed for tumor growth, and partially normalizes tumor vessels with abnormal, hyperpermeable properties caused by VEGF-A, thereby improving the interstitial fluid pressure of the tumor (10).

EGFR-TKIs are thought to inhibit VEGF-A activity through cross-talk between their respective signaling pathways (11). In preclinical studies, combined VEGF and EGFR inhibiton was found to postpone the development of EGFR-TKI resistance, and was still effective in EGFR-TKI-resistant tumors (12,13). Moreover, in the phase II JO25567 study, erlotinib plus bevacizumab (EB) provided a 6.3-month extension in median PFS compared with erlotinib alone (E) in patients with advanced *EGFR* mutation-positive non-squamous NSCLC (14). EB significantly prolonged median PFS {16.0 [95% confidence interval (CI): 13.9–18.1] months} compared with E [9.7 (95% CI: 5.7–11.1) months], without unexpected adverse effects; hazard ratio (HR) 0.54 (95% CI: 0.36–0.79; P=0.0015). To understand the additional effect of EB, we analyzed biomarkers that are thought to correlate with EB efficacy.

Plasma VEGF-A (pVEGFA) is one of the most frequently investigated biomarkers for bevacizumab. The phase III studies, AVAGAST, AVADO, and AViTA, demonstrated that high pVEGFA levels correlate with longer PFS and/or OS in patients with advanced gastric cancer (GC), metastatic breast cancer (mBC), and pancreatic cancer (PAC) treated with bevacizumab (15-17). However, this correlation has not been consistently replicated across different studies and cancer types (18). Various circulating angiogenic factors (CAFs) other than VEGF-A have also been analyzed for

their association with bevacizumab treatment outcomes; soluble VEGF receptor 1 (sVEGFR1), interleukin-8 (IL-8), and angiopoietin 2 were reported to have some predictive potential, but not for patients with NSCLC (19).

Tumor tissue expression levels of VEGF pathway genes and proteins have been studied to find biomarkers for bevacizumab, but few correlations have been demonstrated. *VEGF-A* gene expression has been suggested as a surrogate marker for angiogenesis (20). Recent studies suggest the importance of separate evaluation of the *VEGF-A* splice isoforms, *VEGF-Axxxa* and *xxxb*, to predict bevacizumab treatment outcomes in patients with BC and colorectal cancer (CRC). Although bevacizumab binds both isoforms, *VEGF-Axxxb* has an anti-angiogenic profile that differs from pro-angiogenic *VEGF-Axxxa* (21,22). Low tumor tissue expression levels of baseline VEGFR1 and co-receptor neuropilin 1 have been reported to correlate with better bevacizumab treatment outcomes in several phase III studies in patients with GC, CRC, and mBC (15,19,23).

Tumor endothelial cells (ECs) are the main target of VEGF-A; therefore, the tumor angiogenic response to bevacizumab treatment is thought to be influenced by polymorphisms in angiogenesis-related genes. Several studies have reported that polymorphisms in angiogenesis-related genes affect PFS, OS, and/or severe hypertension in bevacizumab-treated patients with various cancer types, including NSCLC, renal-cell carcinoma, mBC, and PAC (19,24).

Although several biomarkers for bevacizumab treatment efficacy look promising, a consistent predictive biomarker has not been validated across any cancer type. Since NSCLC is categorized into various subtypes based on genotype and histology, biomarker analyses may also be needed within NSCLC subtypes. Therefore, the JO25567 study is thought to be advantageous for biomarker analyses for bevacizumab due to the relatively homogeneous patient population selected (Japanese patients with previously untreated *EGFR*-positive non-squamous NSCLC). In the present study, we performed biomarker analyses for EB treatment using data from the JO25667 study, aiming to: (I) confirm the association between bevacizumab treatment outcomes and pVEGFA level; (II) screen possible predictive biomarker candidates for bevacizumab, including CAFs, messenger RNAs (mRNAs) and proteins in tumor tissues, and polymorphisms in angiogenesis-related genes; (III) understand the underlying mechanisms of bevacizumab efficacy when given as EB. We present this article in accordance with the REMARK reporting checklist (available at https://tlcr.amegroups.com/article/view/10.21037/tlcr-22-632/rc).

Methods

Study design and patients

The study design and patient population for the JO25567 study have been reported previously (14). Briefly, JO25567 was a multicenter, randomized, open-label, phase II study examining the addition of EB as a first-line therapy in patients with NSCLC harboring activating *EGFR* mutations. Between February 21, 2011 and March 5, 2012, 154 patients were enrolled from 30 centers in Japan and randomly assigned 1:1 to receive either erlotinib 150 mg once daily plus bevacizumab 15 mg/kg every 3 weeks or erlotinib 150 mg once daily. The primary endpoint was PFS determined by an independent review committee. Secondary endpoints were OS, tumor response, quality of life, and safety. An exploratory objective was to seek predictive biomarkers for EB treatment outcomes.

Eligible patients had histologically and/or cytologically confirmed stage IIIB/IV or postoperative recurrent non-squamous NSCLC with an activating *EGFR* mutation (either exon 19 deletion or L858R mutation). Key exclusion criteria included confirmation of T790M mutation and the presence of brain metastases. Detailed inclusion and exclusion criteria have been published (14).

The study was performed in accordance with the Declaration of Helsinki (as revised in 2013) and Good Clinical Practice guidelines. The study protocol was reviewed and approved by the institutional review boards of the participating institutions, and written informed consent was obtained from all patients. The study was registered with the Japan Pharmaceutical Information Center (No. JapicCTI-111390).

Procedures for biomarker analyses

Informed consent was obtained from patients before sample collection for each type of biomarker analysis. Five types of biomarker were analyzed (*Figure 1*): pVEGFA, CAFs in serum, polymorphisms of angiogenesis-related genes, and mRNAs and proteins in tumor tissue specimens.

Tumor tissue specimens were removed at diagnosis or during surgery and the formalin-fixed and paraffin-embedded (FFPE) samples were stored at room temperature. Blood samples for the analysis of CAFs were taken

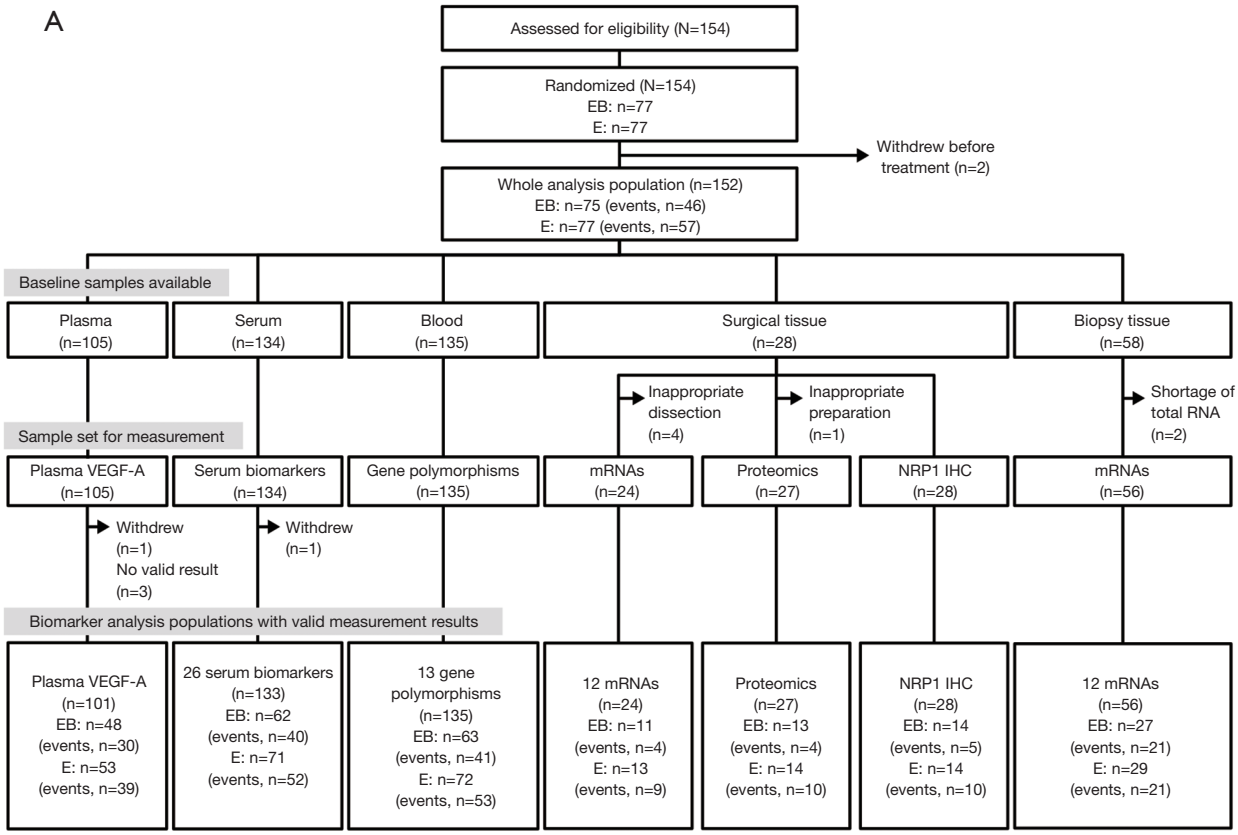

Figure 1 Overview of the study population and analyzed biomarkers. (A) Study profile. (B) List of analyzed biomarkers. EB, combination therapy of erlotinib 150 mg/day and bevacizumab 15 mg/kg every 3 weeks; E, 150 mg/day erlotinib monotherapy; VEGF-A, vascular endothelial growth factor-A; mRNA, messenger RNA; NRP1, neuropilin 1; IHC, immunohistochemistry; VCAM1, vascular cell adhesion molecule 1; ICAM1, intercellular adhesion molecule 1; IL-8, interleukin-8; G-CSF, granulocyte colony-stimulating factor; PDGF-BB, platelet-derived growth factor BB; HGF, hepatocyte growth factor; PECAM1, platelet/endothelial cell adhesion molecule 1; PlGF, placental growth factor; HB-EGF, heparin-binding epidermal growth factor-like growth factor; TGF-α, transforming growth factor-α; FGF, fibroblast growth factor; EGF, epidermal growth factor; sVEGFR, soluble vascular endothelial growth factor receptor; sEGFR, soluble epidermal growth factor receptor; MIP-1β, macrophage inflammatory protein-1β; SNP, single nucleotide polymorphism; VEGFR, vascular endothelial growth factor receptor; CXCR2, CXC chemokine receptor 2; IL-1β, interleukin-1β; VNTR, variable number of tandem repeats; IL-1RN, interleukin-1 receptor antagonist; HIF-1α, hypoxia-inducible factor-1α; HIF-1β, hypoxia-inducible factor-1β.

before the initiation of the first treatment administration; ethylenediamine tetraacetic acid (EDTA) plasma and serum were prepared and stored frozen at –70 ℃ before analysis. For genotyping, a 5 mL blood sample was taken from each patient between day 1 of treatment cycle 1 and the final visit, and stored below –70 ℃.

pVEGFA

pVEGFA was analyzed in duplicate by multiplex enzyme-linked immunosorbent assay (ELISA) methods using Immunological Multiparametric Chip Technique (IMPACT) technology (Roche Diagnostics GmbH, Mannheim, Germany) (18,25). The analysis was performed according to User Guidelines ONC 2.03 version 02 for IMPACT by Roche Diagnostics GmbH.

Quantification of ligands and soluble receptors in serum

The concentrations of secreted proteins in serum samples were determined using commercially available bead-based sandwich immunoassay kits. Vascular cell adhesion molecule 1, intercellular adhesion molecule 1 (ICAM1), macrophage inflammatory protein-1β (MIP-1β), and E-selectin were assayed using the Procarta Cytokine Assay Kit (PN-PC1002) (Panomics, Inc., Fremont, CA, USA). IL-8, granulocyte colony-stimulating factor (G-CSF), VEGF, platelet-derived growth factor BB (PDGF-BB), hepatocyte growth factor (HGF), angiopoietin-2, follistatin, leptin, and platelet/endothelial cell adhesion molecule 1 (PECAM-1) were determined using the Bio-Plex Pro Human Angiogenesis Assay Panel (Bio-Rad Laboratories, 171-A4011M, CA, USA). Placental growth factor (PlGF), epiregulin, heparin-binding epidermal growth factor-like growth factor (HB-EGF), tenascin-C, transforming growth factor (TGF)-α, amphiregulin, betacellulin, basic fibroblast growth factor (bFGF), and EGF were measured using the WideScreen Human Cancer Panel 2 (BPHCPP002-BASE) (Merck, Kenilworth, NJ, USA). Levels of soluble receptors sVEGFR-1, sVEGFR-2, sVEGFR-3, and sEGFR were determined using the Human Soluble Cytokine Receptor Panel 4-plex (HSCR-32K 4-plex) (Millipore, St. Charles, MO, USA). All assays were performed according to each manufacturer's instructions. Measurements were run using a Bio-Plex Suspension Array Full System (Bio-Rad, Hercules, CA, USA).

Genotyping

Key polymorphisms in angiogenesis-related genes were analyzed by LSI Medience Co. (Tokyo, Japan). Genes were extracted from blood samples and amplified by polymerase chain reaction (PCR) using the GeneAmp® PCR System 9700 Dual 96 Well system (Applied Biosystems®, Foster City, CA, USA). A total of 12 single nucleotide polymorphisms (SNPs) were detected by standard PCR reaction and DNA direct sequencing methods in seven genes [*VEGF*, *VEGFR1*, *VEGFR2*, *IL-8*, CXC chemokine receptor 2 (*CXCR2*), *ICAM1*, and *IL-1β*], using a 3130xl Genetic Analyzer (Applied Biosystems®, Foster City, CA, USA). A variable number tandem repeat (VNTR) was analyzed by electrophoresis in the IL-1 receptor antagonist protein (*IL-1RN*) using the Microchip Electrophoresis System for DNA/RNA Analysis MCE®-202 MultiNA (Shimadzu, Kyoto, Japan).

Gene expression analysis by MassARRAY

Total RNA was isolated from FFPE slides of tumor tissue and analyzed using the multiplex analysis of gene expression system, MassARRAY (LungCarta Panel; Sequenom, San Diego, CA, USA). Specifically, the FFPE specimens were divided into sections (4 μm thick), which were placed on slides and deparaffinized. For surgical specimens, one of the serial sections was stained with hematoxylin and eosin as a reference slide. The tumor-rich area of the reference slide was marked by a pathologist. Guided by the slide, the corresponding tumor-rich area was manually dissected in the remaining serial sections to avoid contamination of non-tumor tissue (macrodissection). The macrodissection procedure was not applied for biopsy specimens. Total RNA was extracted, purified, and measured quantitatively using the Quanti-iT™ RiboGreen® RNA Assay Kit (Life Technologies Japan Ltd., Tokyo, Japan) according to the manufacturer's instructions. mRNAs of *VEGF-A*, *VEGFR1*, *VEGFR2*, *prokineticin-2*, *neuropilin 1*, *neuropilin 2*, hypoxia-inducible factor-1α (*HIF-1α*), and *HIF-1β* were analyzed via MassARRAY in reference to beta-actin (*ACTB*), hypoxanthine phosphoribosyltransferase-1 (*HPRT1*), ribosomal protein L13A (*RPL13A*), and 14-3-3 protein

zeta/delta (*YWHAZ*) housekeeping genes, which were used as internal standards.

Immunohistochemistry (IHC) for neuropilin-1

IHC was performed on FFPE slides of tumor tissue at LSI Medience Co. (Tokyo, Japan). The human neuropilin-1 antibody [monoclonal mouse immunoglobulin G1 clone #446915, Code No. MAB38701] was purchased from R&D Systems, Inc. Reference tumor tissue slides of human colon cancer, adenocarcinoma, grade I (Code No. HuCAT116), and human colon cancer, adenocarcinoma, grade II (Code No. HuCAT121) were purchased from US BioMax, Inc. and stored at 4 ℃.

Serial sections were prepared; one section was stained with hematoxylin and eosin and one section was used for IHC with the human neuropilin-1 antibody. The intensity of staining in each tumor cell was assessed among a total of 500 cells. An H-score was calculated as an aggregate of the percentage of stained tumor cells at each intensity for a score between 0 and 3 as follows: H score = (0x%) + (1x%) + (2x%) + (3x%), where 0, 1, 2, and 3 were negative, weak, intermediate, and strong staining, respectively.

Statistical analysis

The modified intent-to-treat population for the efficacy analysis included all patients who had received at least one dose of study treatment and had a tumor assessment at least once after randomization. There were no preplanned sample size or power calculations for the biomarker analyses. Median PFS in each subgroup was estimated using the Kaplan-Meier method; Greenwood's formula was used to calculate 95% CIs.

pVEGFA levels were categorized into high and low using the median as a cutpoint. To assess the predictive value of each biomarker, Cox proportional hazards models were used to determine HRs and 95% CIs with the following terms: EB treatment, biomarker status, and interaction between EB treatment and biomarker status, with or without adjustment by the stratification factors. Patients were stratified according to sex (men *vs.* women), disease stage (stage IIIB *vs.* stage IV *vs.* postoperative relapse), smoking history (never smokers or former light smokers *vs.* others), and type of *EGFR* mutation (exon 19 deletion *vs.* L858R mutation). Multiplicities were not considered in the analyses. Interaction of continuous variable predictors (categorized using median or quartile cutpoints) with treatment was analyzed. Secondarily, the multivariable fractional polynomial interaction (MFPI) procedure and tail-oriented subpopulation treatment effect pattern plot (TO-STEPP) were applied following Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) guidelines (26-28).

The statistical analysis methodology for gene polymorphisms is provided in the Appendix 1. All analyses were conducted using Statistical Analysis Systems, version 9.2 (SAS Institute, Cary, NC, USA) and R version 3.0.3.

Results

Patients and samples

Of the 154 enrolled patients, the data from 152 patients (EB, n=75; E, n=77) were included in the analysis population (*Figure 1*). At the data cutoff date (30 June 2013), 103 progression events had occurred; median follow-up was 20.4 months (14).

The baseline characteristics of patients in each biomarker-analysis population were generally similar to the whole analysis population with respect to age, gender, disease stage, smoking history, and type of *EGFR* mutation (except for those with surgical tissue specimens), and well balanced between treatment groups in each analysis population (Table S1). However, the proportion of patients with recurrent disease was higher in the surgical tissue sample population than the whole analysis population (Table S1).

pVEGFA

We evaluated the predictive value of pVEGFA for EB treatment efficacy in 105 EDTA plasma samples. Median pVEGFA levels were 18.0 pg/mL [minimum (min), 7.7 pg/mL; maximum (max), 95.8 pg/mL] in the EB treatment group and 18.8 pg/mL (min, 9.1 pg/mL; max, 59.2 pg/mL) in the E group.

On Cox analysis, pVEGFA levels were associated with EB treatment outcomes, suggesting that pVEGFA levels may have predictive value (*Figure 2A*,*2B*). EB treatment improved PFS *vs.* E alone for patients with pVEGFA levels below the median in patients with (HR, 0.23; 95% CI: 0.09–0.60) or without (HR, 0.30; 95% CI: 0.14–0.63) adjustment for stratification factors. The risk was reduced by 44% (HR, 0.56; 95% CI: 0.26–1.25) or 27% (HR, 0.73; 95% CI: 0.39–1.39) in patients with pVEGFA levels above the median with or without adjustment for stratification factors,

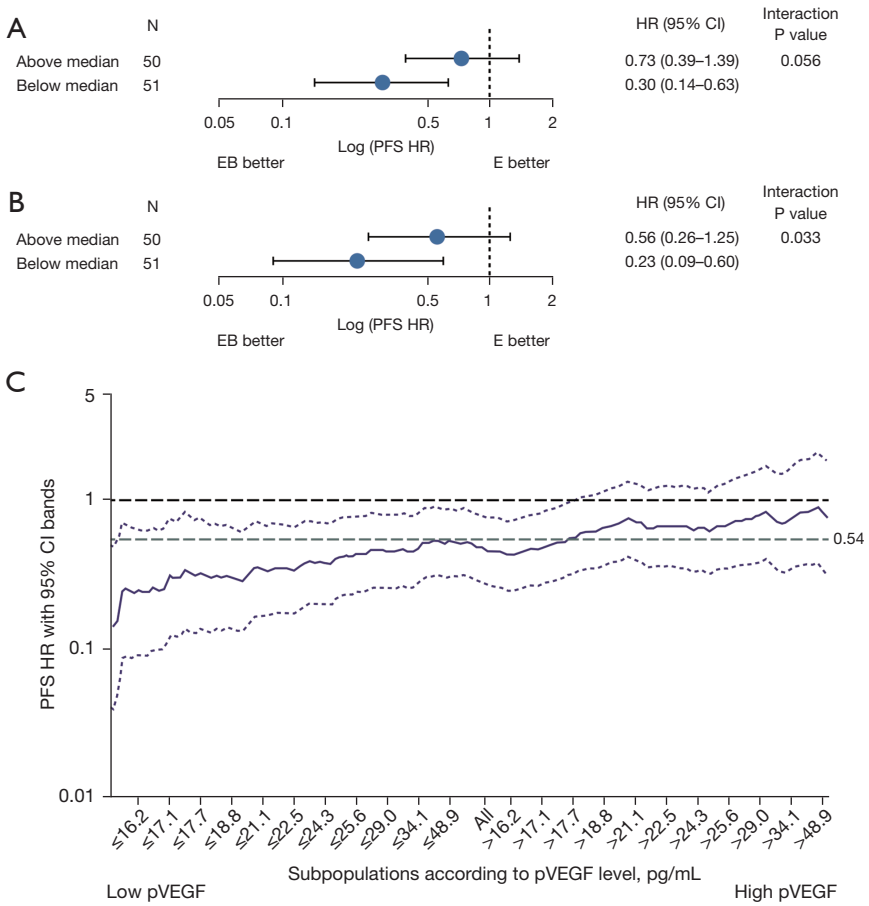

Figure 2 HRs of EB and E alone for PFS according to pVEGF level. (A) Forest plots of HRs for PFS by dichotomized pVEGF levels at the median value (18.57 pg/mL). HRs and interaction P value were calculated by unstratified Cox proportional hazard methodology. (B) Forest plots of HRs for PFS by dichotomized pVEGF levels at the median value (18.57 pg/mL). HRs and interaction P value were calculated by stratified Cox proportional hazard methodology. (C) STEPP of HRs (EB *vs.* E alone) for PFS by pVEGF levels. The TO-STEPP was used. A HR was calculated in each of the subpopulations, which were defined by the range of pVEGF values, and the HR in the overall population is shown in the center of the figure (represented by the grey dotted line). With increasing distance from the center, more patients with high pVEGF values (to the left side) or low pVEGF values (to the right side) were deleted in the subpopulations (25). HR, hazard ratio; CI, confidence interval; PFS, progression-free survival; EB, combination therapy of erlotinib 150 mg/day and bevacizumab 15 mg/kg every 3 weeks; E, 150 mg/day erlotinib monotherapy; pVEGF, plasma vascular endothelial growth factor; STEPP, subpopulation treatment effect pattern plot; TO-STEPP, tail-oriented subpopulation treatment effect pattern plot.

respectively. The interaction P values were 0.033 between EB treatment outcomes and pVEGFA levels with adjustment for stratification factors. The interaction was visualized using TO-STEPP methodology, which demonstrated that the HR was positively related to pVEGFA levels assessed as a continuous variable (*Figure 2C*) (26).

Serum ligands and soluble receptors

We analyzed 26 ligands and soluble receptors in 134 serum samples available among the 152 patients included from JO25567. Cox regression analysis was performed for the interaction of EB treatment outcomes (PFS) with four ligand/receptor subgroups categorized by quartile

cutpoints of the biomarker levels at primary screening. We screened one soluble receptor (sVEGFR3) and four ligands (follistatin, leptin, IL-8, and MIP-1β) as candidate biomarkers, with an interaction P value <0.2. HRs (EB *vs.* E treatment) for PFS in subgroups by biomarker quartiles and interaction P values are shown in *Figure 3*.

Secondly, we evaluated interactions between treatment outcomes and biomarkers using the MFPI approach (27). *Table 1* shows the interaction between EB treatment outcomes and follistatin, leptin, and MIP-1β levels, with P<0.05 without adjustment for stratification factors. When four stratification factors were incorporated in the MFPI analysis as covariates (gender, disease stage, smoking history, and type of *EGFR* mutation), follistatin and leptin levels also had significant interactions (P<0.05) with EB treatment outcomes, but MIP-1β did not. Their relationships were visualized using TO-STEPP methodology (*Figure 4A*,*4B*) (26).

Cutpoints [by methods according to Jiang *et al.* (29)] were estimated at 490.5 (95% CI: 251.6–563.0) and 1,662.4 (95% CI: 1,245.1–5,966.8) pg/mL for follistatin and leptin, respectively, and were supported by STEPP (*Figure 4A*,*4B*). Among the 133 patients analyzed, 35 were above the cutpoint for follistatin and 40 were below the cutpoint for leptin. Even in patients with higher leptin levels (n=93), median PFS was still longer in the EB-treated group [16.0 (95% CI: 13.4–20.9) months] than the E-treated group [11.1 (95% CI: 8.0–18.0) months].

Univariate logistic regression analysis revealed a significant correlation between follistatin levels dichotomized at its cutpoint (490.5 pg/mL) and baseline serum concentrations of 12 out of 25 evaluated serum angiogenesis-related proteins. PECAM-1, G-CSF, IL-8, HGF, HB-EGF, amphiregulin, betacellulin, bFGF, tenascin-C, angiopoietin 2, epiregulin, and PlGF were significant under the threshold q value <0.05 [false discovery rate (FDR) by Benjamini and Hochberg (30)]. Figure S1 shows the distribution of serum concentrations of the 12 proteins divided into two subgroups of patients who had follistatin levels above and below the cutpoint.

Gene expression in tissue specimens

Tumor tissue specimens were collected from 82 patients (58 by biopsy; 24 surgically). MassARRAY analysis for mRNAs of angiogenesis-related genes was performed using the extracted RNA, excluding two biopsy samples due to a shortage of total RNA (<10 ng) extracted. For the statistical analysis, data were used from the 24 surgical samples only, since contamination of mRNA was suspected in the biopsy samples from peripheral normal tissues but not tumor tissues (data not shown). Further analysis for prokineticin-2 was not performed, as the mRNA level was only determined by MassARRAY in 2/24 samples.

Results of the interaction between EB treatment outcomes and mRNA expression levels of *VEGF-A* isoforms and other angiogenesis-related genes using Cox regression analysis are shown in *Figure 5A*. *VEGF-A165a* mRNA was suggested to interact with EB treatment outcomes (P=0.034), but no interactions were observed in the other mRNAs analyzed. Patients with *VEGF-A165a* mRNA levels below the median seemed sensitive to EB treatment efficacy.

Gene polymorphisms

A total of 135 blood samples (from 152 patients) were available for genotype analysis of angiogenesis-related genes. We analyzed 12 SNPs and one VNTR of eight genes including *VEGF-A*, *VEGFR1*, *VEGFR2*, *CXCR2*, *ICAM1*, *IL-1β*, *IL-8*, and *IL-1RN*.

Figure 5B shows the HRs (EB *vs.* E) for PFS in the genotypes analyzed. Patients who were homozygous with the minor allele *VEGF-A* +936C/T (rs3025039) SNP T/T were suggested to have a favorable median PFS with EB treatment compared with those with C/T or C/C; the interaction of its recessive effect with EB treatment was P=0.012 (but only 11 patients had T/T). The minor alleles of *VEGF-A* –2578A/C (rs699947) (A/A, n=15) and *VEGFR1* rs9582036 (G/G, n=6) showed a marginal recessive effect, with P=0.056 and 0.052, respectively (*Figure 5B*). The G allele of *VEGFR1* 3635+319G/T (rs9582036) also showed a marginal dominant effect with P=0.075, but the direction of shift in HR with T/G from major alleles T/T was different from those with G/G (*Figure 5B*). All 135 patients had a T/T genotype in *VEGFR1* +3639C/T (rs7993418). No other significant interactions were observed between EB treatment outcomes and the gene polymorphisms analyzed.

Protein levels in tumor tissues

Neuropilin was evaluated in tumor tissue (n=28) by IHC but no interaction was detected; interaction P values were 0.997 or 0.858 in Cox regression models with or without adjustment for the four stratification factors, where neuropilin H-score levels were categorized as below (n=14) or above the median (n=14). Proteomic analysis was planned but could not be performed due to a shortage of tissue samples for analysis.

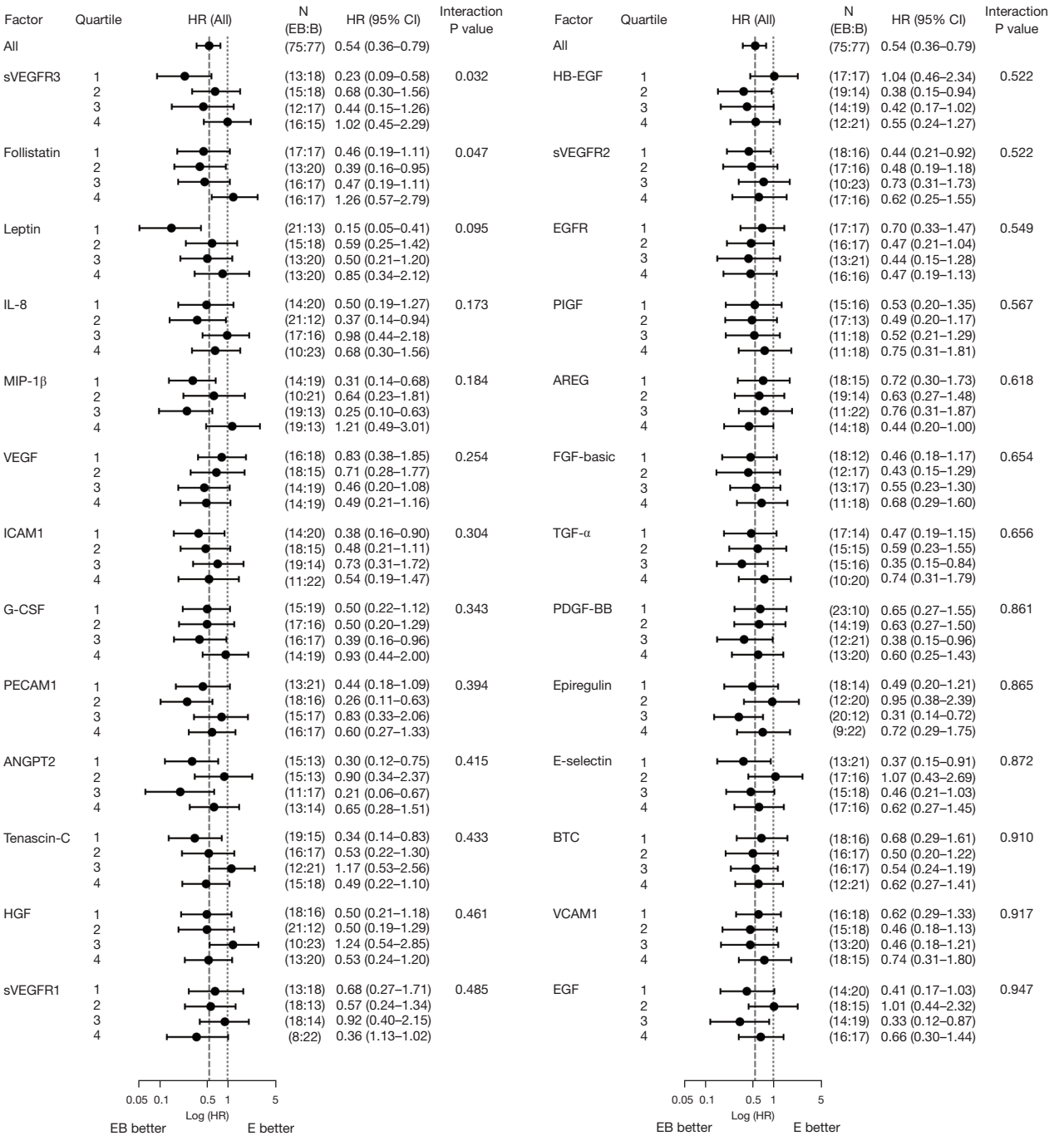

Figure 3 Forest plots of HRs (EB *vs.* E alone) for PFS by quartiles of measured ligands and soluble receptors in serum. HRs and interaction P values were calculated by unstratified Cox proportional hazard methodology. HR, hazard ratio; EB, combination therapy of erlotinib 150 mg/day and bevacizumab 15 mg/kg every 3 weeks; E, 150 mg/day erlotinib monotherapy; CI, confidence interval; sVEGFR, soluble vascular endothelial growth factor receptor; EGFR, epidermal growth factor receptor; HB-EGF, heparin-binding epidermal growth factor-like growth factor; IL-8, interleukin-8; PlGF, placental growth factor; MIP-1β, macrophage inflammatory protein-1β; AREG, amphiregulin; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; ICAM1, intercellular adhesion molecule 1; TGF-α, transforming growth factor-α; G-CSF, granulocyte colony-stimulating factor; PDGF-BB, platelet-derived growth factor BB; PECAM1, platelet/endothelial cell adhesion molecule 1; ANGPT2, angiopoietin 2; BTC, betacellulin; HGF, hepatocyte growth factor; VCAM1, vascular cell adhesion molecule 1; EGF, epidermal growth factor; PFS, progression-free survival.

Table 1 Interaction of bevacizumab treatment with ligands and soluble receptors in serum

Biomarker	Model†	P value of interaction	
		Without covariate	With covariate
Leptin	αX^{-2}	0.0037	0.0049
Follistatin	αX^{3}	0.0148	0.0168
MIP-1β	$\alpha X^{0.5}$	0.0459	0.0548
sVEGFR3	$\alpha \log(X)$	0.0586	0.0593
IL-8	$\alpha X^{-2} + \beta X^{-2} \log(X)$	0.3142	0.1717

†, model chosen by fractional polynomial methodology. MIP-1β, macrophage inflammatory protein-1β; sVEGFR3, soluble vascular endothelial growth factor receptor 3; IL-8, interleukin 8.

Discussion

JO25567 was the first prospective randomized study that demonstrated significant prolongation of PFS by combining bevacizumab with an EGFR-TKI in patients with *EGFR*-mutation positive non-squamous NSCLC. In the current biomarker analysis from JO25567, we observed that baseline levels of pVEGFA, serum follistatin, and leptin were associated with bevacizumab treatment efficacy, when given in combination with erlotinib.

In the current study, patients with pVEGFA levels below the median were considered sensitive to EB treatment; however, this is inconsistent with previous reports showing that higher pVEGFA levels led to better outcomes following bevacizumab treatment in patients with mBC, GC, and PAC (15-17). It is notable that median pVEGFA levels were 18.0 pg/mL in the EB-treated group and 18.8 pg/mL in the E-treated group in the JO25567 study; these are much lower than previous studies. Median pVEGFA was 125 pg/mL in patients with mBC in AVADO and 111 pg/mL in patients with GC in AVAGAST, even though pVEGFA was measured by the same IMPACT assay, which is sensitive to short isoforms (VEGF-A111 and 121) (15,16). Miles *et al.* reported that baseline pVEGFA levels were not predictive of a PFS benefit from bevacizumab in the MERiDiAN phase III trial, which prospectively investigated the correlation of pVEGFA and bevacizumab efficacy in patients with HER2-negative mBC (31). Additionally, a comprehensive reassessment of 12 clinical studies of eight tumors suggested that pVEGFA is not a robust predictive marker of bevacizumab treatment outcomes (18). On the other hand, Bai *et al.* reported a similar trend to the current findings in patients with mCRC, with patients with lower pVEGF-A121 levels gaining more benefit in terms of PFS and OS following bevacizumab treatment (32). They discussed that the roles of VEGF-A121 may be context- and cancer type-dependent. In the case of the current study, the analysis may be affected by the relatively restricted population of Japanese patients who had genetically and histologically homogeneous tumors [non-squamous EGFR active mutation-positive NSCLC (mainly adenocarcinoma)]. Further study will be needed in this population.

In the current study, gene expression analysis in tumor tissues showed that patients with lower *VEGF-A165a* mRNA levels seemed to be sensitive to EB treatment, though the sample size was small (n=24), further supporting pVEGFA as a potent predictive biomarker for bevacizumab treatment outcomes, at least in Japanese patients with NSCLC harboring an *EGFR* mutation. However, Pentheroudakis *et al.* reported that a higher level of *VEGF-Axxxa* mRNA in tumor tissue was associated with adverse prognosis in the absence of bevacizumab but with favorable outcomes when bevacizumab was administered in patients with advanced BC (22). This discrepancy may be derived from the genetically and histologically homogeneous tumor profile in the JO25567 study, as mentioned above.

EB treatment efficacy was reduced in patients with higher serum follistatin levels. The primary function of follistatin, a 31–39 kDa glycosylated cysteine-rich polypeptide, involves binding and neutralizing some members of the TGF-α superfamily, such as activin A (ActA) and myostatin. The interaction between follistatin and ActA results in an almost irreversible binding complex that prevents ActA from interacting with its receptors. Follistatin, widely expressed in various human tissues, is a product of a single gene on chromosome 5q11.2, which is translated into at least two molecular forms by alternative splicing and post-translational modifications i.e., follistatin 288 and 315. The latter is the predominant circulating form, existing mainly in a bound state (33).

Chen *et al.* reported that: (I) elevated follistatin in serum from patients with lung adenocarcinoma is directly produced by tumor cells; (II) serum follistatin is higher in patients with poorly-differentiated adenocarcinoma than in those with well-differentiated tumors; and (III) follistatin may promote the survival of lung adenocarcinoma cells by inhibiting apoptosis (34). Gökmen-Polar *et al.* reported a suggestive result in xenograft models, with the follistatin pathway being one of the top signaling pathways associated with resistance to bevacizumab in a human breast cancer

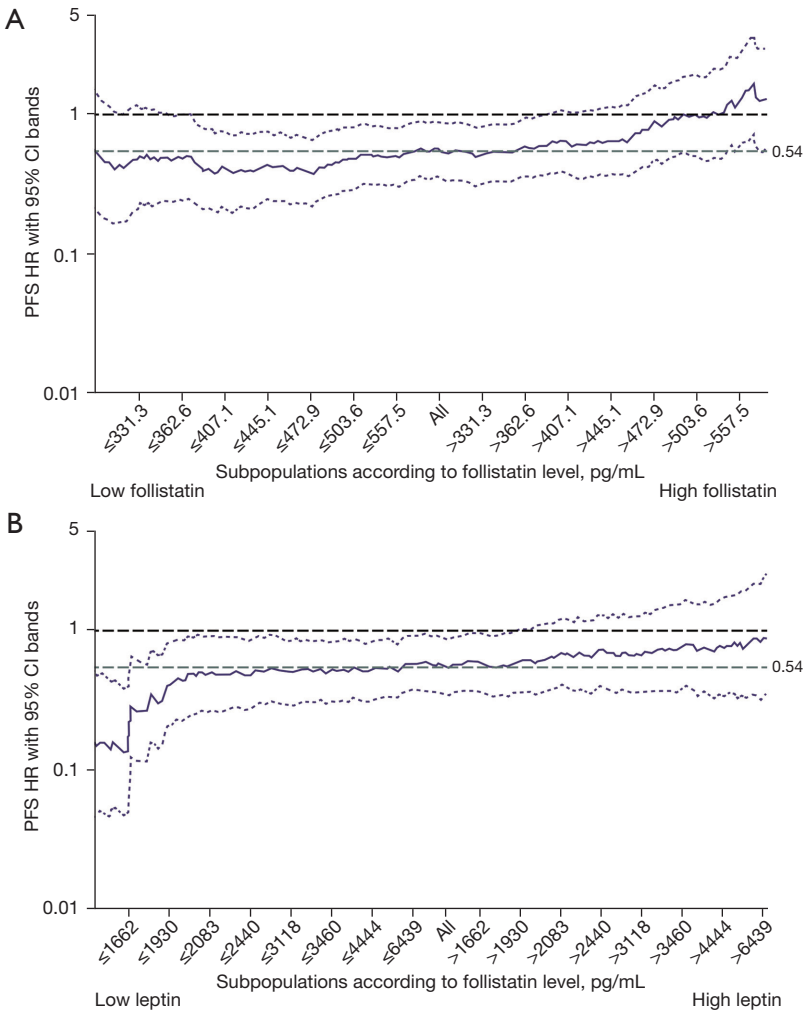

Figure 4 Interaction between outcome of EB treatment on PFS and levels of follistatin and leptin in serum, visualized by TO-STEPPs (25). (A) TO-STEPP of HRs (EB *vs.* E alone) for PFS by serum follistatin. The HR in the overall population is shown in the center of the figure (represented by the grey dotted line). (B) TO-STEPP of HRs for PFS by serum leptin. The HR in the overall population is shown in the center of the figure (represented by the grey dotted line). PFS, progression-free survival; HR, hazard ratio; CI, confidence interval; EB, combination therapy of erlotinib 150 mg/day and bevacizumab 15 mg/kg every 3 weeks; TO-STEPP, tail-oriented subpopulation treatment effect pattern plot; E, 150 mg/day erlotinib monotherapy.

cell line (MCF-7) over expressing the VEGF-A gene (35).

Many investigators have reported that the follistatin/ActA system plays an important role in neovascularization but their findings are controversial. Some have reported that constitutively expressed ActA in ECs inhibits EC proliferation in an autocrine or paracrine manner, to maintain a quiescent endothelium, and that follistatin stimulates EC proliferation and angiogenesis by neutralizing the growth inhibitory effect of ActA (36,37). Conversely, an anti-angiogenic effect of follistatin has also been reported: ActA has been shown to increase the expression of VEGF-A, VEGFR1, and VEGFR2 in an autocrine or paracrine manner, and follistatin reduced the expression of VEGFR1 and VEGFR2 by blocking ActA (38). In addition, ActA induces apoptosis in various tumor cells, and follistatin blocks its activity (38,39). Combining these findings, follistatin is speculated to inhibit VEGF-A-dependent angiogenesis, but stimulate neovascularization independently from VEGF-A/VEGFRs and promote the survival of tumor cells. This speculation may explain the

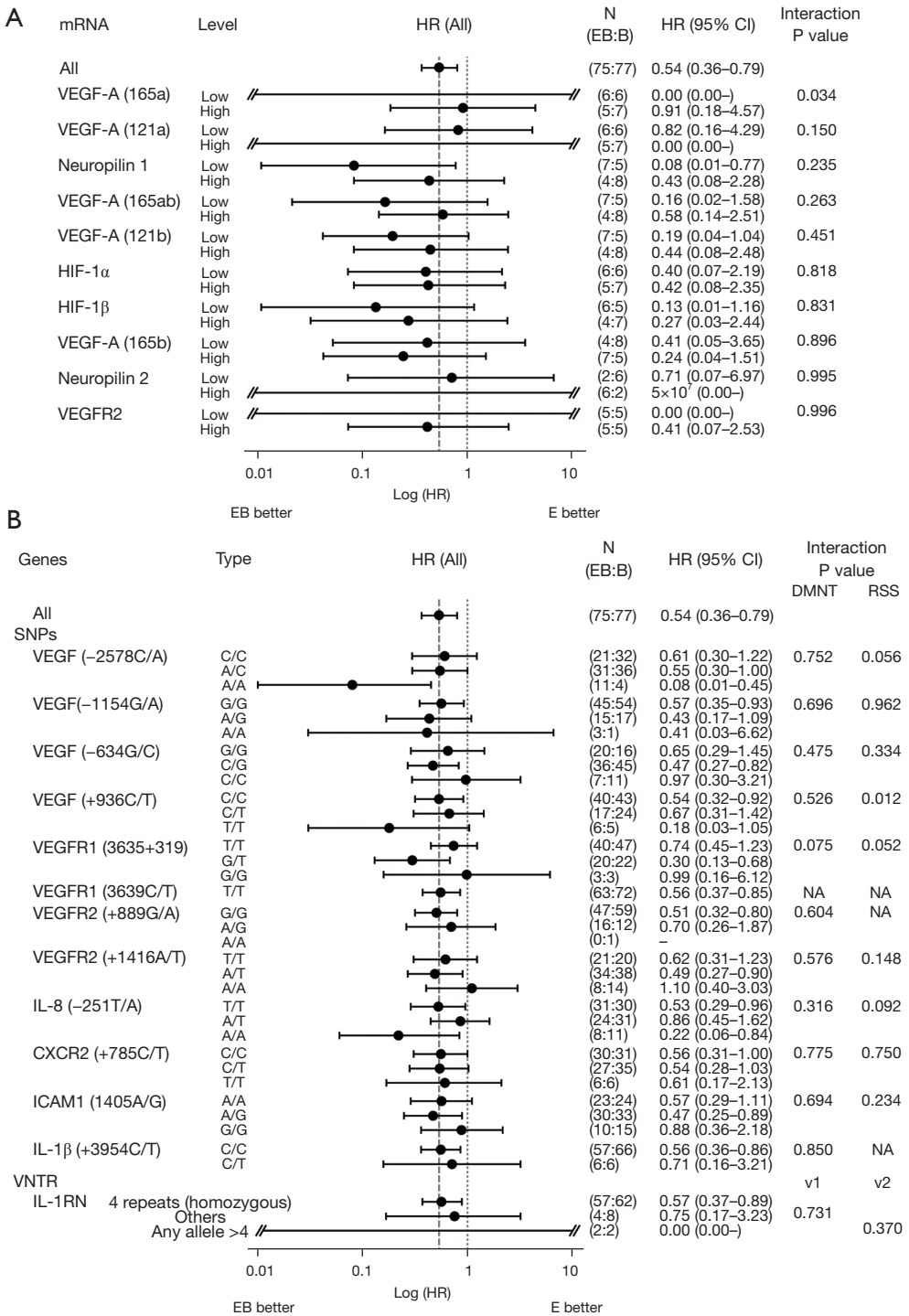

Figure 5 Forest plot of HRs (EB *vs.* E alone) for PFS. (A) by mRNA levels in tumor tissue dichotomized at the median value and (B) by gene polymorphisms. HRs and interaction P value were calculated by unstratified Cox proportional hazards regression analysis. Each genotype polymorphism was transformed into two dummy variables. For SNPs, the V_1 was coded 0 for homo- and heterozygosity of the major allele, and 1 for homozygosity of the minor allele. The V_2 was coded 0 for homozygosity of the major allele and 1 for homo- and heterozygosity of the minor allele. Dominant (DMNT), recessive (RSS), and additive effects of the minor allele are estimated when the interactions of treatment with only V_1, only V_2, and both are detected, respectively. In the figure, V_1 and V_2 were shown as DMNT and RSS

for SNPs, respectively. For VNTR in IL-1RN, the V_1 was coded 0 for homozygote of 4 or more repeats in any alleles and 1 for the others, and the V_2 was coded 1 for homozygote of more than 4 repeats in any alleles and 0 for the other types. mRNA, messenger RNA; HR, hazard ratio; EB, combination therapy of erlotinib 150 mg/day and bevacizumab 15 mg/kg every 3 weeks; E, 150 mg/day erlotinib monotherapy; CI, confidence interval; VEGF-A, vascular endothelial growth factor-A; HIF-1α, hypoxia inducible factor-1α; HIF-1β, hypoxia inducible factor-1β; VEGFR, vascular endothelial growth factor receptor; DMNT, DNA methyl transferase; RSS, residual sum of squares; SNP, single nucleotide polymorphism; VEGF, vascular endothelial growth factor; NA, not applicable; IL-8, interleukin-8; CXCR2, CXC chemokine receptor 2; ICAM1, intercellular adhesion molecule 1; IL-1β, interleukin-1β; VNTR, variable number of tandem repeats; v, version; IL-1RN, interleukin-1 receptor antagonist; PFS, progression-free survival; V_1, first dummy variable; V_2, second dummy variable.

limited efficacy of bevacizumab in patients with high serum follistatin in the present study.

The production of multiple growth factors, including angiogenic factors, has been reported in NSCLC cells (40,41). Frezzetti *et al.* reported that VEGF-A induced the secretion of a variety of angiogenic factors, including follistatin, HGF, angiopoietin-2, G-CSF, IL-8, leptin, PECAM-1, PDGF-BB, and VEGF-A itself in NSCLC cells (42). They hypothesized that the VEGF-A feed-forward loop can induce the formation of a network of multiple growth factors sustaining angiogenesis in NSCLC cells (42). Such an angiogenic condition is expected to reduce sensitivity to anti-VEGF-A agents. In the present study, baseline serum concentrations of 12 proteins out of 25 analyzed angiogenesis-related ligands and receptors were significantly higher (FDR <0.05) in patients with serum follistatin above the cutpoint (≥490.5 pg/mL) than those in other patients (Figure S1). Follistatin and the 12 proteins detected include the same factors reported by Frezzetti *et al.*, except leptin and VEGF-A (42). Although the participation of the VEGF-A feed-forward loop is not clear in the present study, high follistatin serum concentration may be a candidate tumor biomarker for the production of multiple angiogenic factors, and may be one explanation for the restricted efficacy of EB treatment in the patients with higher follistatin levels.

Approximately 40 patients with lower baseline serum levels of leptin were more sensitive to EB treatment than those with higher leptin levels. Leptin is a small adipokine (146 amino acids, 16 kDa) encoded by the obesity (*ob*) gene, which is primarily produced by fat cells (43). Serum leptin concentrations are highly correlated with body fat content and its production by adipocytes relates to nutritional status and acute phase reaction or chronic inflammation (44). In oncology, leptin has been investigated as an angiogenic factor that stimulates proliferation and reduces apoptosis in vascular ECs through leptin receptor expression (43). Leptin also causes the proliferation of various tumor cells such as BC and CRC, where leptin is known as an upstream regulator of VEGF-A and VEGFR2. Failure of initially promising anti-angiogenic therapies, and resistance to anti-angiogenic drugs mainly targeting VEGF-A/VEGFR2, including bevacizumab, are currently relevant problems (43). Compensatory and redundant effects of other angiogenic factors such as leptin are being investigated to clarify these failures.

Leptin levels are correlated with low fat mass or body mass index, and are thought to be inversely correlated with poor nutritional status. Patients with advanced NSCLC frequently suffer weight loss and malnutrition, where a low level of serum leptin is observed (44). Furthermore, patients with NSCLC and cachexia or anorexia are known to have significantly low serum leptin concentrations (44). The correlation with leptin levels and prognosis is unclear (45).

The relationship between serum leptin levels and local leptin levels produced in tumor tissue is not entirely known, but low serum leptin levels are expected to be caused by low levels of both local and systemic leptin production. In fact, the cutpoint of 1,662.4 pg/mL (approximately 1.7 ng/mL) evaluated in the current study was much lower than those considered in three representative studies in patients with NSCLC with comparatively large sample sizes: 9.3±10.8 ng/mL (n=101), 7.11±0.91 ng/mL (n=76) and 9.3±9.2 ng/mL (n=66) (44,46,47).

Patients with low leptin levels are thought to be sensitive to bevacizumab treatment, where leptin contributes little to local tumor angiogenesis, and they are anticipated to have anorexia or cachexia with a worse prognosis (43,44). Bevacizumab could inhibit tumor progression and induce apoptosis efficiently, which could lead to a reduction in the production of proinflammatory cytokines and/or growth factors in tumor cells, mediating cachexia and/or anorexia. These synergistic effects are speculated to improve EB treatment outcomes in patients with low levels of serum leptin.

In the current study, patients who are homozygous with

minor allele VEGF-A rs3025039 SNP (T/T) and rs699947 (A/A) are suggested to be sensitive to EB treatment, as reported by other groups (19). However, we could not conclude that these SNPs are candidate predictors due to the small number of carriers with homozygous minor alleles. The minor allele (G) of SNP rs9582036 in VEGFR1 has been reported to be associated with improved EB treatment outcomes (OS and PFS) in patients with metastatic PAC (24). The marginally dominant effects of VEGFR1 rs9582036 on EB treatment outcomes were suggested in the present study but the effects were inconsistent between patients with G/G and G/T (*Figure 5B*). Low sensitivity to bevacizumab was reported in carriers of the VEGFR1 SNP rs7993418 C/C, but the study did not provide any information, since no patient had a C allele (24).

Since the present study was planned as an exploratory biomarker study, there are several limitations. First, there were no preplanned sample sizes or statistical significance levels for each biomarker analysis. Second, no external/internal validation studies were performed. Following the promising results of the JO25567 study, several phase III studies demonstrated consistent PFS improvement with EGFR-TKI treatment in combination with VEGF signaling inhibitors compared with EGFR-TKI monotherapy (48-52). We expect our results will provide a basis for the validation studies and contribute toward understanding the therapeutic positioning of the combination therapy among various solutions to improve EGFR-TKI outcomes for patients with *EGFR* mutation-positive NSCLC. Further biomarker studies are eagerly awaited.

Conclusions

In conclusion, our analysis of potential predictive biomarkers for favorable EB treatment outcomes in patients with *EGFR*-positive NSCLC reveal that (I) low levels of pVEGFA are associated with better EB treatment outcomes; (II) low leptin levels in baseline serum may be a potential biomarker for sensitivity to EB treatment; (III) high serum follistatin is a biomarker candidate for resistance to EB treatment and production of multiple growth factors; and (IV) a clinically meaningful predictive capacity could not be concluded from the angiogenesis-related gene polymorphisms analyzed and mRNAs/proteins in tumor tissue due to a shortage of samples from the patient population of interest.

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Appendix 1

List of investigators

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Methods

Statistical analysis for gene polymorphisms

SNPs are coded by two dummy variables in the analysis, focusing on the minor allele (the less common allele). The first dummy variable takes a value of 1 if the patient has a SNP genotype with at least one copy of the minor allele (dominant effect of the minor allele) and the second takes value 1 if the subject has two copies of the minor allele (recessive effect of the minor allele). This coding allows for consideration of both dominant and recessive genetic effects in the model. If both dummy variables show significance, it means there is an additive effect.

MFPI approach

The MFPI analysis was performed using the following method by Royston and Sauerbrei (27):

Step 1. Let Z denote a continuous variable of the biomarker, and Z was transformed into Z^{p1} for the fractional polynomials-1 (FP1) model. The powers p_1 were chosen from a set, S = {p_1 =–2, –1, –0.5, 0, 0.5, 1, 2, 3}, where Z^{p1} denotes ln(Z) if p_1=0. In order to choose the best FP1 model, the best fitted p_1 was selected, while minimizing the model fit statistics for the likelihood ratio test based on the Cox proportional hazard model including the following covariates: treatment arm (0 or 1), Z^{p1} and the interaction between treatment arm and Z^{p1}.

Step 2. Z was transformed into Z^{p1} and Z^{p2} for the fractional polynomials-2 (FP2) model. The powers p_1 and p_2 were also chosen from a set, S = {–2, –1, –0.5, 0, 0.5, 1, 2, 3}. If p_i was 0, Z^{pi} denotes ln(Z), where i =1 or 2. Z^{p2} denotes Z^{p1} ln(Z) if p_1 = p_2. In order to choose the best FP2 model, the best combination of p_1 and p_2 was selected, while minimizing the model fit statistics for the likelihood ratio test based on the Cox proportional hazard model including the following covariates: treatment arm (0 or 1), Z^{p1}, Z^{p2} and two interaction terms between treatment arm and Z^{p1} or Z^{p2}.

Step 3. Determine which model is better between the best FP1 in Step 1 and the best FP2 in step 2 based on the comparison of the model fit statistics for the likelihood ratio test between the two models, using a χ^2 test with 3 degrees of freedom (df).

Step 4. The interaction P value was estimated based on the difference of the model fit statistics for the likelihood ratio test between the models with and without the interaction term(s) in Cox proportional hazard models, using a χ^2 test with 1 df if FP1 was selected, or a χ^2 test with 2 df if FP2 was selected.

Step 5. We also performed MFPI analysis to estimate the interaction P value when adjusting for the stratification factors (gender, disease stage, smoking history, and type of *EGFR* mutation). These factors were incorporated in the Cox proportional hazards model as categorical covariates along with the continuous biomarker covariates, and MFPI was conducted from step 1 to step 4 described above.

STEPP

STEPP methodology was used to visualize the interaction between bevacizumab plus erlotinib treatment and a continuous valuable biomarker (26). Two types of STEPP pattern have been proposed, named as sliding window STEPP (SW-STEPP) and TO-STEPP by Bonetti and Gelber (26). We selected TO-STEPP in the current study since it has been reported to be more stable than SW-STEPP (3). TO-STEPP was performed using the following method by Bonetti and Gelber (26):

Step 1. Let the subpopulations be defined with respect to a continuous biomarker value Z*, and let Z_i* be the value of such covariate for patient i. Considering a set of increasing values of Z* {z_1, z_2, ..., z_g}, with the exception of the duplicated values, we constructed an increasing collection of subpopulations P_l, l =1, 2, ..., g by including in P_l the patients for whom $Z_i^* \geq z_l$. Similarly, we constructed the subpopulations P_l, $l = g + 1, ..., 2g–1$ by including in Pl the patients for whom $Z_i^* > z_{l-g}$. Let p

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denote the number of subpopulations after excluding the subpopulations involving less than 30 patients.

Step 2. Let $\hat{\beta}_l$ and $\hat{\sigma}_l$ denote the estimated logarithm of the HR and standard error in the subpopulation P_l, (l=1, ..., p), respectively. We defined the 95% confidence band as $\left\{\beta_l* \in \hat{\beta}_l* \pm \gamma 1.96\sigma_l, l = 1,..., p\right\}$, where β_l* was the component of $\hat{\beta}_l$. The value of γ was estimated to meet the following equation by Monte Carlo simulation; $\mathrm{P}\left[\cap l = {}^{p} 1\left\{\beta_l* \in \hat{\beta}_l* \pm \gamma 1.96\sigma_l\right\}\right] = 0.95$.

Step 3. The treatment effects in each subpopulation and confidence band were plotted, where the horizontal axis showed the biomarker values, and the vertical axis showed the treatment effect (logarithm of HR). A lower value in the vertical axis denoted the better bevacizumab treatment effect.

Cutpoint for dichotomizing a continuous biomarker

To dichotomize a continuous biomarker, we estimated the cutpoint value for potential biomarkers referring to the methods by Jiang *et al.* as follows (29):

Step 1. Let c be any cutoff value of the biomarker. The likelihood ratio test statistic S(c) for treatment effect (HR for PFS in patients treated with bevacizumab and erlotinib combination therapy compared with E) was calculated in the subpopulation with biomarker value below or above c for all potential cutpoints by using a Cox proportional hazards model. The optimal cutpoint $\hat{c}$ was estimated as the one corresponding to the maximum S(c).

Step 2. Confidence interval of $\hat{c}$ was estimated by the bootstrap method. Let A_j (j =1, ..., 1,000) be each bootstrap sample from the observed data. As per the same methods mentioned above, cutpoint $\hat{c}_j$ was estimated in each A_j. The 95% confidence intervals of $\hat{c}$ were estimated based on the empirical distribution of $\hat{c}_j$.

Logistic regression analysis

Correlations between follistatin levels dichotomized at the cutpoint and baseline serum concentrations of angiogenesis-related proteins were evaluated by univariate logistic regression analysis. A Wald test was used to evaluate the statistical significance of coefficients in the model. The FDR was estimated using Benjamini-Hochberg methods (30).

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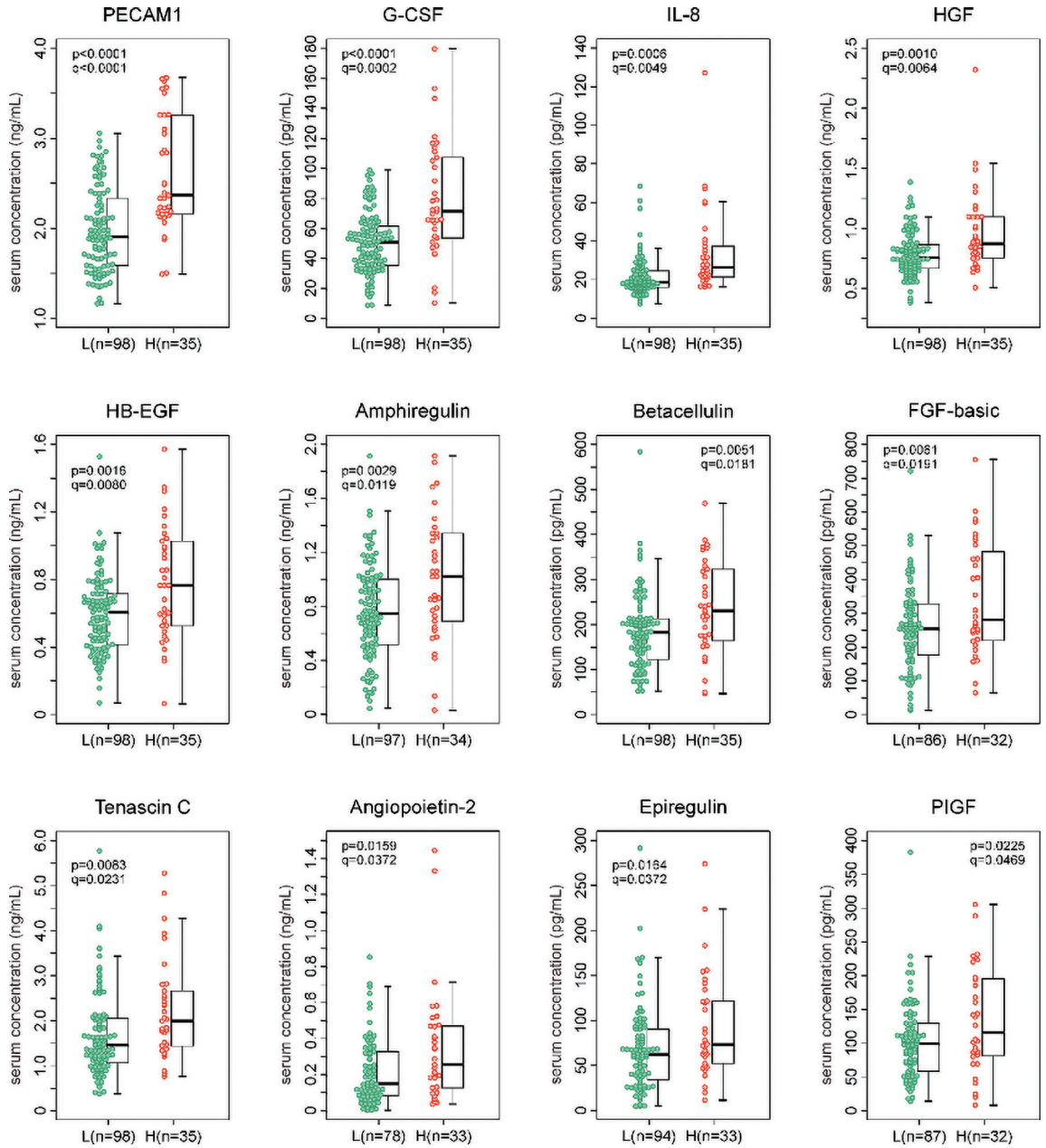

Figure S1 Distributions of serum angiogenesis-related proteins in subgroups dichotomized at the cutpoint of serum follistatin. L and H represent the patients with follistatin level below and above (≥) the cutpoint, respectively. The cutpoint was estimated at 490.5 pg/mL based on the interaction of follistatin with the PFS prolongation effects of EB treatment. Univariate logistic regression analysis was performed. All test results were shown as Wald test P value and q value (FDR) by Benjamini-Hochberg methods in each figure. There were 12 proteins significantly associated with H-follistatin (q<0.05) among 25 angiogenesis-related proteins analyzed. The box plot shows the summary statistics of serum concentrations of the proteins in each subgroup. The bottom and top of the box are the 1Q and 3Q. The horizontal bar within each box represents the median. The upper whisker extends from the 3Q to the highest value within 1.5× the IQR (the distance between 3Q and 1Q). The lower whisker extends from the 1Q to the lowest value within 1.5× IQR. PECAM1, platelet/endothelial cell adhesion molecule 1; G-CSF, granulocyte colony-stimulating factor; IL-8, interleukin-8; HGF, hepatocyte growth factor; HB-EGF, heparin-binding epidermal growth factor-like growth factor; FGF, fibroblast growth factor; PlGF, placental growth factor; PFS, progression-free survival; EB, combination therapy of erlotinib 150 mg/day and bevacizumab 15 mg/kg every 3 weeks; FDR, false discovery rate; 1Q, 25th percentile; 3Q, 75th percentile; IQR, interquartile range.

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Table S1 Baseline demographics and clinical characteristics in each analysis

Characteristics	Whole analysis population (n=152)		pVEGFA (n=101)		Serum (n=133)		Tissue mRNA (n=24)		SNPs/VNTR (n=135)		NRP1 IHC (n=28)	
	EB (n=75)	E (n=77)	EB (n=48)	E (n=53)	EB (n=62)	E (n=71)	EB (n=11)	E (n=13)	EB (n=63)	E (n=72)	EB (n=14)	E (n=14)
Age (years)												
Median	67.0	67.0	70.5	67.0	68.0	68.0	71.0	70.0	68.0	67.5	69.5	69.0
<75 years	63 (84.0)	62 (80.5)	38 (79.2)	41 (77.4)	52 (83.9)	56 (78.9)	9 (81.8)	8 (61.5)	53 (84.1)	57 (79.2)	12 (85.7)	9 (64.3)
≥75 years	12 (16.0)	15 (19.5)	10 (20.8)	12 (22.6)	10 (16.1)	15 (21.1)	2 (18.2)	5 (38.5)	10 (15.9)	15 (20.8)	2 (14.3)	5 (35.7)
Sex												
Male	30 (40.0)	26 (33.8)	21 (43.8)	20 (37.7)	26 (41.9)	23 (32.4)	5 (45.5)	7 (53.8)	26 (41.3)	24 (33.3)	6 (42.9)	7 (50.0)
Female	45 (60.0)	51 (66.2)	27 (56.3)	33 (62.3)	36 (58.1)	48 (67.6)	6 (54.5)	6 (46.2)	37 (58.7)	48 (66.7)	8 (57.1)	7 (50.0)
Smoking status												
Never/former light	51 (68.0)	51 (66.2)	32 (66.7)	34 (64.2)	40 (64.5)	47 (66.2)	7 (63.6)	7 (53.8)	41 (65.1)	47 (65.3)	10 (71.4)	8 (57.1)
Other	24 (32.0)	26 (33.8)	16 (33.3)	19 (35.8)	22 (35.5)	24 (33.8)	4 (36.4)	6 (46.2)	22 (34.9)	25 (34.7)	4 (28.6)	6 (42.9)
Clinical stage												
IIB	1 (1.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
IV	60 (80.0)	62 (80.5)	39 (81.3)	40 (75.5)	50 (80.6)	56 (78.9)	1 (9.1)	2 (15.4)	51 (81.0)	57 (79.2)	2 (14.3)	2 (14.3)
Recurrence	14 (18.7)	15 (19.5)	9 (18.8)	13 (24.5)	12 (19.4)	15 (21.1)	10 (90.9)	11 (84.6)	12 (19.0)	15 (20.8)	12 (85.7)	12 (85.7)
EGFR mutation type												
Exon 19d	40 (53.3)	40 (51.9)	27 (56.3)	31 (58.5)	34 (54.8)	38 (53.5)	6 (54.5)	6 (46.2)	34 (54.0)	38 (52.8)	7 (50.0)	7 (50.0)
L858R	35 (46.7)	37 (48.1)	21 (43.8)	22 (41.5)	28 (45.2)	33 (46.5)	5 (45.5)	7 (53.8)	29 (46.0)	34 (47.2)	7 (50.0)	7 (50.0)

Data are for n (%), unless otherwise specified. pVEGFA, plasma vascular endothelial growth factor-A; mRNA, messenger RNA; SNP, single nucleotide polymorphism; VNTR, variable number of tandem repeats; NRP1, neuropilin 1; IHC, immunohistochemistry; EB, combination therapy of erlotinib 150 mg/day and bevacizumab 15 mg/kg every 3 weeks; E, 150 mg/day erlotinib monotherapy; EGFR, epidermal growth factor receptor.

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