Biomarkers for anti-vascular endothelial growth factor drugs

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Abstract. Angiogenesis is regulated by interactions between vascular endothelial growth factors (VEGFs) and VEGF receptors. VEGF-A, VEGF-D, placental growth factor (PlGF) and plasminogen activator inhibitor-1 (PAI-1) have tumor angiogenic activity. VEGF-A and PAI-1 levels in the blood may impact the activity of bevacizumab, and VEGF-D levels may similarly diminish the efficacy of ramucirumab. However, the dynamics of these angiogenic biomarkers for anti-VEGF therapy have not been well established; therefore, they were evaluated in this retrospective study, which included two cohorts. Cohort 1 included patients who were treated with cytotoxic agents and bevacizumab as first-line chemotherapy, and Cohort 2 comprised patients who were treated with cytotoxic agents and anti-VEGF drugs (bevacizumab, ramucirumab or aflibercept) as second-line chemotherapy. VEGF-A, VEGF-D, PlGF and PAI-1 levels were measured before starting chemotherapy and were re-assessed every 1-2 months until disease progression. Bevacizumab had reduced benefit as a first-line chemotherapeutant in patients with very low or very high levels of VEGF-A. Bevacizumab increased VEGF-A and PlGF levels, but not VEGF-D or PAI-1. Anti-VEGF drugs offered the greatest benefit to patients with high PAI-1 before first- and second-line chemotherapy. PAI-1 levels were not affected by anti-VEGF drugs. Since ramucirumab increased VEGF-D, it offered less benefit to patients with high VEGF-D in second-line chemotherapy. Conversely, aflibercept offered greater benefits to patients with high VEGF-D, without increasing VEGF-D. These biomarkers may be useful for the prediction of drug efficacy and may predict resistance to anti-VEGF drugs.

Introduction

Fluorouracil-based chemotherapy (combined with oxaliplatin or irinotecan) plus anti-epidermal growth factor receptor/vascular endothelial growth factor (anti-EGFR/VEGF) therapy is the standard first-line treatment for metastatic colorectal cancer, with an overall median survival of about 30 months (1-3). Progression-free survival (PFS) of first-line treatment is about a year (3,4); thus, second- or third-line treatment assumes great importance. Cetuximab (5), bevacizumab (6), ramucirumab (7) and aflibercept (8) show survival benefits when used as second-line chemotherapeutants. *RAS* is a predictive marker for anti-EGFR therapy; however, no promising biomarkers have been established for anti-VEGF therapy.

Angiogenesis is regulated by interactions between vascular endothelial growth factors (VEGFs) and VEGF receptors (VEGFRs) and is essential for cancer growth and metastasis (9-11). VEGF-A is the central regulator of tumor angiogenesis, endothelial proliferation, and survival (12,13). VEGF-A binds with high affinity to two structurally similar tyrosine kinase receptors, VEGFR-1 and VEGFR-2, both of which are expressed in tumor vasculature (14). Blockade of the VEGF-A/VEGFR-2 interaction inhibits tumor angiogenesis and growth. Plasminogen activator inhibitor-1 (PAI-1) has angiogenic activity and contributes to tumor progression, tumor invasion, and metastasis (15). High levels of PAI-1 degrade prognoses of patients with various types of cancers (16), including colorectal cancer (17).

At present, three anti-VEGF drugs are available to block the VEGF pathway in different ways. Bevacizumab is a humanized monoclonal antibody that binds to VEGF-A and blocks its activation (4). Ramucirumab is a humanized IgG1 monoclonal antibody that recognizes VEGFR-2, preventing binding of agonists, VEGF-A, VEGF-C, and VEGF-D, and blocking VEGFR-2 activation (7). Aflibercept is a recombinant fusion protein containing a VEGF-binding domain, and it antagonizes the activity of VEGF-A, VEGF-B, and placental growth factor (PlGF) (8). Bevacizumab is used for first-line to third-line treatment, and ramucirumab or aflibercept in combination with FOLFIRI is an effective second-line treatment for

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patients with metastatic colorectal cancer. However, dynamics and contributions of angiogenic biomarkers to anti-VEGF therapy have not been well established. In this retrospective study, we evaluated those dynamics and contributions to anti-VEGF therapy.

Materials and methods

Patients and study design. We conducted a retrospective study of patients with metastatic colorectal cancer who were treated with anti-VEGF-drugs between May 2015 and July 2021. This study included two cohorts. Cohort 1 comprised patients who were treated with cytotoxic agents and bevacizumab as first-line chemotherapy, and Cohort 2 included patients who were treated with cytotoxic agents and anti-VEGF drugs (bevacizumab, ramucirumab, or aflibercept) as second-line chemotherapy. We included patients who participated in a bio-bank project at our institution. This project was approved by local ethics review boards (28-03-738) and written informed consent was obtained from all patients who participated in this project. Inclusion criteria were: histologically confirmed adenocarcinoma of the colon or rectum, patients 20-80 years of age, Eastern Cooperative Oncology Group performance status of 0-1, and adequate organ function (white blood cell count $\geq 3.0 \times 10^9$ cells/l, $\geq 1.5 \times 10^9$ neutrophils/l, platelets $\geq 100 \times 10^9$/l, hemoglobin ≥ 10.0 g/dl, serum bilirubin ≤ 1.5x upper limit of normal; alanine aminotransferase and aspartate amino transferase ≤ 2.5x upper limit of normal, and serum creatinine ≤ 1.5x upper limit of normal), known *RAS* and *BRAF* status (mutant or wild-type), and blood samples stocked in the bio-bank. The presence of at least one measurable reference lesion following the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 was also required. Patients with a history of another malignancy within the past 5 years were excluded. All Cohort 1 patients were chemo-naïve. Eligible patients of Cohort 2 had to have experienced disease progression within 6 months of the last dose of first-line combination therapy with oxaliplatin and a fluoropyrimidine for meta-static disease and had to have received at least one cycle of doublet therapy. Exclusion criteria included brain metastases, poorly controlled hypertension, or any arterial thrombotic or thromboembolic events within 12 months prior to starting chemotherapy. The study was conducted according to ethical guidelines of the Declaration of Helsinki, and the protocol was approved by local ethics review boards (B-2021-467). Information about the right to opt-out was posted on the websites of Main hospital of Nippon Medical School

Sample collection. In the bio-bank project, blood samples (10 ml in BD Vacutainer EDTA tube: Becton Dickinson) were obtained from participants every 1-2 months during chemo-therapy. Blood samples were centrifuged at 1,900 g for 10 min and the upper layer of each sample was transferred to another tube and stored at -80°C until analysis.

Measurement of VEGF-A, VEGF-D, PlGF, and PAI-1. Plasma samples stored from the start to the end of chemotherapy every 2 months were used for measurement of angiogenic factors. VEGF-A, VEGF-D, PlGF, and PAI-1 were measured using commercially available enzyme-linked immunosorbent

assay (ELISA) kits (VEGF-A: Human VEGF Quantikine kit, VEGF-D: VEGF-D Duoset ELISA kit, PlGF: Human PlGF Quantikine kit, PAI-1: Human Serpin E1/PAI-1 Duoset ELISA kit. All kits were from R & D Systems, Minneapolis, MN, USA) and were used according to the manufacturer's proto-cols. To measure PAI-1 concentrations, samples were diluted 200-fold.

Evaluation of clinical responses. Tumor responses were assessed by computed tomography (CT) following RECIST 1.1 criteria, 3 months after starting chemotherapy. After the initial assessment, CT was performed every 3 months until disease progression. Patients who achieved complete responses (CR) or partial responses (PR) were categorized as responders, and those who achieved stable disease (SD) or progressive disease (PD) were considered non-responders. Carcinoembryonic antigen (CEA) and CA19-9 were assayed monthly throughout chemotherapy. The normal CEA level was <5.0 ng/ml and the normal CA19-9 level was <37 U/ml.

Statistical analysis. Statistical analysis was performed using R version 4.1.2 (The R Foundation for Statistical Computing, Vienna, Austria). The Mann-Whitney U test was used to compare differences in each angiogenic factor. Multiple comparisons of the dynamics of each angiogenic factors were tested with the Dunn's test after the Kruskal-Wallis test. To evaluate impacts of VEGFs on the cytoreductive effect, patients were divided into high, medium-high, medium-low, and low groups with quartile values for each angiogenic factor. Clinical responses were tested using Fisher's exact tests. To evaluate impact of VEGFs on survival, patients were divided into two groups, high and low, with the median as the cut-off for each angiogenic factor. Progression free survival (PFS) and overall survival (OS) were tested using Kaplan-Meier analysis followed by the log-rank test and two-stage test.

Results

Patients. Thirty-one patients were included in Cohort 1 and 40 patients in Cohort 2 (Fig. 1). Twelve of 31 Cohort 1 patients had not received second-line chemotherapy. Four Cohort 1 patients were continuing first-line chemotherapy. Two patients were administered anti-EGFR agents as second-line chemotherapy, and the remaining 13 patients were included in Cohort 2. Patient characteristics are shown in Table I and adverse events are listed in Table SI. In Cohort 1, 17 patients had one metastatic site, 9 patients had 2 metastatic sites, 5 patients had three or more meta-static sites total numbers of cases; liver: 18 cases, lung: 9 cases, peritoneum: 11 cases, others such as lymph node or bone: 12 cases). In Cohort 2, 26 patients had one meta-static site, 10 patients had 2 metastatic sites, and 4 patients had three or more metastatic sites (total number of cases: liver: 23 cases, lung: 12 cases, peritoneum: 9 cases, others: 12 cases). Twenty-two patients (71.0%) with *RAS* mutations were included in Cohort 1 and 22 more (55.0%) in Cohort 2. No patients with *BRAF* mutations were included in either cohort. Among 40 patients belonging to Cohort 2, 8 patients received bevacizumab, 18 received ramucirumab, and 14 received aflibercept. In first-line chemotherapy, 21 of the

Table I. Baseline characteristics.

Variables	Cohort 1 (N=31)	Cohort 2 (N=40)
Median age, years (IQR)	64 (12.5)	64 (12.0)
Sex, n (male: female)	15:16	26:14
ECOG performance status, n (0:1)	19:12	30:10
Tumor location, n (right: left)	9:22	7:33
CEA, n (<10: ≥10 ng/ml)	11:20	12:28
Number of metastatic sites, n (1:2: ≥3)	17:9:5	26: 10:4
RAS status (tissue), n (wild-type: mutant)	9:22	18:22
BRAF status (tissue), n (wild-type: mutant)	31:0	40:0
First line chemotherapy, n		
FOLFOX + bevacizumab	-	21
FOLFOX + anti-EGFRs or none	-	19
PFS, n (<6: ≥6 months)	9:22	15:25
Biomarker		
Median VEGF-A, pg/ml (IQR)	56.7 (130.8)	342.9 (682.1)
Median VEGF-D, pg/ml (IQR)	339.8 (534.0)	459.5 (610.3)
Median PlGF, pg/ml (IQR)	8.3 (5.1)	16.9 (12.4)
Median PAI-1, ng/ml (IQR)	16.1 (14.6)	17.8 (16.5)

IQR, interquartile range; ECOG, Eastern Cooperative Oncology Group; PFS, progression-free survival; VEGF, vascular endothelial growth factor; PlGF, placental growth factor; PAI-1, plasminogen activator inhibitor-1.
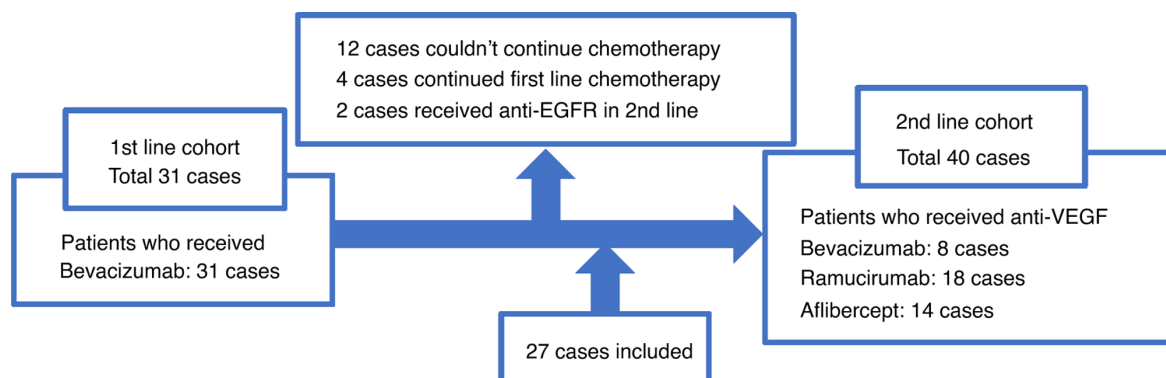

Figure 1. Flowchart of patient enrollment. VEGF, vascular endothelial growth factor.

40 Cohort 2 patients received bevacizumab and 19 received anti-EGFR drugs or no molecular target drugs.

Cohort 1

Outcomes of chemotherapy. In Cohort 1, median cycles of chemotherapies administered were 14 (IQR: 16). Six patients (19%) achieved a CR, 12 (39%) achieved a PR, 9 (29%) experienced SD, and four (13%) experienced PD. Median follow-up was 26.5 months (IQR 10.0). Median OS was 26.6 months and median PFS was 12.4 months (Fig. 2A and B).

VEGF-A, VEGF-D, PlGF, and PAI-1 levels. At the start of first-line chemotherapy, there were no correlations between the four angiogenic factors.

Median VEGF-A level before treatment was 56.7 pg/ml (IQR: 131.0). There was no relationship between VEGF-A level and tumor *RAS* status, or the number of metastatic sites and tumor location (right or left side). VEGF-A level increased significantly one month after starting chemotherapy and continued to rise during treatment (Fig. 3A). At the end of chemotherapy, VEGF-A level (median: 566.8 pg/ml, IQR: 335.9) was significantly higher than before chemotherapy (P<0.0001, Fig. 3B).

Median VEGF-D level before treatment was 340.0 pg/ml (IQR: 534.0). There was no relationship between VEGF-D level and tumor *RAS* status, or the number of metastatic sites and tumor side. Bevacizumab had no impact on VEGF-D (Fig. 3C), and VEGF-D level at the end of therapy (median: 429.4 pg/ml, IQR: 543.0) was the same as before chemotherapy (P=0.29, Fig. 3D).

Median PlGF level before chemotherapy was 8.3 pg/ml (IQR: 5.1). There was no relationship between PlGF level and tumor *RAS* status, or between the number of metastatic sites
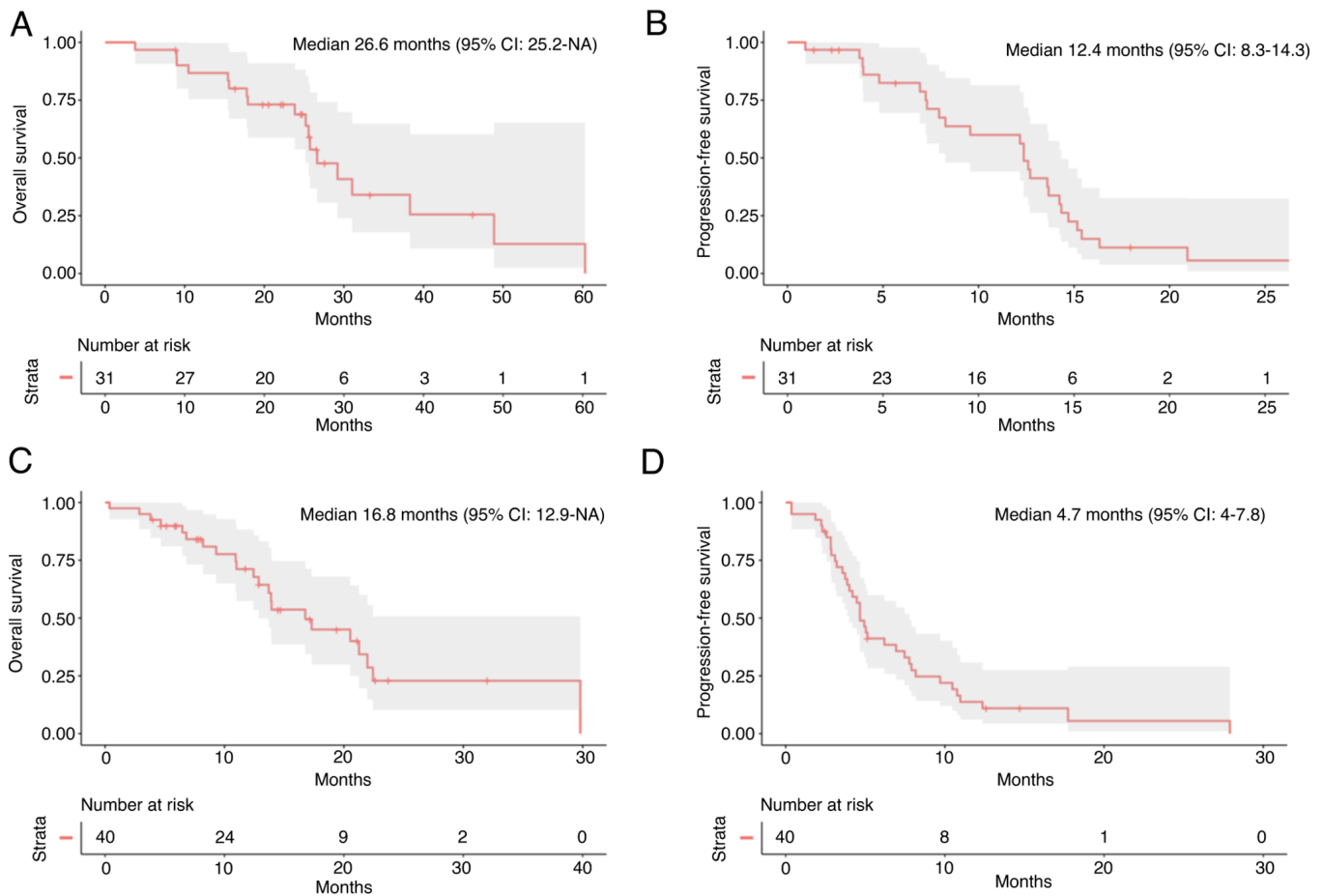
Figure 2. Overall survival and progression-free survival as analyzed using the Kaplan-Meier method in cohort 1 and cohort 2 patients. (A) Overall survival and (B) progression-free survival of cohort 1 patients. (C) Overall survival and (D) progression-free survival of cohort 2 patients.

and tumor side. PlGF level increased significantly one month after starting chemotherapy and continued to rise during chemotherapy (Fig. 3E). PlGF levels at the end of therapy (median: 18.6 pg/ml, IQR: 11.3) were significantly higher than before (P<0.0001, Fig. 3F).

Median PAI-1 level before treatment was 16.1 ng/ml (IQR: 14.6). It had no relationship with VEGF-A level, tumor *RAS* status, the number of metastatic sites, or tumor side. Bevacizumab had no impact on PAI-1 levels (Fig. 3G) and PAI-1 levels at the end of therapy (median: 14.2 ng/ml, IQR: 19.7) were unchanged (P=0.81, Fig. 3H).

Impact of VEGF-A, VEGF-D, PlGF, and PAI-1 levels on the cytoreductive effect and survival. With regard to VEGF-A levels, 72.2% (13/18) of responders (CR or PR) belonged to the medium-low and medium-high quartiles. Conversely, 23.1% (3/13) of non-responders (SD or PD) belonged to the medium-low and medium-high quartiles (Table II, P=0.05). VEGF-D, PlGF, and PAI-1 levels did not influence the cytoreductive effect (Table II). There were no differences in dynamics of those four angiogenic factors between responders and non-responders (Fig. S1).

The low VEGF-A group had significantly shorter OS (P=0.05, Fig. 4A). The low PlGF group had significantly shorter OS (P=0.036, Fig. 4E) by log-rank test; however, non-significant by two-stage test (P=0.86). PFS was not affected by VEGF-A and PlGF (P=0.58, P=0.39, Fig. 4B and F). PAI-1

level had a small impact on OS (P=0.057, Fig. 4G), and the low PAI-1 group had significantly reduced PFS (P=0.0005, Fig. 4H). VEGF-D level did not affect OS or PFS (P=0.23, P=0.19, Fig. 4C and D).

Cohort 2
Outcomes of chemotherapy. In Cohort 2, the median cycles of chemotherapy were 8 with ramucirumab (IQR: 11.5) and with aflibercept (IQR: 9.25). One patient (2.5%) achieved CR, 3 (7.5%) achieved PR, 25 (62.5%) experienced SD, and 11 (27.5%) experienced PD. Median follow-up was 12.5 months. Median OS was 16.8 months (Fig. 2C) and median PFS was 4.7 months (Fig. 2D).

VEGF-A, VEGF-D, PlGF, and PAI-1 levels. Median VEGF-A, VEGF-D, PlGF and PAI-1 levels of all Cohort 2 patients before treatment were 342.9 pg/ml (IQR: 682.1), 480.0 pg/ml (IQR: 610.2), 16.9 pg/ml (IQR: 12.4) and 17.8 ng/ml (IQR: 16.5). VEGF-A and PlGF levels of patients before second-line chemotherapy treated with bevacizumab during first-line chemotherapy were significantly higher than those of patients treated without bevacizumab (P<0.00001, P<0.0001 Fig. 5A and C). Conversely, VEGF-D and PAI-1 levels before second-line chemotherapy of patients treated with bevacizumab in first-line chemotherapy were equal to those of patients treated without bevacizumab (P=0.86, P=0.2, Fig. 5B and D).

Table II. Impact of angiogenic factors on the cytoreductive effect.

Angiogenic factors	Low	Medium low	Medium high	High	P-value
VEGF-A					0.05
Responder	2	7	6	3	
Non-responder	6	1	2	4	
VEGF-D					0.46
Responder	4	5	4	2	
Non-responder	2	1	2	4	
PlGF					0.73
Responder	3	3	3	2	
Non-responder	1	1	2	3	
PAI-1					0.25
Responder	4	6	6	2	
Non-responder	4	2	2	5	

Fisher's exact test was used for all angiogenic factors. The cut-off values were as follows: VEGF-A: Low, <25.8 pg/ml; medium low, ≥25.8 and <56.7 pg/ml; medium high, ≥56.7 and <166.2 pg/ml; high, ≥166.2 pg/ml. VEGF-D: Low, <199.2 pg/ml; medium low, ≥199.2 and <309.7 pg/ml; medium high, ≥309.7 and <764.7 pg/ml; high, ≥764.7 pg/ml. PlGF: Low, <6.1 pg/ml; medium low, ≥6.1 and <6.7 pg/ml; medium high, ≥6.7 and <11.4 pg/ml; high, ≥11.4 pg/ml. PAI-1: Low, <12.0 ng/ml; medium low, ≥12.0 and <16.1 pg/ml; medium high, ≥16.1 and <27.0 pg/ml; high, ≥27.0 pg/ml. PAI-1, plasminogen activator inhibitor-1; PlGF, placental growth factor; VEGF, vascular endothelial growth factor.

In 5 of 7 patients with high levels of VEGF-A at the start of second-line therapy, VEGF-A level decreased at the end thereof. In all patients with low levels of VEGF-A at the start of second-line therapy, VEGF-A level increased until the end of treatment (Fig. 5E).

In patients treated with ramucirumab, VEGF-D increased over time (Fig. 5F); however, in patients treated with bevacizumab or aflibercept, there was no obvious increase. Also, PlGF increased gradually in patients treated with ramucirumab and aflibercept (Fig. 5G and H). Only 8 patients received bevacizumab as a second-line. In these patients, PlGF increased; however, the difference was not significant.

Impact of VEGF-A, VEGF-D, PlGF, and PAI-1 levels on survival. VEGF-A (P=0.91, P=0.93, Fig. 6A and B), VEGF-D (P=0.51, P=0.14, Fig. 6C and D) and PlGF (P=0.65, P=0.5, Fig. 6E and F) levels had no impact on OS or PFS. Low PAI-1 patients had significantly shorter OS (P=0.017, Fig. 6G); however, PAI-1 level had no impact on PFS (P=0.55, Fig. 6H).

In patients treated with ramucirumab, patients with high VEGF-D levels had non-significantly shorter OS (P=0.068, Fig. 7A) and significantly shorter PFS (P=0.017, Fig. 7B). VEGF-A, PlGF, and PAI-1 had no impact on OS (P=0.61, P=0.79, P=0.41) and PFS (P=0.85, P=0.27, P=0.30). In patients treated with aflibercept, the high VEGF-D group had non-significantly longer OS (P=0.058, Fig. 7C) and significantly longer PFS (P=0.026, Fig. 7D). VEGF-A, PlGF, and PAI-1 had no impact on OS (P=0.61, P=0.90, P=0.26) or PFS (P=0.41, P=0.42, P=0.059). Fig. 7E and F show that PlGF levels had no impact on OS or PFS of patients who were treated with aflibercept.

VEGF-A, VEGF-D, PlGF, and PAI-1 levels at the end of second-line chemotherapy. At the end of second-line chemotherapy, VEGF-A, VEGF-D, and PlGF levels were significantly higher than baseline (P<0.0001, P=0.022, P<0.0001 Fig. 8A-C); however, PAI-1 levels did not increase (P=0.89, Fig. 8D).

Discussion

In the present study, there were three valuable findings. First, bevacizumab increases VEGF-A and PlGF levels, but not VEGF-D or PAI-1 levels. Conversely, anti-EGFRs have no impact on levels of these four growth factors. Second, anti-VEGF drugs have greater benefit for patients with high PAI-1 levels before starting first- and second-line chemotherapy, and PAI-1 level is not affected by anti-VEGF drugs. Third, VEGF-D may be a useful biomarker for drug selection in second-line chemotherapy.

It is noteworthy that VEGF and PlGF levels increase one month after starting bevacizumab. It has been reported that VEGF-A and PlGF levels are high in patients who had received prior bevacizumab (18). However, the present study shows that VEGF and PlGF levels increase one month after starting bevacizumab and maintain high levels during drug administration. Bevacizumab inhibits angiogenesis by binding VEGF-A (19). Thus, bevacizumab is less beneficial for patients with low VEGF-A levels before chemotherapy, as indicated in Cohort 1. However, the sustainable increase of VEGF-A induced by bevacizumab may provoke acquired resistance to bevacizumab. Indeed, most responders had medium-low or medium-high levels of VEGF-A before starting chemotherapy, indicating that adequate VEGF-A levels provide benefits for patients treated with bevacizumab. There appears to be no benefit if the initial VEGF-A levels are too high or too low. Conversely, high VEGF-A levels may not provide a benefit in patients treated with ramucirumab. This hypothesis is supported by the fact that ramucirumab has better outcomes in bevacizumab-naïve patients than in patients whose first-line treatment included bevacizumab (20). We believe that the VEGF-A increase induced by bevacizumab has an unfavorable impact on efficacy of ramucirumab.
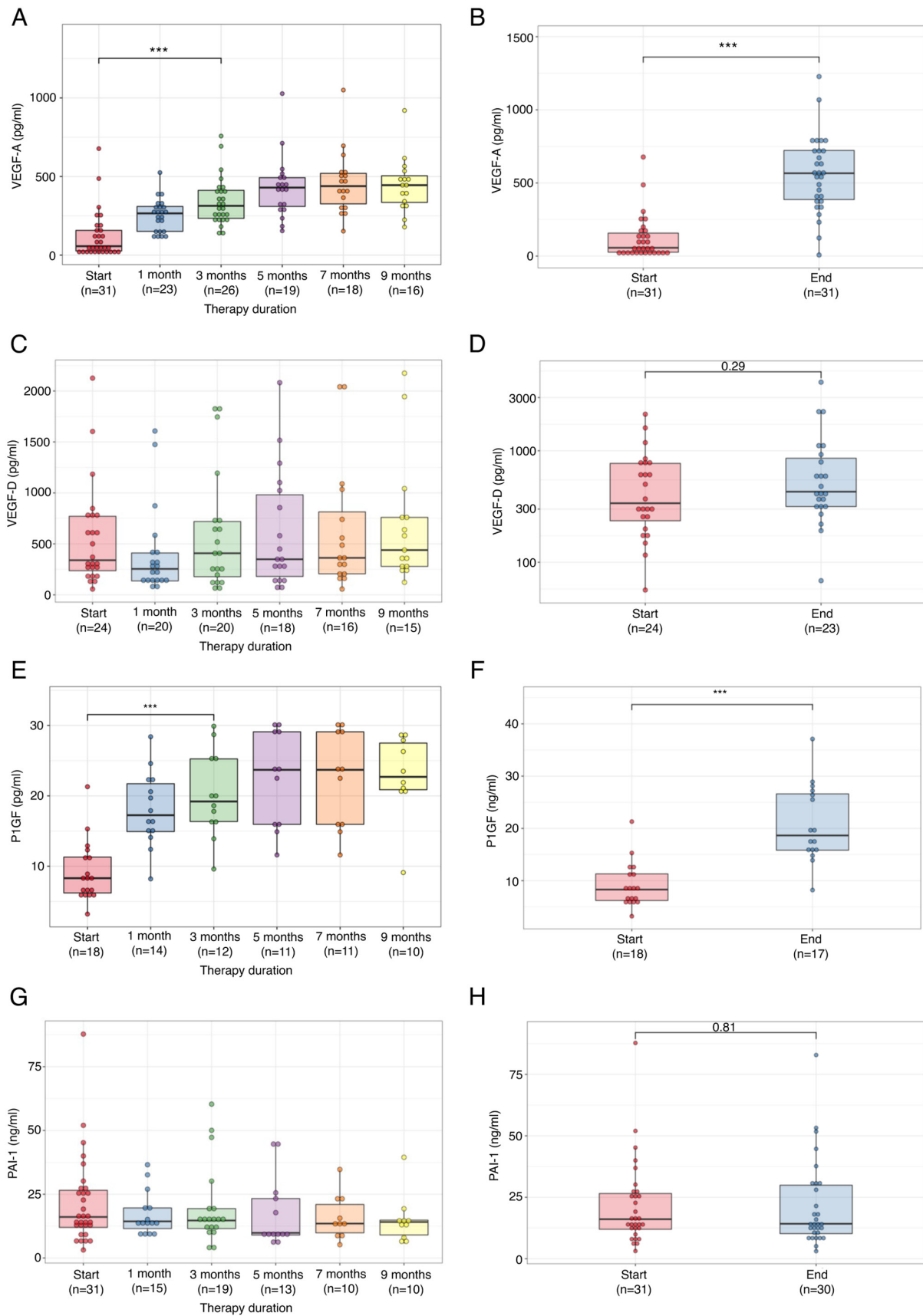
Figure 3. Dynamics of angiogenic factors in cohort 1. (A) Time-course of changing VEGF-A levels of cohort 1 patients (Dunn's test after the Kruskal-Wallis test). (B) Comparison between VEGF-A levels before first-line chemotherapy and those thereafter (Mann-Whitney U test). (C) Time-course of changing VEGF-D levels of cohort 1 patients (Dunn's test after the Kruskal-Wallis test). (D) Comparison between VEGF-D levels before first-line chemotherapy and those thereafter (Mann-Whitney U test). (E) Time-course of changing PlGF levels of cohort 1 patients (Dunn's test after the Kruskal-Wallis test). (F) Comparison between PlGF levels before first-line chemotherapy and those thereafter (Mann-Whitney U test). (G) Time-course of changing PAI-1 levels of cohort 1 patients (Dunn's test after the Kruskal-Wallis test). (H) Comparison between PAI-1 levels before first-line chemotherapy and those thereafter (Mann-Whitney U test). ***P<0.0001. PAI-1, plasminogen activator inhibitor-1; PlGF, placental growth factor; VEGF, vascular endothelial growth factor.
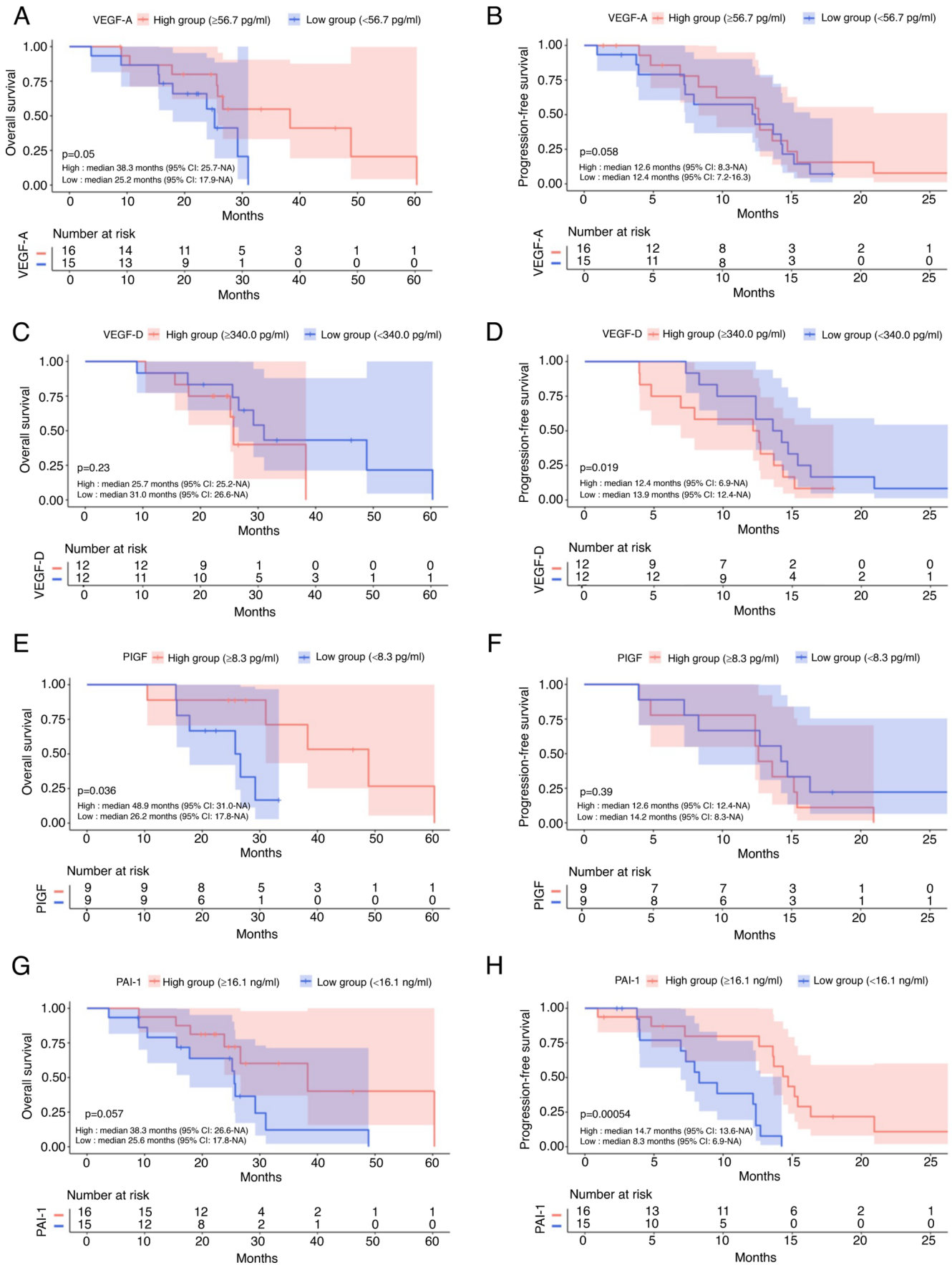
Figure 4. Overall survival and progression-free survival analysis in cohort 1 grouped by the level of each angiogenic factor using the Kaplan-Meier method followed by a log-rank test. (A) Overall survival and (B) progression-free survival of cohort 1 patients grouped by VEGF-A levels. (C) Overall survival and (D) progression-free survival of cohort 1 patients grouped by VEGF-D levels. (E) Overall survival and (F) progression-free survival of cohort 1 patients grouped by PlGF levels. (G) Overall survival and (H) progression-free survival of cohort 1 patients grouped by PAI-1 levels. PAI-1, plasminogen activator inhibitor-1; PlGF, placental growth factor; VEGF, vascular endothelial growth factor; NA, not applicable.
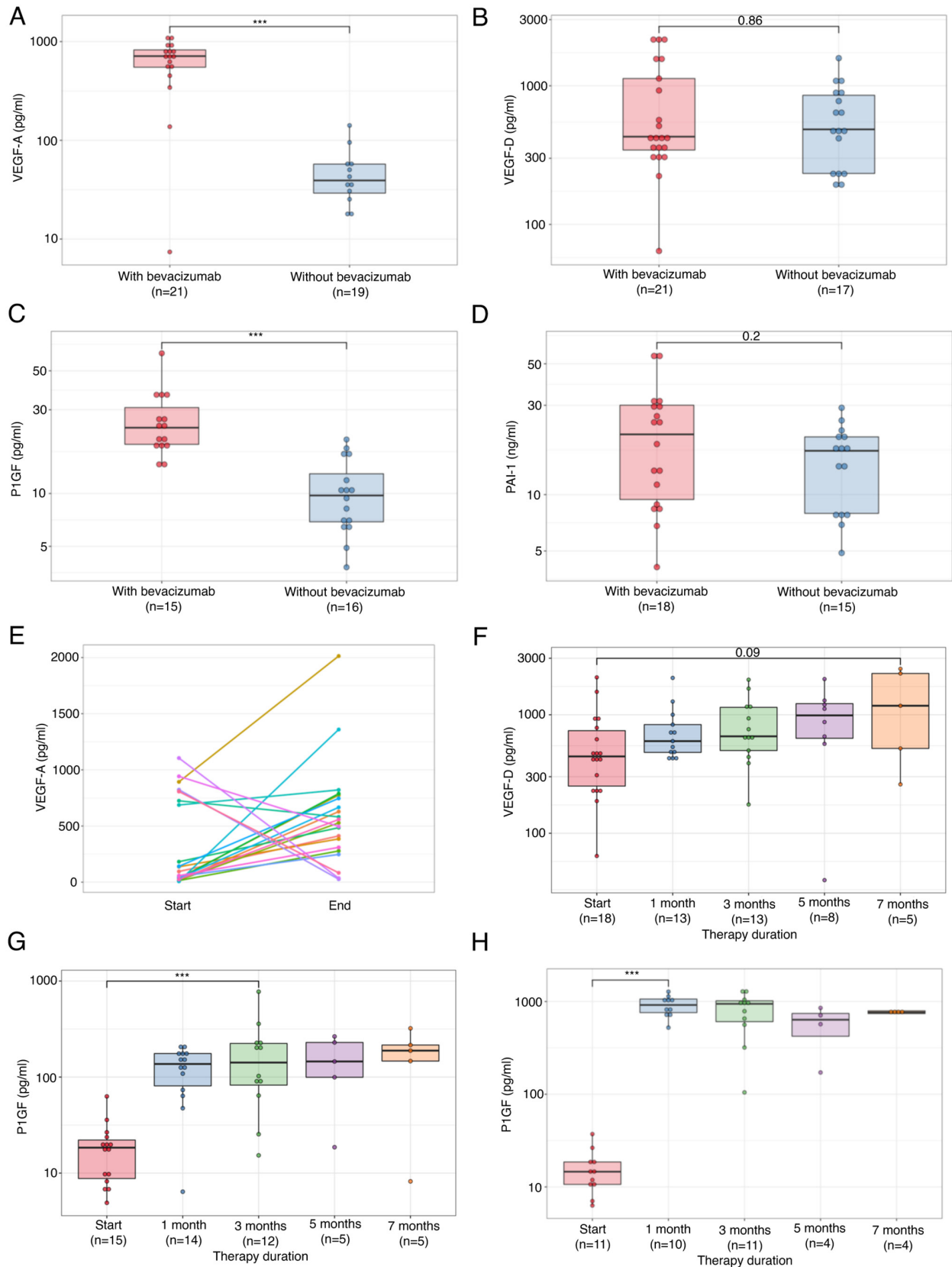
Figure 5. Dynamics of angiogenic factors in cohort 2 patients. (A) Changing VEGF-A levels at the start of second-line chemotherapy of cohort 2 patients who received bevacizumab or anti-EGFR drugs (Mann-Whitney U test). (B) Changing VEGF-D levels at the start of second-line chemotherapy of cohort 2 patients who received bevacizumab or anti-EGFR drugs (Mann-Whitney U test). (C) Changing PlGF levels at the start of second-line chemotherapy of cohort 2 patients who received bevacizumab or anti-EGFR drugs (Mann-Whitney U test). (D) Changing PAI-1 levels at the start of second-line chemotherapy of cohort 2 patients who received bevacizumab or anti-EGFR drugs (Mann-Whitney U test). (E) Comparison of VEGF-A levels before and after second-line chemotherapy. (F) Time-course of changing VEGF-D levels of patients treated with ramucirumab in second-line chemotherapy (Dunn's test after the Kruskal-Wallis test). (G) Time-course of changing PlGF levels of patients treated with ramucirumab in second-line chemotherapy (Dunn's test after the Kruskal-Wallis test). (H) Time-course of changing PlGF levels of patients treated with aflibercept in second-line chemotherapy (Dunn's test after the Kruskal-Wallis test). $^{***}P<0.0001$. PAI-1, plasminogen activator inhibitor-1; PlGF, placental growth factor; VEGF, vascular endothelial growth factor.
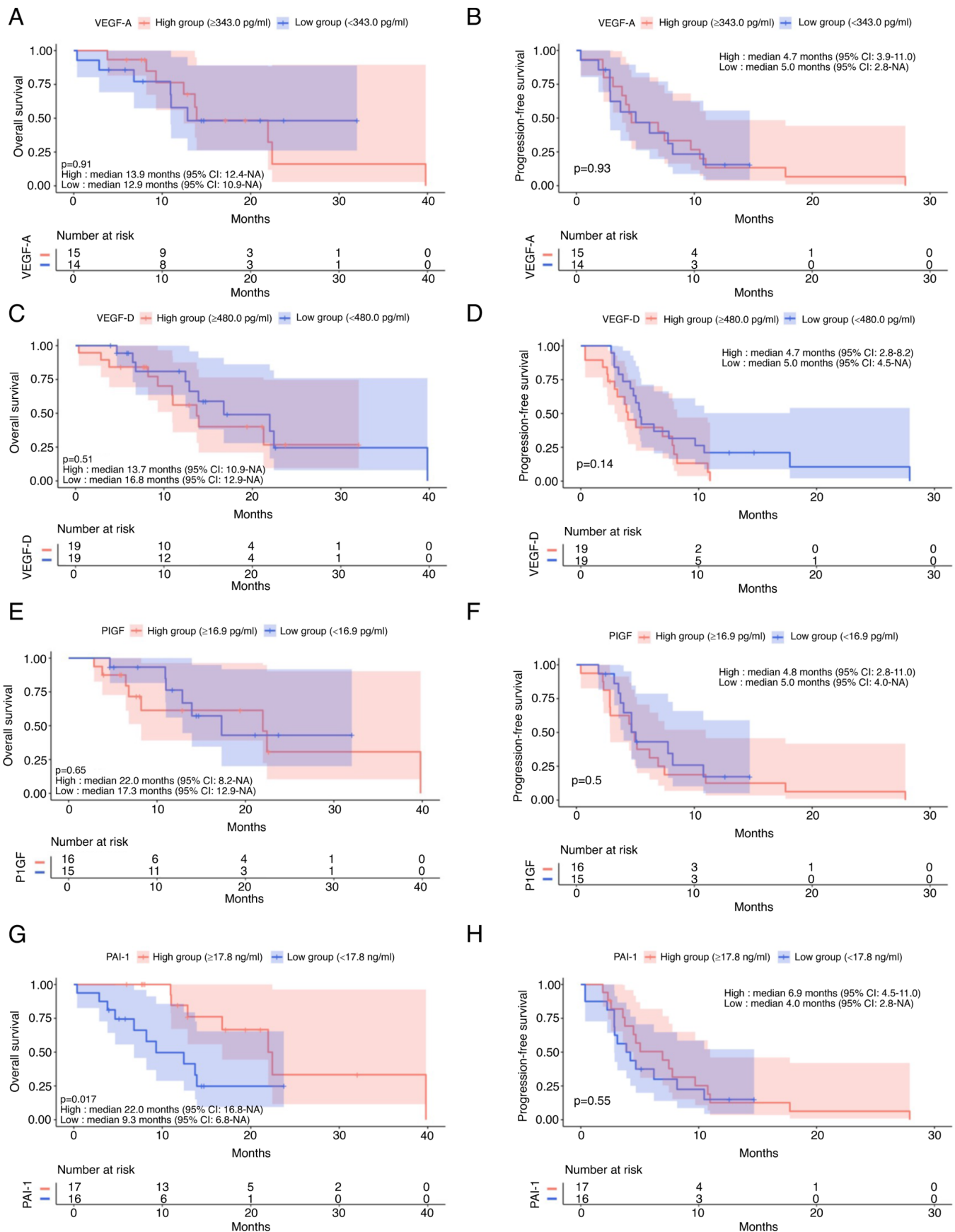
Figure 6. Overall survival and progression-free survival analysis in cohort 2 grouped by the level of each angiogenic factor using the Kaplan-Meier method followed by the log-rank test. (A) Overall survival and (B) progression-free survival of cohort 2 patients grouped by VEGF-A level. (C) Overall survival and (D) progression-free survival of cohort 2 patients grouped by VEGF-D level. (E) Overall survival and (F) progression-free survival of cohort 2 patients grouped by PlGF level. (G) Overall survival and (H) progression-free survival of cohort 2 patients grouped by PAI-1 level. PAI-1, plasminogen activator inhibitor-1; PlGF, placental growth factor; VEGF, vascular endothelial growth factor; NA, not applicable.
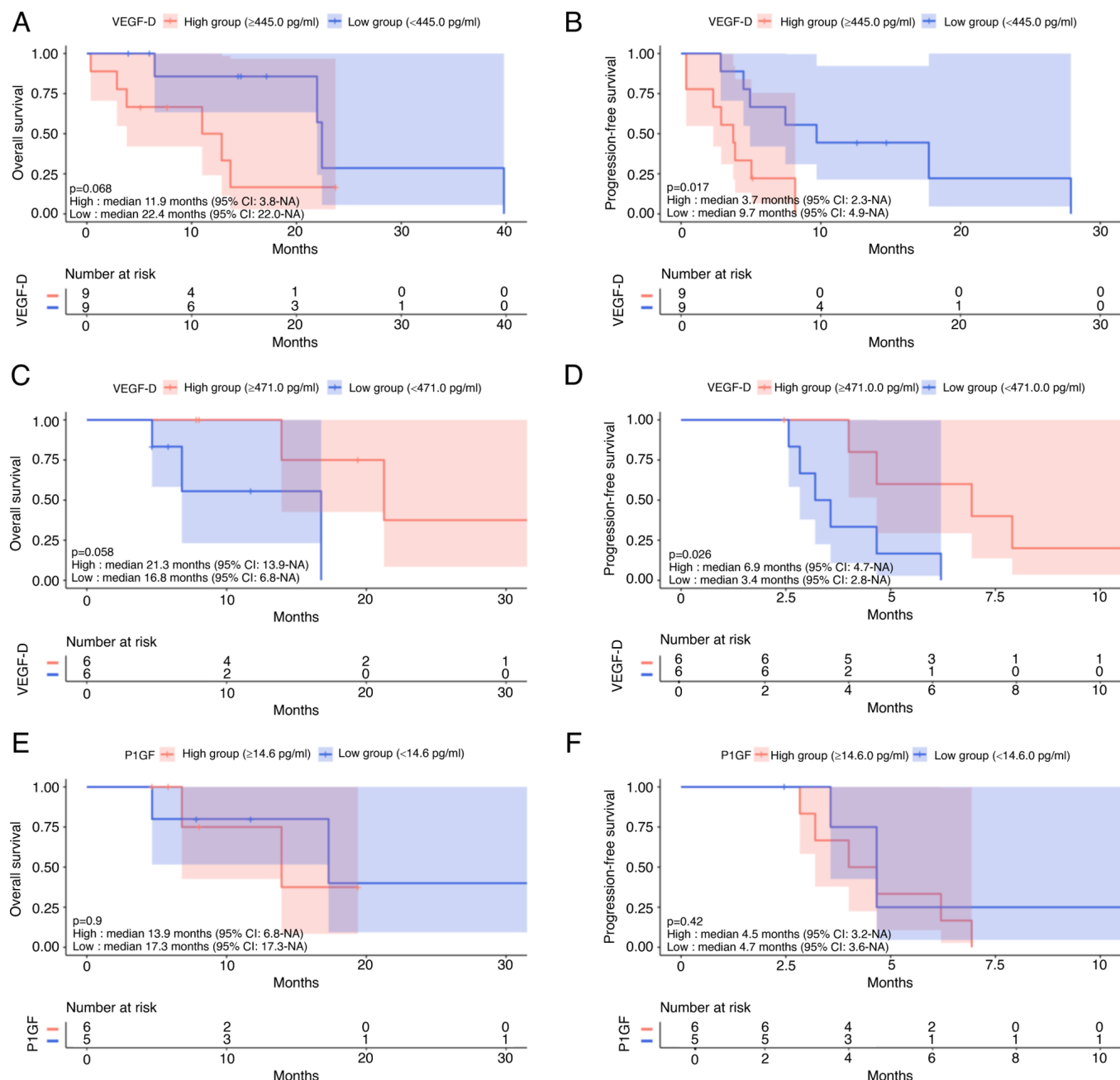
Figure 7. Overall survival and progression-free survival analysis in patients treated with ramucirumab and aflibercept, grouped by the level of each angiogenic factor using the Kaplan-Meier method followed by the log-rank test. (A) Overall survival and (B) progression-free survival of patients who received ramucirumab, grouped by VEGF-D level. (C) Overall survival and (D) progression-free survival of patients who received aflibercept, grouped by VEGF-D level. (E) Overall survival and (F) progression-free survival of patients who received aflibercept, grouped by PlGF levels. PlGF, placental growth factor; VEGF, vascular endothelial growth factor; NA, not applicable.

Anti-VEGF drugs have greater benefit for patients with high PAI-1 levels before chemotherapy in either the first or second line. This is the first study showing that high PAI-1 levels are a favorable prognostic factor for patients receiving second-line chemotherapy with ramucirumab or aflibercept. It has been reported that high PAI-1 levels are a poor prognostic factor for stage I-IV colorectal cancer patients who are not receiving chemotherapy (17). Previous studies have already reported that high PAI-1 levels are an unfavorable prognostic factor for patients receiving bevacizumab (21-23), in contrast to results of the present study. Tumor angiogenesis requires PAI-1 (24), and PAI-1 has a dose-dependent effect on tumor angiogenesis (25).

Inhibition of PAI-1 limits tumor angiogenesis (26), indicating that patients with high PAI-1 levels have hyper-vascular tumors that can respond to anti-VEGFs. However, patients with high PAI-1 levels had better PFS, but the same OS during the first line. Conversely, in the present study, patients with high PAI-1 levels had better OS, but similar PFS in the second line. Thus, we need further studies to clarify or resolve this contradiction.

In the present study, we measured PAI-1 repeatedly and showed that bevacizumab had no impact on PAI-1 levels. In previous studies, bevacizumab decreased PAI-1 levels in patients with lung cancer (21), metastatic solid cancers (22), or colorectal cancer (23); however, PAI-1 levels were
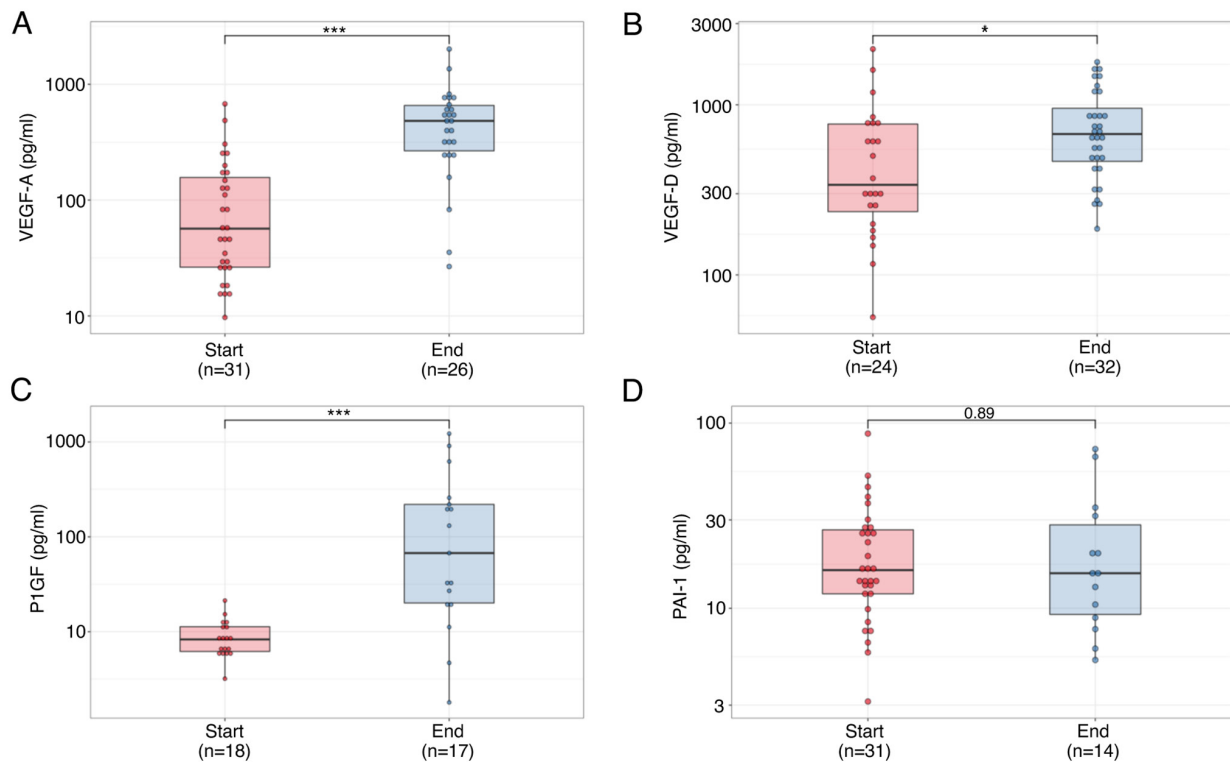
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Figure 8. Comparison of levels of angiogenic factors between the start of first-line chemotherapy and at the end of second-line chemotherapy. (A) Comparison of VEGF-A levels before first-line chemotherapy and after second-line chemotherapy (Mann-Whitney U test). (B) Comparison of VEGF-D level before and after first-line chemotherapy (Mann-Whitney U test). (C) Comparison of PlGF levels before and after first-line chemotherapy (Mann-Whitney U test). (D) Comparison of PAI-1 levels before and after first-line chemotherapy (Mann-Whitney U test). ***P<0.0001, *P<0.05. PAI-1, plasminogen activator inhibitor-1; PlGF, placental growth factor; VEGF, vascular endothelial growth factor.

measured only once after starting chemotherapy in these studies. PAI-1 antigen is mainly detected in fibroblasts and endothelial cells (27), indicating that PAI-1 levels are strongly affected by the microenvironment of cancer cells; thus, an increase or decrease of PAI-1 has a complex mechanism. In the present study, tumor progression or shrinkage had no effect on PAI-1 levels and there was no association between VEGF-A and PAI-1 levels. Thus, PAI-1 inhibitors are accepted as anti-cancer drugs by virtue of their anti-angiogenic effects.

VEGF-D may be a useful biomarker for drug selection in second-line chemotherapy. Ramucirumab has less benefit for patients with high VEGF-D levels and ramucirumab increases VEGF-D. Conversely, aflibercept has greater benefit for patients with high VEGF-D levels without increasing VEGF-D. VEGF-D has no effect on benefits of bevacizumab and is not affected by bevacizumab. In the present study, patients with low VEGF-D levels had significantly better PFS and non-significantly better OS. Tabernero et al (28) reported that ramucirumab has a favorable impact on patients with high levels of VEGF-D (>115 pg/ml) before chemotherapy, but no other studies have reported an association between ramucirumab and VEGF-D levels. In the present study, VEGF-D level was ≤115 pg/ml in only one of 18 patients who were treated with ramucirumab. As with the association between bevacizumab and VEGF-A level, too high a level of VEGF-D may restrict the efficacy of ramucirumab. Ramucirumab increased VEGF-D levels one month after starting chemotherapy and sustained the elevation during the second line; however, bevacizumab

and aflibercept did not. Although no studies, including that by Tabernero et al (28), reported VEGF-D dynamics after starting chemotherapy, including ramucirumab, this increase is easy to understand in that VEGF-D elevation caused acquired resistance to ramucirumab. The fact that VEGF-A level did not show a distinctive trend after administration of ramucirumab, supports this hypothesis. Interestingly, patients with high levels of VEGF-D had greater benefit from aflibercept. In the biomarker study, VELOUR, VEGF-D was not measured (18); thus, the present study is the first to report a clear association between VEGF-D level and efficacy of aflibercept. PlGF had no impact on the effect of aflibercept, similar to the results of a previous study (18).

Results of the present study suggest that moderate levels of VEGF-A are necessary for a favorable effect of bevacizumab, and moderate levels of VEGF-D are necessary for reasonable efficacy of ramucirumab. Bevacizumab inhibits angiogenesis by blocking VEGF-A, and ramucirumab inhibits it by blocking VEGF-D. Not surprisingly, bevacizumab has little effect in patients with low VEGF-A levels and ramucirumab does not help patients with low VEGF-D levels. However, it is surprising that bevacizumab may be less effective for patients with very high levels of VEGF-A, and ramucirumab, likewise, may be less useful for patients with very high levels of VEGF-D.

This study had several limitations. This is a retrospective, single-center study that included a small number of patients. Thus, regimens of chemotherapy other than anti-VEGFs were not standardized. It is unclear whether VEGF-A, VEGF-D,

PlGF, or PAI-1 impacted the prognosis of patients receiving anti-EGFR therapy, because those were the only patients we included. The present study failed to identify an optimal level of VEGF-A and VEGF-D because it included small numbers of patients; thus, further study is needed.

In conclusion, there is an optimal level of VEGF-A for a favorable effect of bevacizumab and also of VEGF-D for positive outcomes with ramucirumab. VEGF-A and PlGF levels are increased by bevacizumab and VEGF-D levels are increased by ramucirumab. Anti-VEGF drugs have benefits for patients with high PAI-1 levels and PAI-1 levels are not affected by anti-VEGFs. These biomarkers may be useful for predicting drug efficacy and interpreting resistance to anti-VEGF drugs. Presently, two prospective studies (the Brave Ace study and the Ukit study) which evaluate the utility of biomarkers, including VEGFs, in patients treated with anti-VEGF drugs in second-line chemotherapy, are ongoing. These two studies have larger sample sizes compared with our study; thus, they may provide additional information on the efficacy of VEGFs.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on request.

Authors' contributions

TY, SKu and HY designed the study. TY, SKu, HS, AM, RO, SS, YY, GT, TI, KT, TM, KY, SKa and KU acquired, analyzed and interpreted the data. TY and SKu wrote the manuscript. AM and RO were involved in drafting the manuscript. HY revised the manuscript critically. TY and SKu confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Our bio-bank project was approved by Medical Ethics Committee of Nippon Medical School (Bunkyo-ku, Tokyo, Japan; 28-03-738) and written informed consent was obtained from all patients. The present study was approved by the Medical Ethics Committee of Nippon Medical School (Bunkyo-ku, Tokyo, Japan; B-2021-467). Information about the right to opt-out for the present study was posted on the websites of Nippon Medical School. All experimental procedures were performed according to regulations and internal biosafety and bioethics guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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