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Potential of VEGFR2 expression as a predictive marker of PD-1 blockade in patients with advanced NSCLC

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Abstract. Angiogenesis serves a crucial role in cancer progression. Vascular endothelial growth factor (VEGF) exhibits an immunosuppressive function in patients with cancer. However, it remains unclear whether expression of VEGF in tumor tissue can predict the outcome of programmed death-1 blockade in patients with advanced non-small cell lung cancer (NSCLC). A training (n=32) and validation (n=76) cohort of patients with advanced NSCLC who received first-line pembrolizumab were enrolled. Immunohistochemical staining for VEGF receptor 2 (VEGFR2) and tumor-infiltrating lymphocytes (TILs; CD4, CD8 and FOXP3) was performed in tumor specimens of both cohorts and association with clinical outcomes was assessed. The percentages of high VEGFR2 expression were 34.3% (11/32) in training cohort and 25.0% (19/76) in validation cohort. No statistically significant difference in objective response between high and low VEGFR2 expression was observed for training (27.2 vs. 45.0%) and validation (31.2 vs. 35.7%) cohorts. The positive rate of intratumoral FOXP3 was significantly associated with high VEGFR2 expression for validation cohort, but not training cohort. In validation cohort, high VEGFR2 expression in patients with non-adenocarcinoma (non-AC) was significantly correlated with positive FOXP3 TILs in intratumoral and stromal sites, but not CD4 and CD8. High VEGFR2 expression in both cohorts indicated a significantly worse overall survival (OS) than low VEGFR2 expression. VEGFR2 was identified as an independent prognostic marker associated with worse OS. High VEGFR2 expression was a significant marker for predicting worse OS in patients treated with first-line pembrolizumab, particularly in those with non-AC.

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Introduction

Currently, immunotherapy is considered a standard care for human cancer and contributes to improving the outcome following diagnosis. Programmed death-1 (PD-1) blockade serves a crucial role to improve a survival time in cancer treatment. Although exploratory studies have been performed to discover an optimal predictive marker of PD-1 blockade treatment, there are no established markers associated with the efficacy of PD-1 blockade aside from programmed death ligand-1 (PD-L1) expression in tumor specimens (1,2). The expression of PD-L1 has been identified as a predictive marker for certain types of human neoplasm, such as non-small cell lung cancer (NSCLC). Since PD-L1 expression is not completely accurate biomarker for predicting the efficacy of immune checkpoint inhibitors (ICIs), novel predictors of PD-1 blockade should be identified to improve treatment outcome. Aside from PD-L1 expression, there are numerous useful markers for predicting the efficacy of ICIs, such as tumor mutation burden (TMB), microsatellite instability-high/mismatch repair-deficient (MSI-H/dMMR), major histocompatibility complex molecules and T cell receptor (2). PD-1 blockade is associated with improved response and prolonged survival in patients with advanced NSCLC harboring high TMB and metastatic colorectal cancer with MSI-H/Dmmr (2). However, it is difficult to predict the response and outcome of PD-1 blockade using current biomarkers. Therefore, the discovery of new biomarkers for ICIs treatment is necessary to improve the outcome of patients with cancer who receive PD-1 therapy.

Recently, a combination of antiangiogenic agents and ICIs such as pembrolizumab plus ramucirumab has emerged as an effective treatment for cancer (3). Proangiogenic factors, such as vascular endothelial growth factor (VEGF), cause an immunosuppressive tumor microenvironment, which increases the proliferation of FOXP3 and myeloid-derived suppressor cells (3,4). Based on preclinical data, the addition of angiogenic inhibitors to ICIs has been successful in treatment of several types of human cancer, including hepatocellular and

clear cell renal carcinoma and non-squamous NSCLC (4-7). As a combination of ICIs, multi-targeted or selective VEGF receptors (VEGFRs), tyrosine kinase inhibitors and anti-VEGF monoclonal antibodies provide a significant survival benefit compared with treatment with single agents (5-8). The combination of bevacizumab and atezolizumab with chemotherapy has been approved for advanced NSCLC (7). Moreover, a phase I expansion study of a combination of ramucirumab (anti-VEGFR2 monoclonal antibody) and pembrolizumab was performed in patients with previously untreated advanced NSCLC with PD-L1 expression ≥1% (9). The objective response rate (ORR; 42.3 vs. 27.2%) and median progression-free survival (PFS; 9.3 vs. 5.4 months) for combination of VEGFR2 inhibitor with pembrolizumab are greater than those for pembrolizumab monotherapy (9,10). A potential increase in activity of tumor immune cells by inhibiting VEGFR2 has been suggested (8,9); however, it is uncertain whether VEGFR2 expression in tumor cells is a useful biomarker for predicting the efficacy of PD-1 blockade. Furthermore, ramucirumab, a VEGFR2 inhibitor, has been clinically identified as a standard treatment for patients with previously treated NSCLC and inhibition of VEGFR2 plays a crucial role in the suppression of tumor growth (11). A recent study reported that VEGFR2 expression is associated with worse prognosis in patients with surgically resected NSCLC (12). As the combination of certain therapeutic agents with PD-1 blockade is known to be effective for patients with advanced NSCLC, immunotherapy in addition to VEGF inhibitor is expected to be an effective treatment for advanced NSCLC (13,14). Further studies are needed to elucidate the prognostic significance of VEGFR2 expression as a predictor of PD-1 blockade.

The present clinicopathological study aimed to elucidate the predictive role of VEGFR2 expression in patients with advanced NSCLC who received PD-1 blockade as a first-line treatment, based on correlation with the number of tumor-infiltrating lymphocytes (TILs) in intratumoral and stromal tissue.

Materials and methods

Patients. A total of 207 patients with advanced or metastatic NSCLC were treated with pembrolizumab monotherapy as the first-line treatment at our institution (Saitama Medical University, Hidaka, Japan) from May 2017 to March 2021. The inclusion criteria was as follows; having a therapeutic history of first-line pembrolizumab and enough tumor tissue for immunohistochemistry. Of these, 99 patients did not have sufficient tumor specimens for immunohistochemistry before pembrolizumab treatment. Thus, a total of 108 patients (nmale=86, nfemale=22; age range 37-85 years), was eligible for the study. Of these, 32 patients were analyzed as training cohort for investigation and 76 patients were evaluated as validation cohort. The patients who received first-line pembrolizumab from May 2017 to November 2018 were registered as training cohort, whereas, those from December 2018 to March 2021 were allocated as validation cohort. Clinical data such as age, sex, performance status (PS), and smoking history were extracted from medical records. The present study was approved by the Institutional Ethics Committee of the International Medical Center of Saitama Medical University (approval no. 19-075). The requirement for written informed consent for use of human tissue was waived by the ethics committee of Saitama Medical University owing to the retrospective nature of the study.

Therapeutic schedule and evaluation. For first-line monotherapy in all patients, 200 mg/day pembrolizumab was administered intravenously. Physical examination, complete blood count, biochemical testing such as liver and renal dysfunction and electrolytes, and adverse events were measured by the chief physician. Toxicity was graded based on the Common Terminology Criteria for Adverse Events, version 4.0 (15). Tumor response was examined according to Response Evaluation Criteria in Solid Tumors version 1.1 (16). Objective response rate (ORR) and disease control rate (DCR) were assessed. DCR was defined as the percentage of complete response (CR), partial response (PR) and stable disease (SD).

Immunohistochemical staining. Immunohistochemical staining was performed as previously described (17). VEGFR2 (1:100; Cell Signaling Technology, Inc.; cat #2472) was scored according to the stained tumor area (biopsy and surgical sample) as follows: 1, ≤10; 2, 11-24; 3, 25-49 and 4, ≥50% staining. High and low expression were defined by scores of 1-3 and 4, respectively, for VEGFR2, as previously described (17). The sections were evaluated using a light microscope (x200 and x400 magnification) in a blinded fashion by at least two authors. In the case of discrepancies, both investigators evaluated the slides simultaneously until they reached a final consensus. The investigators were blinded to patient outcome.

Multiplex immunohistochemistry (mIHC; OPAL™) staining, image acquisition and data analysis. Tumor specimens were formalin-fixed and paraffin-embedded (fixative concentration, 10%; temperature, room; duration 30 min.). Then, three sections with the largest area of viable tumor cells were selected. Afterwards, 5-μm-thick sections of tissue were deparaffinized and rehydrated using xylene and ethanol for mIHC staining. Next, slides were treated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. To expose antigens, sections were autoclaved in 10 mmol/l sodium citrate buffer (pH 6.0) for 121˚C and 20 min, followed by microwave treatment at 98˚C for 15 min and cooled for 30 min. After rinsing in 0.05 M tris-buffered saline containing 0.1% Tween 20, sections were incubated at 4˚C overnight with mouse monoclonal CD4 (Leica Biosystems; clone 4B12; 1:100; high pH retrieval; cat. NCL-L-CD4-368), CD8 (Dako; Agilent Technologies, Inc.; clone C8/144B; 1:150 high pH retrieval; cat. M7103), FOXP3 (Abcam; 1:50; clone 236A/E7; pH 6 retrieval) (cat. Ab20034) and pan cytokeratin (Abcam; clone AE1/AE3; 1:100; pH 6) (cat. Ab27988). Blocking reagent (Antibody Diluent, Akoya) was incubated at room temperature, 10 min. Secondary antibody (2 drops of Opal Polymer HRP, Akoya) (cat. NEL811001KT) was incubated for room temperature. 10 min. using Akoya: NEL 811001KT opal-7-color Manual IHC KIT (opal Polymer HRP MS+Rb). Chromogen detection reagent for HRP/DAB was Akoya: NEL 811001KT opal-7-color Manual IHC KIT (1Xplus Amplification Diluent) and counterstain was incubated for room temperature, 5 min. using Spectral DAPI solution.

Immunofluorescence signals were visualized using OPAL 7-color IHC kit (Akoya Biosciences, Inc.) tyramide signal amplification dyes 520, 540, 570, 620, 650 and 690 and counterstained with Spectral DAPI. The Mantra imaging platform (Akoya Biosciences, Inc.) was used for imaging with Mantra snap software (version 1.0.3) for data acquisition (http://www.perkinelmer.com). Color separation, tissue and cell segmentation and cell phenotyping were performed using inForm® Software v2.5.1 (Akoya Biosciences, Inc.) to extract image data. Slides were evaluated for the presence of TILs in the tumor and stroma. mIHC staining and data analysis were performed as previously described (18).

All slides were scanned at 20x magnification to achieve high-powered imaging at a resolution of 0.5 μm/pixel using Phenochart (Akoya Biosciences, Inc.). High-powered imaging was used to assess intratumoral area with lymphocytic infiltrate, tumor margin and stromal area. An algorithm was designed based on pattern recognition of pan cytokeratin-positive (tumor) and -negative areas (stroma). Cell segmentation was performed on all cells counterstained with DAPI. TIL distribution scoring for was performed on the 20x pre-scanned images of each patient. A total of three high-powered images of tumor parenchyma and stroma with highest TIL density were selected to grade TIL density, OPAL-positive TIL count and percentage. Multiple images (3 images) from the tumor and stroma were quantified. The cell count of TILs was determined by normalizing to 1,000 cells after counting all cells Images were analyzed on inForm 2.5.1 software (Akoya Biosciences, MA). Type of microscope was Mantra2 multispectral microscopy (Akoya Biosciences, Marlborough, MA) with magnification: 20x objective. Fluorescence images were acquired on Mantra2 multispectral microscopy (Akoya Biosciences, Marlborough, MA) with 20x objective.

Statistical analysis. Student's t (unpaired t) and χ^2 test were used for continuous and categorical variables, respectively. P<0.05 was considered to indicate a statistically significant difference. Correlation between TIL measurement and variables were analyzed using Pearson's rank test. Tumor PD-L1 expression was counted as a tumor proportional score and classified as high or low based on median expression value. Median TIL count in the tumor and stroma was used to define high and low expression. Progression-free survival (PFS) was defined as the time from initial ICI treatment to disease progression or death. Overall survival (OS) was defined as the time from initial ICI treatment to death from any cause. Kaplan-Meier method was used to estimate survival as a function of time and survival differences were analyzed using log-rank test. Univariate and multivariate analyses were performed using logistic regression. All statistical analyses were performed using GraphPad Prism (v.8.0; GraphPad Software, Inc.) and JMP 14.0 (SAS Institute, Inc.).

Results

Patient demographics according to VEGFR2 expression in training and validation cohorts. Patient demographic according to VEGFR2 expression in training cohort and validation cohort are shown in Table I. In the training cohort of 32 patients, PS was 0, 1, 2, and 3 in 10 (31.2%), 14 (43.8%), 7 (21.8%) and 1 (3.2%) patients, respectively. A histology of adenocarcinoma (AC) and non-AC was observed in 17 (53.1%) and 15 (46.9%) patients, respectively. In the validation cohort of 76 patients (nmale=86, nfemale=22; median age, 70 years; age range, 37-85 years), smoking history was observed in 96 (88.8%) patients. PS was 0, 1, 2 and 3 in 37 (34.2%), 53 (49.1%), 12 (11.1%) and 6 (5.6%) patients, respectively. Histological types of AC and non-AC (squamous cell carcinoma and other) were identified in 61 (56.5%), 22 (20.4%), and 25 (23.1%) patients, respectively. Regarding PD-L1 expression, 62 (57.4%) and 46 (42.6%) displayed levels ≥50% and <50%, respectively.

VEGFR2 was highly expressed in lung cancer and closely correlated with histology of non-AC. Immunohistochemical examination was performed on all tumor specimens. Representative images of VEGFR2, CD4, CD8 and FOXP3 expression are shown in Fig. 1. Immunostaining for VEGFR2 was performed on cell membranes and cytoplasm of the tumor specimens. The percentages of high expression of VEGFR2 in training and validation cohort were 34.3% (11/32) and 25.0% (19/76), respectively. The incidence of scoring 1, 2, 3, and 4 was 9 (28.1%), 7 (21.9%), 5 (15.6%), and 11 (34.4%) for training cohort, respectively, and 18 (23.7%), 16 (21.1%), 23 (30.2%), and 19 (25.0%) for validation cohort, respectively. High VEGFR2 expression was significantly associated with sex in the training cohort and histological type in the non-AC group for validation cohort (Table I).

Table II shows ORR and disease control rate (DCR) according to VEGFR2 expression levels. ORR and DCR were 38.7 and 67.7 for training cohort and 31.2 and 79.1% for validation cohort. No statistically significant difference in ORR of the patients between high and low VEGFR2 expression was observed in the training (27.2 vs. 45.0) and validation (31.2 vs. 35.7%) cohorts. In training cohort, patients with high VEGFR2 expression yielded a significantly lower DCR than those with low VEGFR2 expression (36.3 vs. 85.0) but not in validation cohort (75.0 vs. 80.3%).

High VEGFR2 expression was closely associated with positive FOXP3, but not CD4 and CD8. In training cohort (n=32), median cell count for CD4, CD8, and FOXP3/1,000 cells was 1.4 (range, 0-126), 2.7 (0-166) and 4.7 (0-65) in intratumoral sites, respectively, and 7.4 (0-214), 16.9 (0-212) and 9.5 (0-116) in stromal sites, respectively (data not shown). No statistically significant difference in positive percentage of CD4, CD8, and FOXP3 TILs was observed between high and low VEGFR2 expression in intratumoral (Fig. 2A) and stromal sites (Fig. 2B). For validation cohort, median cell counts for CD4, CD8 and FOXP3 TILs/1,000 cells were 3.1 (range, 0-589), 9.2 (0-221) and 5.9 (0-658) in intratumoral lesions, respectively, and 6.7 (0-442), 19.7 (0-363) and 8.6 (0-205) in the stromal lesions, respectively. Positive rate of intratumoral, but not stromal, FOXP3 (Fig. 2C and D) was significantly associated with high VEGFR2 expression in all patients. Positive FOXP3 expression exhibited a significant association with high VEGFR2 expression in intratumoral, but not stromal, lesions in patients with AC (Fig. 2E and F). A statistically significant association was observed between high VEGFR2 expression and positive intratumoral/stromal FOXP3 in patients with non-AC (Fig. 2G and H).

Table I. Characteristics according to VEGFR2 in patients receiving pembrolizumab.

	Training cohort				Validation cohort			
Characteristic	All patients (n=32)	High (n=11)	Low (n=21)	P-value	All patients (n=76)	High (n=19)	Low (n=57)	P-value
Age, <75/≥75 years	20/12	8/3	12/9	0.467	42/34	14/5	28/29	0.069
Sex, male/female	23/8	11/0	12/ 8	0.028[a]	62/14	17/2	45/12	0.496
ECOG PS 0-1 /2-3	24/8	9/2	15/6	0.680	61/15	13/6	48/9	0.182
Smoking (BI), <900/≥900	10/22	4/7	6/15	0.702	37/39	12/7	25/32	0.188
Histology, AC/Non-AC	17/15	6/5	11/10	>0.999	41/35	6/13	35/22	0.033[a]
Brain meta, yes/no	14/18	5/6	9/12	>0.999	22/54	7/12	15/42	0.394
Bone meta								
Yes/No	14/18	6/5	8/13	0.465	21/55	3/16	18/39	0.242
Response								
PR/Non-PR	12/20	3/8	9/12	0.467	25/51	5/14	20/37	0.579
PD-L1								
1-49 /50-100%	11/21	4/7	7/14	>0.999	20/56	3/16	17/40	0.367
Prior RT								
Yes/No	13/19	3/8	10/11	0.450	28/48	5/14	23/34	0.411
G3/4 irAE								
Yes/No	7/25	1/10	6/15	0.374	16/60	2/17	14/43	0.329
Albumin								
High/Low	16/16	4/7	12/9	0.457	46/30	11/8	35/22	0.792
CRP								
High/Low	17/15	6/5	11/10	>0.999	33/43	9/10	24/33	0.791

[a]P<0.05. ECOG, Eastern Cooperative Oncology Group; PS, performance status; PD-L1, programmed death ligand-1; irAE, immune-related adverse event; AC, adenocarcinoma; PR, partial response; CRP, C-reactive protein; meta, metastasis; RT, radiation therapy; pembro, pembrolizumab; comb, combined platinum-based chemotherapy with PD-1 blockade; VEGFR, vascular endothelial growth factor receptor; BI, brinkman index.

Table II. ORR and DCR.

Response	Training cohort (n=32)	Validation cohort (n=76)
CR	0.000	0.000
PR	12.000	25.000
SD	9.000	32.000
PD	10.000	15.000
NE	1.000	4.000
ORR, %	38.700	34.700

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; ORR, objective response; DCR, disease control rate; VEGFR2, vascular endothelial growth factor receptor 2.

High VEGFR2 expression was significantly associated with worse outcome. Kaplan-Meier curves based on expression of VEGFR2 were constructed for all patients (Fig. 3). In training cohort, median PFS and OS were 143 and 485 days, respectively. A total of 27 patients experienced tumor recurrence and 22 died due to progressive disease (data not shown). Patients with high VEGFR2 expression showed a significantly worse OS, but not PFS, than those with low VEGFR2 expression (Fig. 3A and B). Univariate and multivariate survival analyses were performed according to VEGFR2 expression in validation cohort. The median PFS and OS were 324 and 731 days, respectively. A total of 47 patients experienced tumor recurrence and 37 died as a result of progressive disease (Table II). Univariate analysis revealed PS for PFS and PS and VEGFR2 as significant predictor for OS. PS, VEGFR2 and VEGFC were selected for subsequent multivariate analysis. Multivariate analysis confirmed that PS was an independent prognostic factor for PFS and PS and VEGFR2 were identified as significant predictors of OS (Table III). A sub-analysis revealed that high expression of VEGFR2 was significantly associated with shorter PFS and OS in 35 patients without AC but not in 41 patients with AC (Fig. 3C and D).

Discussion

To the best of our knowledge, the present clinicopathological study is the first to evaluate the prognostic significance of angiogenic markers in patients with advanced NSCLC who received first-line pembrolizumab monotherapy. There was an association between high VEGFR2 expression and regulatory

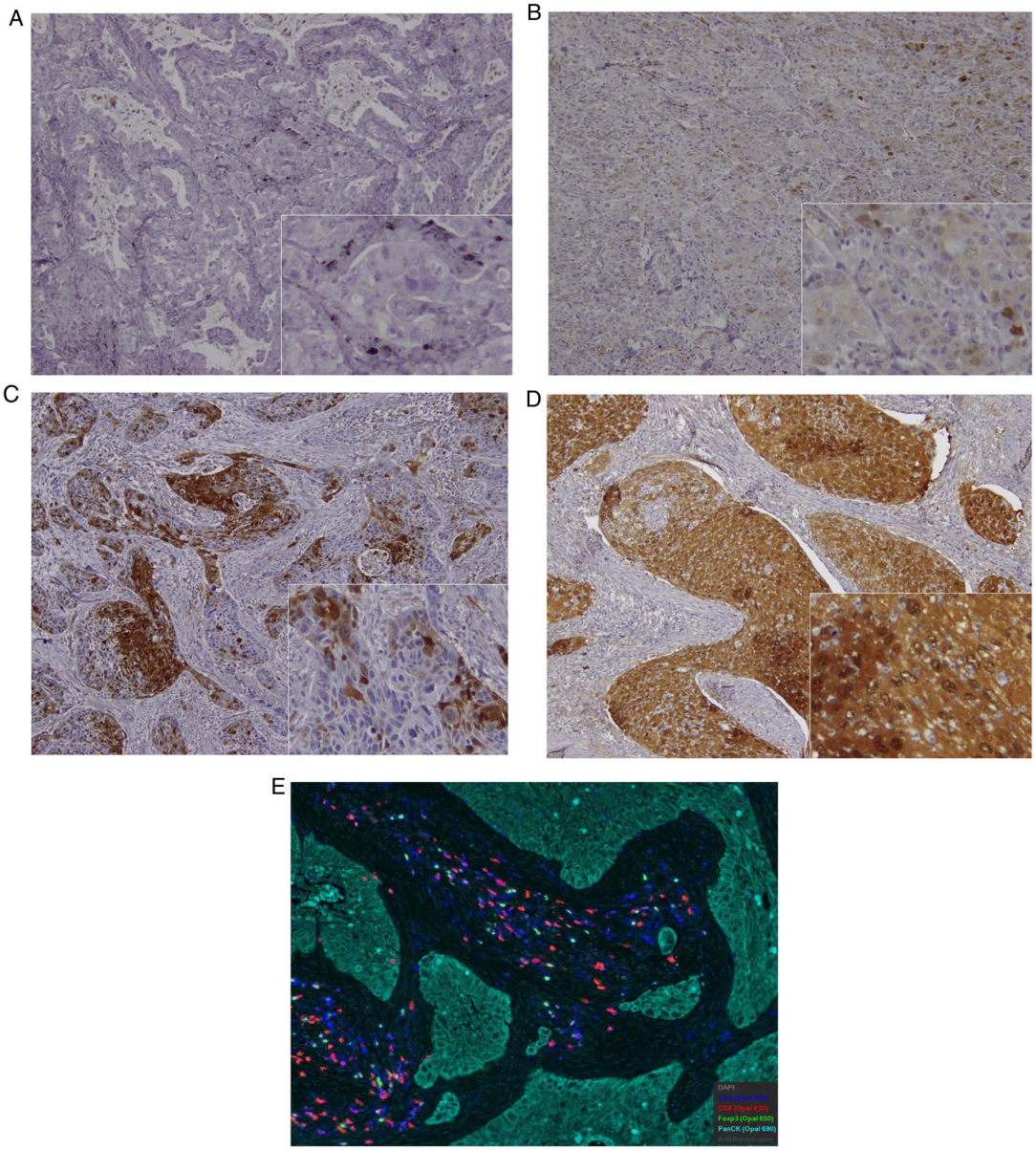

Figure 1. Immunochemistry assay for VEGFR2 expression in non-small cell lung cancer tissue. VEGFR2 was strongly stained on cell membranes and cytoplasm of tumor specimens. Representative images of (A) 1, (B) 2, (C) 3 and (D) 4 VEGFR2 expression score. (E) Immunofluorescence by multiplex immunohistochemistry staining. CD4 (blue), CD8 (red) and FOXP3 (green) lymphocytes were observed in the stroma and intratumoral sites of tumor specimen. Low magnification, x200; high magnification, x400. VEGFR, vascular endothelial growth factor receptor; CK, cytokeratin.

T lymphocytes in tumor specimens with advanced NSCLC and high VEGFR2 expression was an independent marker for predicting worse OS in patients who received first-line pembrolizumab monotherapy, particularly in those with non-AC. VEGFR2 was highly expressed in patients with NSCLC and played a negative role in the efficacy of PD-1 blockade treatment. VEGFR2 expression was associated with the immunosuppressive tumor environment and tended to be resistant to PD-1 blockade treatment in patients with NSCLC with non-AC histology. However, the reason for this phenomenon remains unclear. The present study explored the clinical significance of VEGFR2 expression by training cohort. High VEGFR2 expression was associated with poor OS following PD-1 blockade administration. A significant association between high VEGFR2 expression and worse outcome following pembrolizumab treatment was confirmed by validation cohort. However, high VEGFR2 expression was associated with high FOXP3 in validation cohort, whereas there was not significant relationship between the expression of VEGFR2 and FOXP3 in training cohort. The association of TILs with VEGFR2 may be weak in human tumor specimens. Further investigation is warranted to elucidate the therapeutic significance of VEGFR2 inhibitors in addition to PD-1 blockade in patients with NSCLC with high FOXP3 levels in tumor specimens.

A review reported that the synergistic effects between VEGFR2-targeting therapy and immunotherapy in previous studies (3,4,19) and suppression of VEGFR2 in T cells decreases infiltration of regulatory T cells (Tregs) into tumor tissue (19). Experimental studies have demonstrated that VEGFR2 is selectively expressed by $FOXP3^{high}$ but not $FOXP3^{low}$ Treg (20) and blockade of VEGF is associated with inhibition of the

Table III. Univariate and multivariate survival analysis in 76 patients with pembrolizumab treatment.

	Progression-free survival					Overall survival				
	Univariate analysis		Multivariate analysis			Univariate analysis		Multivariate analysis		
Variable	MST, days	P-value	HR	95% CI	P-value	MST, days	P-value	HR	95% CI	P-value
Age, <75/≥75 years	402/307	0.380				731/829	0.881			
Sex, male/female	324/258	0.558				581/829	0.454			
ECOG PS, 0-1/2-3	336/203	0.027[a]	1.462	0.991-2.067	0.046[a]	829/203	<0.001[a]	1.813	1.247-2.571	0.002[a]
Smoking (BI), <900/≥900	336/324	0.610				731/1046	0.928			
Histology, AC/non-AC	470/310	0.282				829/731	0.745			
Brain metastasis, yes/no	470/307	0.564				731/1046	0.663			
Bone metastasis, yes/no	216/402	0.116				537/829	0.307			
Prior RT, yes/no	363/307	0.731				522/731	0.887			
CRP, high/low	303/336	0.451				522/731	0.186			
Albumin, high/low	363/307	0.251				829/521	0.073			
PD-L1 expression, 1-49/50-100%	324/307	0.927				581/731	0.974			
VEGFR2, high/low	303/363	0.199	1.251	0.873-1.736	0.221	314/1046	0.015[a]	1.592	1.088-2.281	0.017[a]

[a]Statistically significant. MST, median survival time; ECOG PS, Eastern Cooperative Oncology Group performance status; BI, Brinkman index; AC, adenocarcinoma; RT, radiation therapy; CRP, C-reactive protein; PD-L1, programmed death ligand-1; HR, hazard ratio; 95% CI, 95% confidence interval; NR, not reached; VEGFR2, vascular endothelial growth factor receptor 2.

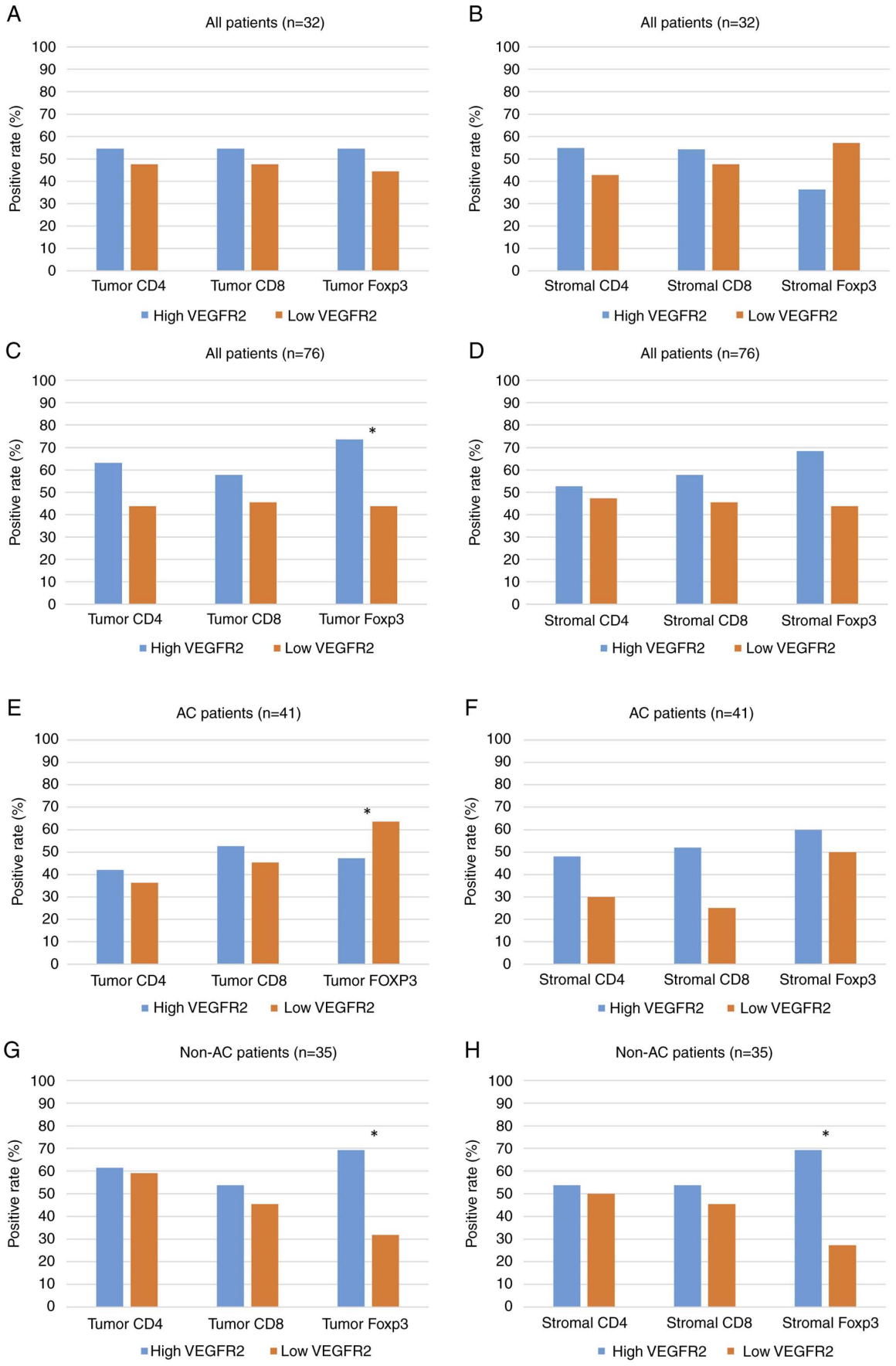

Figure 2. Positive rate for CD4, CD8 and FOXP3 tumor-infiltrating lymphocytes according to expression of VEGFR2. There was no significant difference in positive rate for CD4, CD8 and FOXP3 according to VEGFR2 expression in (A) intratumoral and (B) stromal sites of patients in training cohort (n=32). In validation cohort, positive rate of CD4, CD8 and FOXP3 according to VEGFR2 expression was compared in the intratumoral (C) and stromal sites (D) of all patients, in the intratumoral (E) and stromal sites (F) of AC patients, and in the intratumoral (G) and stromal sites (H) of non-AC patients. VEGFR, vascular endothelial growth factor receptor; AC, adenocarcinoma. *P<0.05 vs. low VEGFR2.

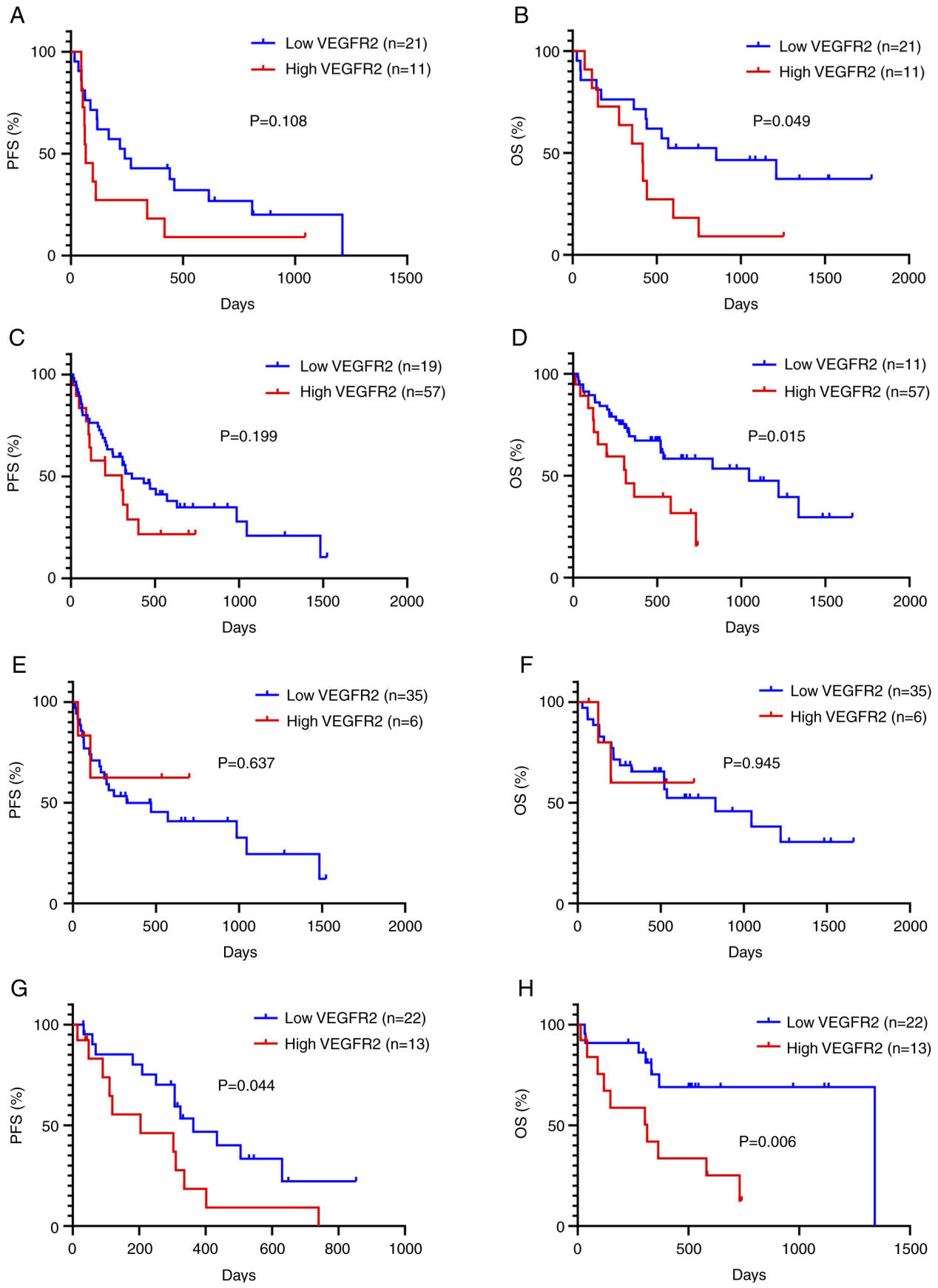

Figure 3. Kaplan-Meier curves of PFS and OS according to VEGFR2 expression. In training cohort (n=32), there was no statistically significant difference in (A) PFS according to VEGFR2 expression, however, the patients with high VEGFR2 expression exhibited a significantly worse (B) OS than those with low VEGFR2. In validation cohort, no statistically significant difference in (C) PFS was observed all patients between high and low VEGFR2 expression and (D) OS was significantly worse in high than in low VEGFR2 expression. There was no statistically significant difference in (E) PFS and (F) OS according to VEGFR2 expression in patients with AC. Non-AC patients with high VEGFR2 expression displayed a significant worse PFS (G) and OS (H) than those with low VEGFR2. VEGFR, vascular endothelial growth factor receptor; PFS, progression-free survival; OS, overall survival; AC, adenocarcinoma.

immunosuppressive phenotype of VEGFR2$^+$ myeloid cells and increased T cell activation (21). The aforementioned reports suggest that inhibition of VEGFR2 enhances the efficacy of ICIs in patients with cancer (19-21). Recently, Shibaki *et al* (22) demonstrated that high serum VEGF is significantly associated with worse prognosis following PD-1 blockade treatment in 235 patients with advanced NSCLC. To the best of our knowledge, however, no studies have reported the association between VEGF expression in tumor specimens and the efficacy of PD-1 blockade. VEGF-ligand antibodies exhibits non-specific staining within tumor tissue, whereas, VEGFR2 is clearly stained in small tissues such as biopsy samples. Here, FOXP3 increased in tumor tissue when VEGFR2 was highly expressed and VEGFR2 mobilized FOXP3 entry into intratumoral lesions with non-AC histology. Histological analysis showed that expression of VEGF was not associated with the mobilization of FOXP3 TILs in AC tumor tissue, whereas VEGFR2 increased FOXP3 TILs infiltration into non-AC intratumoral and stromal tissue. The reason for this difference is unclear. Survival analysis demonstrated that high VEGFR2 expression was associated with worse outcomes in patients with non-AC. Considering the potential to increase FOXP3 by upregulating VEGFR2 (20,21), the present study suggested an association between FOXP3 and VEGFR2 expression in tumor specimens.

Clinical studies reporting the efficacy of pembrolizumab with VEGFR2 inhibitor have been performed in patients with different types of cancer (23-25). It has been reported that lenvatinib (a multikinase inhibitor of VEGFR2, VEGFR2 and VEGFR3) + pembrolizumab exhibits anti-tumor activity (ORR, 39.6%) in patients with advanced endometrial cancer (23) and ramucirumab in combination with pembrolizumab yields favorable antitumor activity in patients with advanced gastric or gastro-esophageal junction AC and urothelial carcinoma (ORR, 7 and 13%, respectively) (24), but ramucirumab + pembrolizumab shows limited clinical activity (ORR, 4%) in patients with advanced biliary tract cancer (25). The aforementioned studies showed that the synergistic efficacy of VEGFR2 inhibitor in addition to pembrolizumab may be different based on histological or cancer type. Bevacizumab + atezolizumab is a standard first-line treatment for patients with advanced hepatocellular carcinoma (5). Reckamp *et al* (26) performed a phase II study to evaluate the efficacy of ramucirumab + pembrolizumab compared with investigator's choice of care in patients with advanced NSCLC who previously received chemotherapy + PD-1 blockade. Even following resistance to prior ICI, ramucirumab with pembrolizumab improved OS compared with standard care, with ORR of 22% (26). The results of the aforementioned study suggested that modulation of tumor immune microenvironment by antiangiogenic drug promotes resensitization to PD-1 blockade in patients with advanced NSCLC, although the mechanism remains unclear (26). Thus, inhibition of VEGFR2 may serve a key role in the improvement of immune microenvironment.

The expression of PD-L1 within tumor cells is a useful marker for predicting the efficacy of ICI treatment in patients with advanced NSCLC (10,13,14). It has been reported that ICI therapy is also effective for patients with NSCLC with negative PD-L1 expression and ~20% of patients are expected to achieve long term survival (27,28). Therefore, PD-L1 expression does not predict efficacy and outcome of ICI therapy, and is not suitable for an optimal biomarker to PD-1 blockade treatment.

The present study had several limitations. First, the sample size was relatively limited; thus, the results may have been biased. For immunohistochemistry, only 108 of 207 patients were available. For molecular targeting therapy, the majority of biopsy samples are used for detection of genetic alterations. Thus, more than half of patients were not eligible because of inadequate or unavailable tumor tissue. Base-line testing for molecular characteristics is important for subsequent therapy. Recent research reported that TMB, POLE mutation and alterations in DNA damage repair genes could affect the efficacy of ICI treatment, loss of serine/threonine kinase 11/liver kinase B1 induces primary resistance to PD-1 blockade in patients with *KRAS* mutant lung AC and epidermal growth factor receptor (*EGFR*) mutations are associated with low response rate to PD-1 blockade (2,29-32). Patients with *TP53* and *KRAS* mutant exhibit greater PFS than those with wild-type *TP53* and *KRAS*) treated with pembrolizumab (33). Here, patient molecular profiling before ICI treatment was not adequately investigated. Thus, further study is warranted to evaluate detailed molecular profiling before ICI therapy. Second, it was difficult to evaluate the expression of VEGFR2 in immune cells in stromal tissue. Immune cells, such as lymphocytes, may serve a key role in VEGFR2-mediated resistance to immunotherapy (20,21). It is difficult to detect expression of VEGFR2 for immunohistochemistry is inadequate for detection of stromal immune cells. Here, VEGF-A, B and D as VEGF-ligand markers and VEGFR1 and VEGFR3 as VEGF receptor markers were immunohistochemically examined. Non-specific staining for these markers was wholly observed in the tumor specimens, regardless of clones and methods (data not shown). For immunohistochemical staining of small samples, such as a transbronchial lung biopsy, use of VEGF or VEGFR antibodies with non-specific staining should be avoided (34). Therefore, VEGFR2 was selected for accurate immunohistochemistry and other VEGF antibodies were not used.

In conclusion, high expression of VEGFR2 was identified as a significant prognostic marker for predicting worse OS following first-line pembrolizumab monotherapy in patients with advanced NSCLC, particularly in those with non-AC. VEGFR2 may serve a crucial role in predicting the efficacy of PD-1 blockade monotherapy. Moreover, VEGFR2 expression in tumor specimens was associated with levels of regulatory T cells. Further investigation is required to elucidate the therapeutic significance of VEGFR2 inhibition in addition to PD-1 blockade based on expression of FOXP3.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Author's contributions

KKa, OY and TK conceived the study and wrote the manuscript. KH, YM, AS, HI, KKo and MY collected and analyzed data. KKa, HI and HK interpreted data. KKa, OY, TK and HK revised the manuscript. All authors have read and approved the final manuscript. KKa and HI confirm the authenticity of all the raw data

Ethics approval and consent to participate

The present study was approved (19-075) by the Institutional Ethics Committee of the International Medical Center of Saitama Medical University, Hidaka city, Japan. The Ethical Committee waived the need to obtain written informed consent for the use of human tissues to participate from the patients owing to the retrospective nature of the study.

Patient consent for publication

Not applicable.

Competing interests

KKa has received research grants and a speaker honorarium from Ono Pharmaceutical Company, Boehringer Ingelheim, Chugai Pharmaceutical, Taiho Pharmaceutical, Eli Lilly Japan and AstraZeneca. AM and OY received a speaker honorarium from Eli Lilly, Taiho Pharmaceutical, Pfizer, Chugai Pharmaceutical and AstraZeneca. HK has received research grants and a speaker honorarium from Ono Pharmaceutical Company, Bristol-Myers Company, Boehringer Ingelheim, MSD, Daiichi Sankyo Company, Chugai Pharmaceutical, Taiho Pharmaceutical, Merck Biopharma Company, Eli Lilly Japan and AstraZeneca. HI has received research grants and a speaker honorarium from Ono Pharmaceutical Company, AstraZeneca and Bristol-Myers Company.

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