# PD-L1 mRNA expression in EGFR-mutant lung adenocarcinoma

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Abstract. Molecular mechanisms of programmed deathligand 1 (PD-L1) mRNA expression and roles of apoptosis and biomarkers are poorly understood in epidermal growth factor receptor (EGFR)-mutant lung adenocarcinoma patients. Thirty-three patients with recurrent postoperative EGFR-mutant lung adenocarcinoma (exon 19 deletion in 16, L858R in 15, G719C in 2 patients) treated with gefitinib were studied. PD-L1 mRNA expression of formalin-fixed paraffinembedded paratumoral and intratumoral tissues was quantified by PCR. Correlations of PD-L1 mRNA expression with BIM, p53 upregulated modular of apoptosis (PUMA), human epidermal growth factor receptor 2 (HER2), mesenchymalepithelial transition (MET), EGFR, and vascular endothelial growth factor A (VEGFA) were determined. Eleven of the 33 patients (33.3%) and 14/33 patients (42.4%) expressed intratumoral and paratumoral PD-L1 mRNA, respectively. Patients with intratumoral PD-L1 mRNA expression had significantly higher BIM and lower VEGFA expression compared with paratumoral PD-L1 mRNA patients (P=0.049, P=0.009). PD-L1

Abbreviations: PD-L1, programmed death-ligand 1; PD-1, programmed death 1; BIM, BCL2-like 11; NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; PUMA, p53 upregulated modular of apoptosis; HER2, human epidermal growth factor receptor 2; VEGFA, vascular endothelial growth factor A; MET, mesenchymal- epithelial transition; FFPE, formalin-fixed paraffin-embedded; PFS, progression-free survival; OS, overall survival; PCR, polymerase chain reaction; CTC, National Cancer Institute Common Terminology Criteria

*Key words: PD-L1*, non-small cell lung cancer, epidermal growth factor receptor, tyrosine kinase inhibitor

mRNA expression was not associated with the expression of *PUMA*, *HER2*, *EGFR* and *MET* but was positively correlated with *BIM* expression (r=0.41, P=0.017) and inversely correlated with *VEGFA* expression (r=-0.33, P=0.043). Patients with intratumoral *PD-L1* mRNA expression had significantly shorter median progression-free survival (PFS) after gefitinib therapy compared with no *PD-L1* expression (255 vs. 732 days, respectively; P=0.032). Thus, *PD-L1* mRNA expression in *EGFR*-mutant lung adenocarcinoma was associated with *BIM* and *VEGFA* mRNA expression and with shorter PFS after gefitinib therapy.

## Introduction

Activating mutations in epidermal growth factor receptor (EGFR) were reported to be potential targets for the treatment of non-small cell lung cancer (NSCLC) (1,2). EGFR mutation frequency was reported to vary by population type; for example, in North America and Western Europe, approximately 5-10% of adenocarcinoma patients contain mutations, whereas approximately 60-70% of non-smokers in East Asia have EGFR mutations (3,4). EGFR tyrosine kinase inhibitors (EGFR-TKIs) including gefitinib, erlotinib, and afatinib have demonstrated marked radiographic and clinical improvement in patients with EGFR mutations and are recommended for the treatment of EGFR-mutant NSCLC (5,6). A longer progressionfree survival (PFS) was reported in NSCLC patients with such mutations who were treated with an EGFR-TKI as a first-line therapy compared with those receiving platinum-based chemotherapy (7-11). The expression of PD-L1, BCL2L11 (BIM), p53 upregulated modular of apoptosis (PUMA), human epidermal growth factor receptor 2 (HER2), vascular endothelial growth factor A (VEGFA), EGFR and mesenchymal-epithelial transition (MET) were reported to be prognostic factors for patients with EGFR mutations receiving EGFR-TKI therapy (12-18).

Programmed death 1 (PD-1) is a co-inhibitory receptor expressed on activated T and B cells and is involved in tumor immune escape (19-21). The PD-1 ligand, termed programmed death-ligand 1 (PD-L1), has been reported to be overexpressed in many cancers (22). Recent clinical trials have shown promising efficacy for PD-L1 and PD-1 antibody blockade

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in NSCLC (23-25). A recent study reported that PD-L1 was expressed in 19.6-65.3% of NSCLC patients (26-30) and that *EGFR* mutation status was associated with PD-L1 expression as assessed by immunohistochemistry (IHC) (31, 32). Chen *et al* (33) reported three pathways of *EGFR* activation: i) EGF simulation; ii) *EGFR-19 del*; and iii) *EGFR-L858R* mutation, which induced PD-L1 expression. Therefore, constitutive oncogenic pathway activation may upregulate PD-L1 expression. Azuma *et al* (32) reported that high PD-L1 expression was associated with the presence of *EGFR* mutations in surgically resected NSCLC indicating it may be an independent negative prognostic factor.

Several studies have reported an association between *PD-L1* and apoptotic activity and angiogenesis in addition to other prognostic factors of EGFR-TKI (34,35). For this reason, *HER2*, *EGFR* and *MET* genes were selected as prognostic markers of EGFR-TKI, *VEGFA* was selected as an angiogenic marker, and *BIM* and *PUMA* were selected as apoptotic markers. This study investigated the association between *PD-L1* mRNA expression and other prognostic factors for EGFR-TKI therapy, including *BIM*, *PUMA*, *HER2*, *VEGFA*, *EGFR* and *MET* in lung tissue from patients with *EGFR*-mutant NSCLC.

## **Patients and methods**

*Clinical samples*. Samples from 33 patients with recurrent postoperative *EGFR*-mutant lung adenocarcinoma (exon 19 deletion in 16, L858R in 15, G719C in 2 patients) treated with gefitinib between January 2008 and January 2016 were obtained. The inclusion criteria were: i) patients with advanced and postoperative recurrent NSCLC; ii) patients with an *EGFR* mutation (Del 19, L858R mutation, and minor mutation); iii) patients treated with gefitinib; iv) patients aged <80 years; and v) either male or female patients. The exclusion criteria were: i) patients with complications or a history of serious lung disorder; ii) pregnant women, women who may possibly be pregnant, women who hope to be pregnant, lactating women; and iii) men who declined contraception.

mRNA expression of *PD-L1*, *BIM*, *PUMA*, *HER2*, *VEGFA*, *EGFR* and *MET* were investigated by the real-time PCR analysis of 33 formalin-fixed paraffin-embedded (FFPE) slides of intratumoral and paratumoral lung tissue surgical samples.

*mRNA extraction from intratumoral and paratumoral tissues.* Total RNA including miRNA was extracted from FFPE sections of intratumoral and paratumoral lung tissues using a miRNeasy FFPE kit (Qiagen KK, Tokyo, Japan) according to the manufacturer's protocol. Paratumoral tissues were defined as normal lung cells including inflammatory cells, and/or mesenchymal cells in the same section with a 1-2 cm distance from the tumor edge.

Detection of PD-L1, BIM, PUMA, HER2, VEGFA, EGFR and MET. Total RNA was stored at -80°C until use. cDNA was synthesized using PrimeScript RT MasterMix (Perfect Real-Time; Takara Bio, Inc., Otsu, Japan). Quantitative real-time PCR was performed using a Thermal Cycler Dice Real-Time System TP800 (Takara Bio, Inc.), using SYBR Premix Ex Taq II (Tli RNaseH Plus; Takara Bio, Inc.). Each PCR reaction used Perfect Real Time primers (Takara Bio, Inc.) as follows: PD-L1 forward, 5'-CGTCTCCTCCAAATGTGTATCA-3' and reverse, 5'-TGGTAATTCTGGGAGCCATC-3'; BIM-EL forward, 5'-GAGCCACAAGGTAATCCTGAA-3' and reverse, 5'-ATACCCACTGGAGGATCGAG-3'; BIM-L forward, 5'-GACAGAGCCACAAGACAGGA-3' and reverse, 5'-GGA AGCCATTGCACTGAGATA-3'; BIM-S forward, 5'-AGAC AGAGCCACAAGCTTCC-3' and reverse, 5'-TGCATAGT AAGCGTTAAACTCG-3'; PUMA forward, 5'-GACGACC TCAACGCACAGTA-3' and reverse, 5'-GAGATTGTACA GGACCCTCCA-3'; HER2 forward, 5'-GGAAACCTGGA ACTCACCTACCTG-3' and reverse, 5'-AGTGGGACCTGC CTCACTTG-3'; VEGFA forward, 5'-TCACAGGTACAGGG ATGAGGACAC-3' and reverse, 5'-CAAAGCACAGCAAT GTCCTGAAG-3'; EGFR forward, 5'-GTGGCGGGACA TAGTCAGCA-3' and reverse, 5'-CCCATTGGGACAGCT TGGA-3'; MET forward, 5'-TCCCATCAACAGGACTACA CACTT-3' and reverse, 5'-GCTGCAGGTATAGGCAGTGA CAA-3; and GAPDH forward, 5'-GCACCGTCAAGGCTGA GAAC-3' and reverse, 5'-TGGTGAAGACGCCAGTGGA-3'.

Quantification of PD-L1, BIM, PUMA, HER2, VEGFA, EGFR and MET expression. The targets were obtained from the same mRNA preparations. The relative expression of PD-L1, BIM, PUMA, HER2, VEGFR, EGFR and MET in mRNA isolated from tissue sections of intratumoral and paratumoral lung tissues, normalized to the reference gene (GAPDH), were calculated using the KCL22 or H2228 cell line for calibration (35-37). PD-L1 negative was defined as no detection of PD-L1 mRNA in this study.

Validation between PD-L1 mRNA levels and IHC. We confirmed the validity between PD-L1 mRNA levels and PD-L1 expression by IHC. PD-L1 IHC was performed using an automated IHC assay (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) with rabbit anti-human PD-L1 antibody (clone 28-8, cat. no. ab205921; Epitomics; Abcam, Burlingame, CA, USA). Tumor PD-L1 protein expression was confirmed when staining of the tumor-cell membrane (at any intensity) was observed at a prespecified expression in a section that included at least 100 tumor cells that could be evaluated.

*Clinical outcomes.* We retrospectively analyzed the clinical characteristics, response rate, and disease control rate for gefitinib in patients with and without *PD-L1*. We then estimated the PFS and overall survival (OS). OS was defined as the interval from the date of diagnosis until death from any cause. The PFS of patients treated with gefitinib was assessed from the date of induction of gefitinib therapy until the first sign of disease progression, as determined by computed tomographic or magnetic resonance imaging, according to the Response Evaluation Criteria in Solid Tumors (RECIST) criteria.

Statistical analysis. Statistical analyses were performed using SPSS software for Windows, version 12.0 (SPSS Inc., Tokyo, Japan). Differences in the relative expression of *PD-L1*, *BIM*, *PUMA*, *HER2*, *VEGFA*, *EGFR* and *MET* between patients with and without *PD-L1* expression were compared with the Wilcoxon rank-sum test. Survival curves were plotted using

Table I. Characteristics of patients (n=33).

Parameters	Values
Age (years) range Mean	25-82 64.7
Sex Male Female	26 7
ECOG Performance status 0 1 2	21 10 2
Histological pattern Ad Clinical stage	33
Rec EGFR mutation at primary site 19del L858R G719C	33 16 15 2
Line of gefitinib therapy First Second Third	16 16 1

ECOG, Eastern Cooperative Oncology Group; Rec, recurrence after surgical resection; Ad, adenocarcinoma; *EGFR*, epidermal growth factor receptor; L858R, exon 21 L858R; 19del, exon 19 deletion; G719C, exon 18 G719C.

the Kaplan-Meier method, and the log-rank test was used for statistical analysis. A P-value <0.05 indicated a statistically significant difference.

We used univariate analysis and multivariate Cox regression analysis to identify factors associated with a shorter PFS and OS. The investigated prognostic factors were age, sex (male vs. female), performance status (PS; 2 vs. 1 vs. 0), brain metastasis (yes vs. no), bone metastasis (yes vs. no), pulmonary metastasis (yes vs. no), pleura metastasis (yes vs. no), liver metastasis (yes vs. no), lymph node metastasis (yes vs. no), *EGFR* mutation [major mutations (L858R and exon 19 deletion) vs. minor mutations (other mutations)], smoking history [pack(s)-year], and intratumoral *PD-L1* expression (yes vs. no).

This single-center study was conducted at Toho University Omori Medical Center (Tokyo, Japan) and was approved by its Human Genome/Gene Analysis Research Ethics Committee (authorization no. 27128).

## Results

*PD-L1 mRNA expression in EGFR-positive NSCLC.* We analyzed *PD-L1* mRNA expression in 33 patients with *EGFR* mutation-positive NSCLC patients who were treated with

Table II. PD-L1 mRNA expression (n=33).

PD-L1 expression	Ν	%
Intratumoral	11ª	33.3
Paratumoral	14 <sup>a</sup>	42.4
Absent	14 <sup>a</sup>	43.4

<sup>a</sup>Six patients had both intratumoral and paratumoral expression. *PD-L1*, programmed death-ligand 1.



Figure 1. The relative expression of *PD-L1* is similar between paratumoral (285.6 $\pm$ 631.3; mean  $\pm$  SD) and intratumoral (67.2 $\pm$ 631.3; mean  $\pm$  SD) tissues. \*P=0.056. Error bars indicate the SD.



Figure 2. *PD-L1* mRNA expression is positively correlated with PD-L1 IHC expression. r=0.44, P=0.015. *PD-L1*, programmed death-ligand 1; IHC, immunohistochemistry.

gefitinib. The patient characteristics are presented in Table I. Intratumoral *PD-L1* mRNA expression was noted in 11 out of 33 patients (33.3%), and paratumoral expression was noted in 14 out of 33 patients (42.4%) (Table II). Six patients had both intratumoral and paratumoral *PD-L1* expression. There was no significant difference in the relative expression of

	Intratumoral PA (mear	<i>D-L1</i> expression 1 ± SD)		Paratumoral <i>P1</i> (mean		
	Positive (n=11)	Negative (n=22)	P-value	Positive (n=14)	Negative (n=19)	P-value
BIM	57.4±87.7	14.5±34.3	0.049	6.1±8.5	4.4±9.1	0.85
PUMA	15.9±13.7	17.3±47.5	0.93	29.8±69.2	42.2±127	0.45
HER2	1071±874	1354±941	0.41	1020±819	1648±1577	0.17
VEGFA	549±294	1440±1032	0.009	1328±1050	1474±1108	0.70
EGFR	34.1±26.2	38.7±45.6	0.76	29.3±27.6	26.1±37.0	0.78
MET	33.9±36.6	22.7±18.9	0.25	9.8±14.8	14.3±15.1	0.41

Table III. Associations of PD-L1 expression with BIM, PUMA, HER2, VEGFA, EGFR, and MET expression (n=33).

PD-L1, programmed death-ligand 1; BIM, BCL2L11; PUMA, p53 upregulated modular of apoptosis; HER2, human epidermal growth factor receptor 2; VEGFA, vascular endothelial growth factor A; EGFR, epidermal growth factor receptor; MET, mesenchymal-epithelial transition.



Figure 3. *PD-L1* mRNA expression is positively correlated with *BIM* expression. r=0.41, P=0.017. *PD-L1*, programmed death-ligand 1; *BIM*, BCL2L11.



Figure 4. *PD-L1* mRNA expression is inversely correlated with *VEGFA* expression. r=-0.33, P=0.043. *PD-L1*, programmed death-ligand 1; *VEGFA*, vascular endothelial growth factor A.

*PD-L1* mRNA between intratumoral and paratumoral tissues (P=0.056) (Fig. 1).

## Table IV. Clinical response after EGFR-TKI therapy (n=33).

Clinical response (%)	Patients with intratumoral <i>PD-L1</i> expression (n=11)	Patients without intratumoral <i>PD-L1</i> expression (n=22)	P-value
Response rate	45.5	59.1	0.45
Disease control rate	91.0	95.5	0.60

EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; *PD-L1*, programmed death-ligand 1.

*Validation between PD-L1 mRNA levels and IHC results.* We confirmed the validity between the PD-L1 mRNA levels and PD-L1 expression by IHC. There was a significant correlation between PD-L1 mRNA levels and PD-L1 IHC expression (r=0.44, P=0.015) (Fig. 2).

Association of PD-L1 expression with BIM, PUMA, HER2, VEGFR, EGFR and MET expression. Patients with intratumoral PD-L1 mRNA expression had significantly higher BIM expression and significantly lower VEGFA expression compared with those without PD-L1 expression (P=0.049 and P=0.009, respectively) (Table III). The expression of PUMA, HER2, EGFR, and MET was not associated with PD-L1 mRNA expression status. Paratumoral PD-L1 mRNA expression was not associated with the expression of BIM, PUMA, HER2, VEGFA, or EGFR (Table III).

*Correlations of PD-L1 mRNA expression with BIM and VEGFA expression.* We assessed the correlations of intratumoral *PD-L1* mRNA expression with *BIM* and *VEGFA* mRNA expression. *PD-L1* mRNA expression was positively correlated with *BIM* expression (r=0.41, P=0.017) (Fig. 3) and inversely correlated with *VEGFA* expression (r=-0.33, P=0.043) (Fig. 4). However, *PD-L1* mRNA expression was not correlated with the expression of *HER2*, *EGFR*, *MET*, and *PUMA* expression (Fig. 5).



Figure 5. *PD-L1* mRNA expression is not correlated with *HER2* (r=-0.11, P=0.56), *EGFR* (r=-0.67, P=0.22), *MET* (r= -0.44, P=0.81), and *PUMA* (r=0.06, P=0.93) expression. *PD-L1*, programmed death-ligand 1; *HER2*, human epidermal growth factor receptor 2; *EGFR*, epidermal growth factor receptor; *MET*, mesenchymal-epithelial transition; *PUMA*, p53 upregulated modular of apoptosis.



Figure 6. Kaplan-Meier curves for PFS. The median PFS was significantly shorter for patients with detectable intratumoral *PD-L1* mRNA expression after gefitinib therapy compared with those lacking *PD-L1* mRNA expression (255 vs. 732 days, respectively). \*P=0.032. PFS, progression-free survival; *PD-L1*, programmed death-ligand 1.



Figure 7. Kaplan-Meier curves for OS. There was no significant difference in the median OS between the groups (1291 vs. 1511 days). P=0.24. OS, overall survival.

*Clinical response and survival.* There were no significant differences in the response rate or disease control rate between patients with (n=11) or without (n=22) intratumoral *PD-L1* expression (Table IV). Patients with intratumoral *PD-L1* mRNA expression had a significantly shorter median PFS after gefitinib therapy compared with those without *PD-L1* 

expression (255 vs. 732 days, respectively; P=0.032) (Fig. 6). However, the median OS did not significantly differ between these groups (1,291 vs. 1,511 days, P=0.24) (Fig. 7).

Multivariate Cox regression analysis revealed that intratumoral *PD-L1* expression was the most important independent indicator of a shorter PFS (hazard ratio, 2.953; 95% confidence interval, 1.270-6.868; P=0.012) (Table V).

Parameters	HR	95% CI	P-value
Univariate Cox Regression Analysis			
Pleura metastasis (yes vs. no)	2.06	0.773-5.484	0.15
Bone metastasis (yes vs. no)	3.86	1.506-9.887	0.005
Intratumoral PD-L1 expression	2.29	1.054-4.953	0.036
Multivariate Cox Regression Analysis			
Pleura metastasis (yes vs. no)	3.47	1.193-10.107	0.02
Bone metastasis (yes vs. no)	5.03	1.830-13.803	0.002
Intratumoral PD-L1 expression	2.95	1.270-6.868	0.012

Ta	l	bl	e	V	Indicators	s of	shorter	PFS	after	gefitinib	treatment.
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However, intratumoral *PD-L1* expression was not an indicator of a shorter OS.

#### Discussion

We investigated the association between *PD-L1* mRNA expression and prognostic factors associated with EGFR-TKI therapy, including *BIM*, *PUMA*, *HER2*, *VEGFA*, *EGFR* and *MET* in the lung tissues of patients with *EGFR*-mutant NSCLC. *PD-L1* mRNA expression in *EGFR*-mutant lung adenocarcinoma was associated with *BIM* and *VEGFA* mRNA expression and with a shorter PFS after gefitinib therapy. To the best of our knowledge, this is the first study of the association of *BIM* and *VEGFA* mRNA expression in human NSCLC clinical samples.

EGFR activation induced PD-L1 expression, indicating that this constitutive oncogenic pathway activation may upregulate PD-L1 (33). IHC analysis revealed that PD-L1 was positive in 53.6-58.8% of tumor specimens in patients with *EGFR*-mutant NSCLC (38-40). In the present study, intratumoral *PD-L1* mRNA expression was noted in 11 out of 33 patients (33.3%). This lower ratio may be explained by the degradation of mRNA in the specimens used in this study. Future studies should examine the correlation between *PD-L1* mRNA expression and PD-L1 protein expression deternined by IHC.

BIM is a proapoptotic protein of the B-cell CLL/ lymphoma 2 (Bcl-2) family of proteins and is a key modulator of apoptosis induced by EGFR-TKI (41). It has been reported that BIM upregulation is related to the expression of PD-L1 by tumor-reactive CD8<sup>+</sup> T cells in patients with malignant melanoma (42). In the present study, patients with detectable *PD-L1* mRNA expression had significantly higher *BIM* expression (P=0.049), and *PD-L1* mRNA expression was positively correlated with *BIM* expression. Recently, Dronca *et al* (34) reported that BIM, regulated by PD-1 and PD-L1, was crucial for T-cell activation and apoptosis, especially in effector CD8<sup>+</sup> T cells from melanoma patients, and that T-cell BIM levels reflected the patient response to anti-PD-1 cancer therapy. Future studies should examine the association between PD-L1 and BIM.

Although VEGF pathway activation is most commonly associated with increased angiogenesis, recent studies reported that increased angiogenesis promoted an immunosuppressive tumor microenvironment (43-45). Other studies suggested that VEGF inhibition increased the number of tumor-infiltrating lymphocytes (46). Joseph *et al* reported that PD-L1 expression assessed by IHC was inversely correlated with the expression of VEGFA, VEGFR1, and VEGFR2 in clear cell renal carcinoma (35). In the present study, *VEGFA* expression was significantly lower in patients with intratumoral *PD-L1* mRNA expression compared with patients lacking *PD-L1* mRNA expression (P=0.009). In addition, the relative *PD-L1* mRNA expression (r= -0.33, P=0.043). These findings indicated that tumors with increased VEGF expression have decreased immune infiltration and therefore, there is less adaptive pressure to express PD-L1.

High IHC staining of PD-L1 was associated with a poor prognosis in several human malignancies, indicating that high intratumoral PD-L1 expression may drive tumor recurrence by preventing antitumor immunity (47,48). PD-L1-positive patients treated with EGFR-TKI had a faster disease progression compared with PD-L1-negative patients (49,50). Data from the present study revealed that as PD-L1 expression increased, VEGFA expression decreased leading to the suppression of angiogenesis, tumor growth and metastasis, which consequently shortened the PFS of EGFR-TKI. In EGFR mutation-positive NSCLC, BIM reflects the expression of PD-L1 because it is a downstream signal of PD-L1; therefore, future clinical applications are expected. PD-L1 expression has been reported to change after EGFR-TKI treatment (51). Han et al (51) reported that intratumoral PD-L1 IHC expression was markedly increased in 38.9% of patients after gefitinib treatment. Since samples were obtained and used before treatment in the present study, there appears to be no correlation between the expression of PD-L1 and EGFR, MET, and HER2. Our future study will investigate the relationship between PD-L1 expression and EGFR, MET and HER2 after EGFR-TKI resistance. Furthermore, it was suggested that PD-L1 expression was also related to BIM-mediated apoptosis and VEGFA-mediated angiogenesis in EGFR-mutated lung cancer.

This study had some limitations. First, it was a retrospective single-center study with a small sample size. We revealed differences in clinical outcome according to PD-L1 expression; however, the number of patients enrolled was too small to consider the association of PD-L1 expression and PFS. Furthermore, the OS was not different between patients with and without PD-L1 expression. Thus, a large-scale multicenter study is required to confirm the validity of our results. Second, there is a possibility of the influence of mRNA deterioration in the specimens used in this study. In addition, since we used a cell line as a control and samples to produce the standard curve, we cannot assess/confirm that the clinical samples were of sufficient high quality to produce meaningful results in the present study.

In conclusion, *PD-L1* mRNA expression in *EGFR*-mutant lung adenocarcinoma was associated with *BIM* and *VEGFA* mRNA expression and with a shorter PFS after gefitinib therapy. The present results should help treatment planning for patients with *EGFR*-mutant NSCLC.

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## Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

## **Author's contributions**

KI, AK, TMi and SH conceived and designed the study. KK, HK, TY and YN performed the experiments. KI and AK wrote the paper. TMa, HO, GS, KS, SS, YT, NT, AI and SH reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

#### Ethics approval and consent to participate

This single-center study was conducted at Toho University Omori Medical Center (Tokyo, Japan) and was approved by its Human Genome/Gene Analysis Research Ethics Committee (authorization no. 27128).

## **Consent for publication**

Not applicable.

## **Competing interests**

The authors declare that they have no competing interests.

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