PD-L1 mRNA expression in EGFR-mutant lung adenocarcinoma

KAZUTOSHI ISOBE[1], ATSUSHI KAKIMOTO[1], TETUO MIKAMI[2], KYOHEI KABURAKI[1], HIROSHI KOBAYASHI[1], TAKAHIRO YOSHIZAWA[1], YUTA NAKANO[1], TAKASHI MAKINO[3], HAJIME OTSUKA[3], GO SANO[1], KEISHI SUGINO[1], SUSUMU SAKAMOTO[1], YUJIRO TAKAI[1], NAOBUMI TOCHIGI[4], AKIRA IYODA[2] and SAKAE HOMMA[1]

Divisions of [1]Respiratory Medicine, [2]Pathology, [3]Chest Surgery and [4]Surgical Pathology, Toho University School of Medicine, Tokyo 143-8541, Japan

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Abstract. Molecular mechanisms of programmed death-ligand 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 paraffin-embedded 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)*, mesenchymal-epithelial 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* 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.

Correspondence to: Dr Kazutoshi Isobe, Division of Respiratory Medicine, Toho University School of Medicine, 6-11-1 Omori-Nishi, Ota-ku, Tokyo 143-8541, Japan
E-mail: kazutoshiisobe@med.toho-u.ac.jp

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

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 progression-free 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

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'-GGAAGCCATTGCACTGAGATA-3'; BIM-S forward, 5'-AGACAGAGCCACAAGCTTCC-3' and reverse, 5'-TGCATAGTAAGCGTTAAACTCG-3'; PUMA forward, 5'-GACGACCTCAACGCACAGTA-3' and reverse, 5'-GAGATTGTACAGGACCCTCCA-3'; HER2 forward, 5'-GGAAACCTGGAACTCACCTACCTG-3' and reverse, 5'-AGTGGGACCTGCCTCACTTG-3'; VEGFA forward, 5'-TCACAGGTACAGGGATGAGGACAC-3' and reverse, 5'-CAAAGCACAGCAATGTCCTGAAG-3'; EGFR forward, 5'-GTGGCGGGACATAGTCAGCA-3' and reverse, 5'-CCCATTGGGACAGCTTGGA-3'; MET forward, 5'-TCCCATCAACAGGACTACACACTT-3' and reverse, 5'-GCTGCAGGTATAGGCAGTGACAA-3; and GAPDH forward, 5'-GCACCGTCAAGGCTGAGAAC-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	25-82
Mean	64.7
Sex	
Male	26
Female	7
ECOG Performance status	
0	21
1	10
2	2
Histological pattern	
Ad	33
Clinical stage	
Rec	33
EGFR mutation at primary site	
19del	16
L858R	15
G719C	2
Line of gefitinib therapy	
First	16
Second	16
Third	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.

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

PD-L1 expression	N	%
Intratumoral	11[a]	33.3
Paratumoral	14[a]	42.4
Absent	14[a]	43.4

[a]Six patients had both intratumoral and paratumoral expression. *PD-L1*, programmed death-ligand 1.

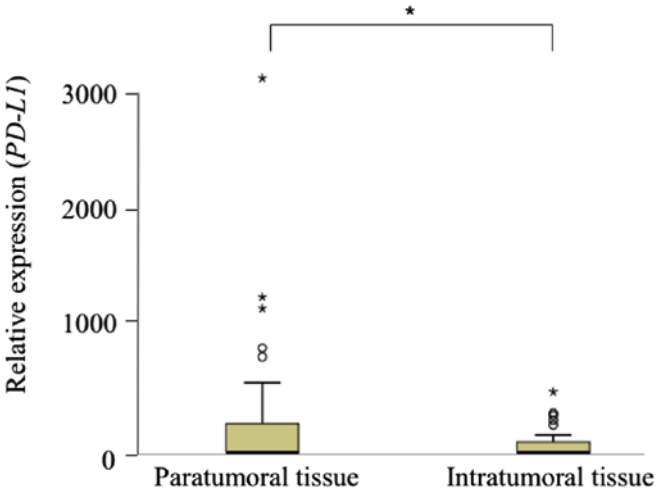

Figure 1. The relative expression of *PD-L1* is similar between paratumoral (285.6±631.3; mean ± SD) and intratumoral (67.2±631.3; mean ± SD) tissues. *P=0.056. Error bars indicate the SD.

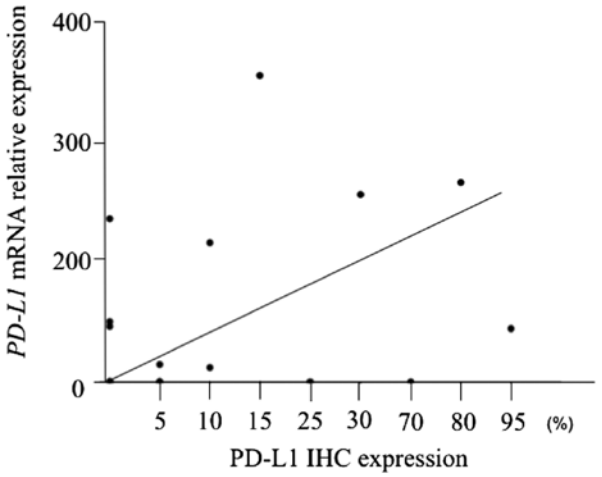

Figure 2. *PD-L1* mRNA expression is positively correlated with PD-L1 IHC expression. r=0.44, P=0.015. *PD-L1*, programmed death-ligand 1; IHC, immunohistochemistry.

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 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

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

	Intratumoral *PD-L1* expression (mean ± SD)			Paratumoral *PD-L1* expression (mean ± SD)		
	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

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.

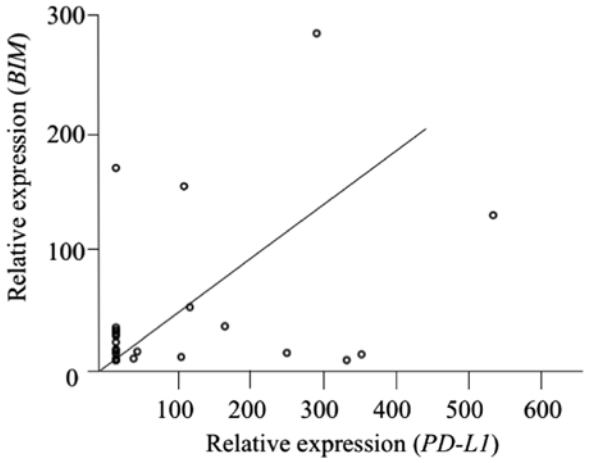

Figure 3. *PD-L1* mRNA expression is positively correlated with *BIM* expression. r=0.41, P=0.017. *PD-L1*, programmed death-ligand 1; *BIM*, BCL2L11.

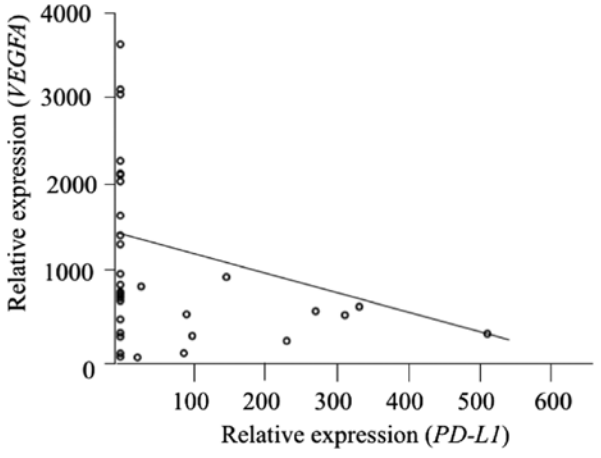

Figure 4. *PD-L1* mRNA expression is inversely correlated with *VEGFA* expression. r=-0.33, P=0.043. *PD-L1*, programmed death-ligand 1; *VEGFA*, vascular endothelial growth factor A.

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 *PD-L1* expression (n=11)	Patients without intratumoral *PD-L1* 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).

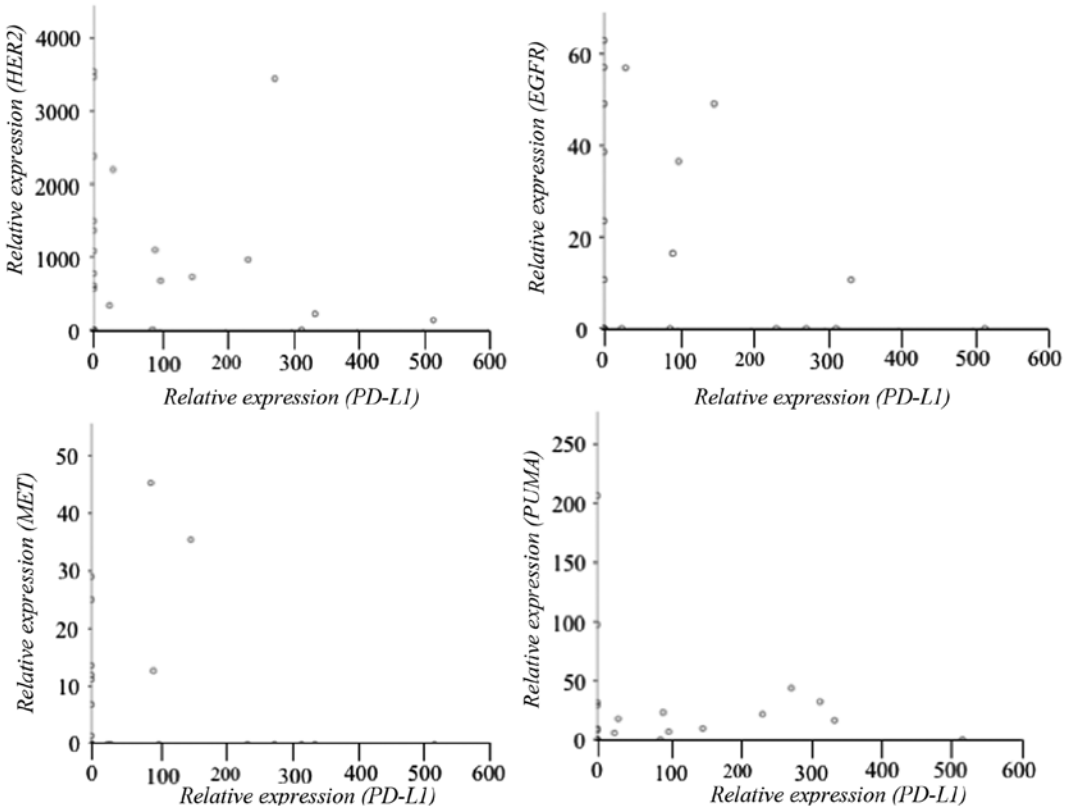

Figure 5. *PD-L1* mRNA expression is not correlated with *HER2* (r=-0.11, P=0.56), *EGFR* (r=-0.67, P=0.22), *MET* (r= -0.44, P=0.81), and *PUMA* (r=0.06, P=0.93) expression. *PD-L1*, programmed death-ligand 1; *HER2*, human epidermal growth factor receptor 2; *EGFR*, epidermal growth factor receptor;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

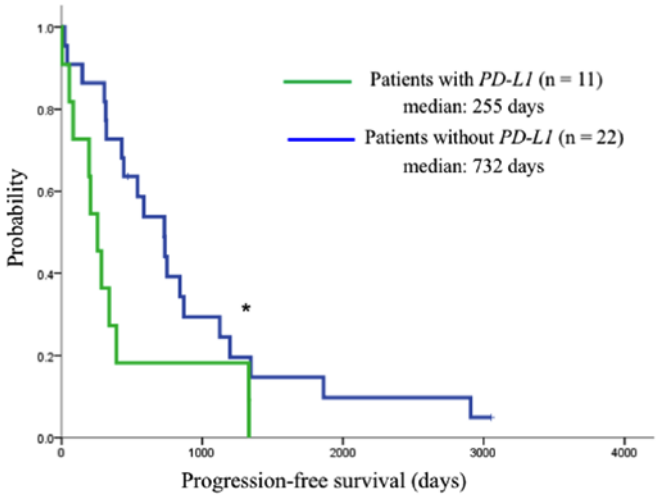

Figure 6. Kaplan-Meier curves for PFS. The median PFS was significantly shorter for patients with detectable intratumoral *PD-L1* mRNA expression after gefitinib therapy compared with those lacking *PD-L1* mRNA expression (255 vs. 732 days, respectively). *P=0.032. PFS, progression-free survival; *PD-L1*,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

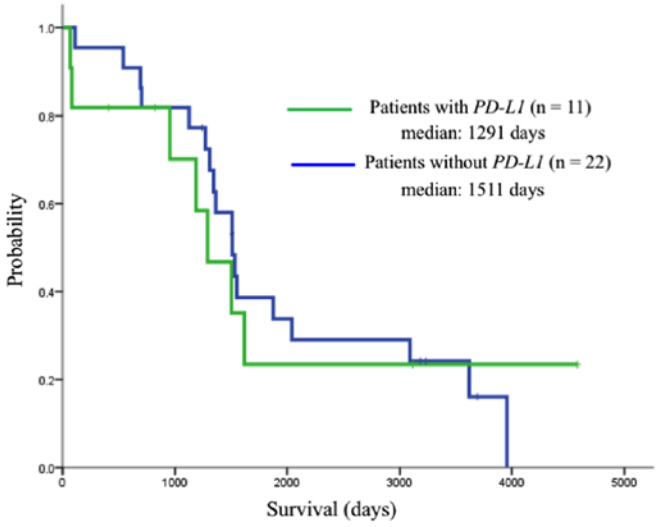

Figure 7. Kaplan-Meier curves for OS. There was no significant difference in the median OS between the groups (1291 vs. 1511 days). P=0.24. OS, overall survival.

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).

Table V. Indicators of shorter PFS after gefitinib treatment.

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

HR, hazard ratio; CI, confidence interval; *PD-L1*, programmed death-ligand 1.

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 determined 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 $CD8^+$ 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 $CD8^+$ 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 was inversely correlated with *VEGFA* 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.

References

1. Paez JG, Jänne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, *et al*: EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. Science 304: 1497-1500, 2004.
2. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, Harris PL, Haserlat SM, Supko JG, Haluska FG, *et al*: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med 350: 2129-2139, 2004.
3. Tan DS, Mok TS and Rebbeck TR: Cancer genomics: Diversity and disparity across ethnicity and geography. J Clin Oncol 34: 91-101, 2016.
4. Tan DS, Yom SS, Tsao MS, Pass HI, Kelly K, Peled N, Yung RC, Wistuba II, Yatabe Y, Unger M, *et al*: The International Association for the Study of Lung Cancer consensus statement on optimizing management of EGFR mutation-positive non-small cell lung cancer: Status in 2016. J Thorac Oncol 11: 946-963, 2016.
5. Masters GA, Temin S, Azzoli CG, Giaccone G, Baker S Jr, Brahmer JR, Ellis PM, Gajra A, Rackear N, Schiller JH, *et al*; Systemic Therapy for Stage IV Non-Small-Cell Lung Cancer: American Society of Clinical Oncology Clinical Practice Guideline Update. J Clin Oncol 33: 3488-3515, 2015.
6. Kuan FC, Kuo LT, Chen MC, Yang CT, Shi CS, Teng D and Lee KD: Overall survival benefits of first-line EGFR tyrosine kinase inhibitors in EGFR-mutated non-small-cell lung cancers: A systematic review and meta-analysis. Br J Cancer 113: 1519-1528, 2015.
7. Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, Sunpaweravong P, Han B, Margono B, Ichinose Y, *et al*: Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med 361: 947-957, 2009.
8. Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, Gemma A, Harada M, Yoshizawa H, Kinoshita I, *et al*; North-East Japan Study Group: Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med 362: 2380-2388, 2010.
9. Zhou C, Wu YL, Chen G, Feng J, Liu XQ, Wang C, Zhang S, Wang J, Zhou S, Ren S, *et al*: Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): A multicentre, open-label, randomised, phase 3 study. Lancet Oncol 12: 735-742, 2011.
10. Yang JC, Wu YL, Schuler M, Sebastian M, Popat S, Yamamoto N, Zhou C, Hu CP, O'Byrne K, Feng J, *et al*: Afatinib versus cisplatin-based chemotherapy for EGFR mutation-positive lung adenocarcinoma (LUX-Lung 3 and LUX-Lung 6): Analysis of overall survival data from two randomised, phase 3 trials. Lancet Oncol 16: 141-151, 2015.
11. Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, Seto T, Satouchi M, Tada H, Hirashima T, *et al*; West Japan Oncology Group: Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): An open label, randomised phase 3 trial. Lancet Oncol 11: 121-128, 2010.
12. Bean GR, Ganesan YT, Dong Y, Takeda S, Liu H, Chan PM, Huang Y, Chodosh LA, Zambetti GP, Hsieh JJ, *et al*: PUMA and BIM are required for oncogene inactivation-induced apoptosis. Sci Signal 6: ra20, 2013.
13. Isobe K, Kakimoto A, Mikami T, Kaburaki K, Kobayashi H, Yoshizawa T, Makino T, Otsuka H, Sano GO, Sugino K, *et al*: Association of BIM deletion polymorphism and BIM-γ RNA expression in NSCLC with EGFR mutation. Cancer Genomics Proteomics 13: 475-482, 2016.
14. Costa C, Molina MA, Drozdowskyj A, Giménez-Capitán A, Bertran-Alamillo J, Karachaliou N, Gervais R, Massuti B, Wei J, Moran T, *et al*: The impact of EGFR T790M mutations and BIM mRNA expression on outcome in patients with EGFR-mutant NSCLC treated with erlotinib or chemotherapy in the randomized phase III EURTAC trial. Clin Cancer Res 20: 2001-2010, 2014.
15. Garofalo M, Romano G, Di Leva G, Nuovo G, Jeon YJ, Ngankeu A, Sun J, Lovat F, Alder H, Condorelli G, *et al*: EGFR and MET receptor tyrosine kinase-altered microRNA expression induces tumorigenesis and gefitinib resistance in lung cancers. Nat Med 18: 74-82, 2011.

16. Naumov GN, Nilsson MB, Cascone T, Briggs A, Straume O, Akslen LA, Lifshits E, Byers LA, Xu L, Wu HK, *et al*: Combined vascular endothelial growth factor receptor and epidermal growth factor receptor (EGFR) blockade inhibits tumor growth in xenograft models of EGFR inhibitor resistance. Clin Cancer Res 15: 3484-3494, 2009.
17. Hirsch FR, Varella-Garcia M and Cappuzzo F: Predictive value of EGFR and HER2 overexpression in advanced non-small-cell lung cancer. Oncogene 28 (Suppl 1): S32-S37, 2009.
18. Rho JK, Choi YJ, Lee JK, Ryoo BY, Na II, Yang SH, Lee SS, Kim CH, Yoo YD and Lee JC: The role of MET activation in determining the sensitivity to epidermal growth factor receptor tyrosine kinase inhibitors. Mol Cancer Res 7: 1736-1743, 2009.
19. Agata Y, Kawasaki A, Nishimura H, Ishida Y, Tsubata T, Yagita H and Honjo T: Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. Int Immunol 8: 765-772, 1996.
20. Nishimura H and Honjo T: PD-1: An inhibitory immunoreceptor involved in peripheral tolerance. Trends Immunol 22: 265-268, 2001.
21. Watanabe N, Gavrieli M, Sedy JR, Yang J, Fallarino F, Loftin SK, Hurchla MA, Zimmerman N, Sim J, Zang X, *et al*: BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. Nat Immunol 4: 670-679, 2003.
22. Keir ME, Butte MJ, Freeman GJ and Sharpe AH: PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol 26: 677-704, 2008.
23. Brahmer J, Reckamp KL, Baas P, Crinò L, Eberhardt WE, Poddubskaya E, Antonia S, Pluzanski A, Vokes EE, Holgado E, *et al*: Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. N Engl J Med 373: 123-135, 2015.
24. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, Chow LQ, Vokes EE, Felip E, Holgado E, *et al*: Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. N Engl J Med 373: 1627-1639, 2015.
25. Herbst RS, Baas P, Kim DW, Felip E, Pérez-Gracia JL, Han JY, Molina J, Kim JH, Arvis CD, Ahn MJ, *et al*: Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): A randomised controlled trial. Lancet 387: 1540-1550, 2016.
26. Afreen S and Dermime S: The immunoinhibitory B7-H1 molecule as a potential target in cancer: Killing many birds with one stone. Hematol Oncol Stem Cell Ther 7: 1-17, 2014.
27. Boland JM, Kwon ED, Harrington SM, Wampfler JA, Tang H, Yang P and Aubry MC: Tumor B7-H1 and B7-H3 expression in squamous cell carcinoma of the lung. Clin Lung Cancer 14: 157-163, 2013.
28. Konishi J, Yamazaki K, Azuma M, Kinoshita I, Dosaka-Akita H and Nishimura M: B7-H1 expression on non-small cell lung cancer cells and its relationship with tumor-infiltrating lymphocytes and their PD-1 expression. Clin Cancer Res 10: 5094-5100, 2004.
29. Chen YY, Wang LB, Zhu HL, Li XY, Zhu YP, Yin YL, Lü FZ, Wang ZL and Qu JM: Relationship between programmed death-ligand 1 and clinicopathological characteristics in non-small cell lung cancer patients. Chin Med Sci J 28: 147-151, 2013.
30. Mu CY, Huang JA, Chen Y, Chen C and Zhang XG: High expression of PD-L1 in lung cancer may contribute to poor prognosis and tumor cells immune escape through suppressing tumor infiltrating dendritic cells maturation. Med Oncol 28: 682-688, 2011.
31. Akbay EA, Koyama S, Carretero J, Altabef A, Tchaicha JH, Christensen CL, Mikse OR, Cherniack AD, Beauchamp EM, Pugh TJ, *et al*: Activation of the PD-1 pathway contributes to immune escape in EGFR-driven lung tumors. Cancer Discov 3: 1355-1363, 2013.
32. Azuma K, Ota K, Kawahara A, Hattori S, Iwama E, Harada T, Matsumoto K, Takayama K, Takamori S, Kage M, *et al*: Association of PD-L1 overexpression with activating EGFR mutations in surgically resected nonsmall-cell lung cancer. Ann Oncol 25: 1935-1940, 2014.
33. Chen N, Fang W, Zhan J, Hong S, Tang Y, Kang S, Zhang Y, He X, Zhou T, Qin T, *et al*: Upregulation of PD-L1 by EGFR activation mediates the immune escape in EGFR-driven NSCLC: Implication for optional immune targeted therapy for NSCLC patients with EGFR mutation. J Thorac Oncol 10: 910-923, 2015.
34. Dronca RS, Liu X, Harrington SM, Chen L, Cao S, Kottschade LA, McWilliams RR, Block MS, Nevala WK, Thompson MA, *et al*: T cell Bim levels reflect responses to anti-PD-1 cancer therapy. JCI Insight 1: 1, 2016.
35. Joseph RW, Parasramka M, Eckel-Passow JE, Serie D, Wu K, Jiang L, Kalari K, Thompson RH, Huu Ho T, Castle EP, *et al*: Inverse association between programmed death ligand 1 and genes in the VEGF pathway in primary clear cell renal cell carcinoma. Cancer Immunol Res 1: 378-385, 2013.
36. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A and Speleman F: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: RESEARCH0034, 2002.
37. Wong ML and Medrano JF: Real-time PCR for mRNA quantitation. Biotechniques 39: 75-85, 2005.
38. D'Incecco A, Andreozzi M, Ludovini V, Rossi E, Capodanno A, Landi L, Tibaldi C, Minuti G, Salvini J, Coppi E, *et al*: PD-1 and PD-L1 expression in molecularly selected non-small-cell lung cancer patients. Br J Cancer 112: 95-102, 2015.
39. Tang Y, Fang W, Zhang Y, Hong S, Kang S, Yan Y, Chen N, Zhan J, He X, Qin T, *et al*: The association between PD-L1 and EGFR status and the prognostic value of PD-L1 in advanced non-small cell lung cancer patients treated with EGFR-TKIs. Oncotarget 6: 14209-14219, 2015.
40. Lin C, Chen X, Li M, Liu J, Qi X, Yang W, Zhang H, Cai Z, Dai Y and Ouyang X: Programmed death-ligand 1 expression predicts tyrosine kinase inhibitor response and better prognosis in a cohort of patients with epidermal growth factor receptor mutation-positive lung adenocarcinoma. Clin Lung Cancer 16: e25-e35, 2015.
41. Gong Y, Somwar R, Politi K, Balak M, Chmielecki J, Jiang X and Pao W: Induction of BIM is essential for apoptosis triggered by EGFR kinase inhibitors in mutant EGFR-dependent lung adenocarcinomas. PLoS Med 4: e294, 2007.
42. Gibbons RM, Liu X, Pulko V, Harrington SM, Krco CJ, Kwon ED and Dong H: B7-H1 limits the entry of effector CD8(+) T cells to the memory pool by upregulating Bim. Oncoimmunology 1: 1061-1073, 2012.
43. Huang Y, Goel S, Duda DG, Fukumura D and Jain RK: Vascular normalization as an emerging strategy to enhance cancer immunotherapy. Cancer Res 73: 2943-2948, 2013.
44. Wei J, Wu A, Kong LY, Wang Y, Fuller G, Fokt I, Melillo G, Priebe W and Heimberger AB: Hypoxia potentiates glioma-mediated immunosuppression. PLoS One 6: e16195, 2011.
45. Ott PA, Hodi FS and Buchbinder EI: Inhibition of immune checkpoints and vascular endothelial growth factor as combination therapy for metastatic melanoma: An overview of rationale, preclinical evidence, and initial clinical data. Front Oncol 5: 202, 2015.
46. Huang Y, Yuan J, Righi E, Kamoun WS, Ancukiewicz M, Nezivar J, Santosuosso M, Martin JD, Martin MR, Vianello F, *et al*: Vascular normalizing doses of antiangiogenic treatment reprogram the immunosuppressive tumor microenvironment and enhance immunotherapy. Proc Natl Acad Sci USA 109: 17561-17566, 2012.
47. Zou W and Chen L: Inhibitory B7-family molecules in the tumour microenvironment. Nat Rev Immunol 8: 467-477, 2008.
48. Chen J, Jiang CC, Jin L and Zhang XD: Regulation of PD-L1: A novel role of pro-survival signalling in cancer. Ann Oncol 27: 409-416, 2016.
49. Okita R, Maeda A, Shimizu K, Nojima Y, Saisho S and Nakata M: PD-L1 overexpression is partially regulated by EGFR/HER2 signaling and associated with poor prognosis in patients with non-small-cell lung cancer. Cancer Immunol Immunother 66: 865-876, 2017.
50. Shimoji M, Shimizu S, Sato K, Suda K, Kobayashi Y, Tomizawa K, Takemoto T and Mitsudomi T: Clinical and pathologic features of lung cancer expressing programmed cell death ligand 1 (PD-L1). Lung Cancer 98: 69-75, 2016.
51. Han JJ, Kim DW, Koh J, Keam B, Kim TM, Jeon YK, Lee SH, Chung DH and Heo DS: Change in PD-L1 expression after acquiring resistance to gefitinib in EGFR-mutant non-small-cell lung cancer. Clin Lung Cancer 17: 263-270.e262, 2016.