PD-L1 mRNA expression in EGFR-mutant lung adenocarcinoma

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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 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.

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) EGFR 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 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'-CGTCTCTCCAAAAATGTGTATCA-3' and reverse, 5'-TGCTATTCTGGGACCCATC-3'; BIM forward, 5'-AGGCCAAAGTGAATCTCTGAA-3' and reverse, 5'-ATACCACTGGAAGTGTTCG-3'; BIM-L forward, 5'-GAAGAGCCGCAAGACAGGGA-3' and reverse, 5'-GGAGTGTGTTAAGGTGCTGA-3'; BIM-S forward, 5'-AGACAGGACAAAGCCTTC-3' and reverse, 5'-TGCTAGTACTAAGCGTTAAACTCG-3'; BIM-EL forward, 5'-TCACAGGTACCGGGATGAAGACAC-3' and reverse, 5'-CAAAGCAGACGACATGTCTCTGAAG-3'; BIM forward, 5'-TGTTGCGGGGACAAGTCTAGTCAGCA-3' and reverse, 5'-CCTATTGGGGACGCTTGA-3'; MET forward, 5'-TCCCTCAACAGGACACTACAACCTT-3' and reverse, 5'-GCTGCAGGTATAGGCAGTGTAACA-3'; and GAPDH forward, 5'-GCACCCTGAAGGCGTGCAAC-3' and reverse, 5'-TGGTGAAGACCGCAGCTGGA-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, VEGFA, 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 treatment (35,36). The targets were those who had PD-L1 expression and those who did not. 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 imaging. The PFS and overall survival (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 as 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...
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), 

\[ EGF R \] mutation at primary site

19del
L858R
G719C

Line of gefitinib therapy
First
Second
Third

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

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
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<tbody>
<tr>
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<td>19del</td>
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<tr>
<td>First</td>
<td>16</td>
</tr>
<tr>
<td>Second</td>
<td>16</td>
</tr>
<tr>
<td>Third</td>
<td>1</td>
</tr>
</tbody>
</table>

ECOG, Eastern Cooperative Oncology Group; Rec, recurrence after surgical resection; Ad, adenocarcinoma; \[ EGF R \], epidermal growth factor receptor; L858R, exon 21 L858R; 19del, exon 19 deletion; G719C, exon 18 G719C.

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

<table>
<thead>
<tr>
<th>PD-L1 expression</th>
<th>N</th>
<th>%</th>
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<tr>
<td>Intratumoral</td>
<td>11</td>
<td>33.3</td>
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<tr>
<td>Paratumoral</td>
<td>14</td>
<td>42.4</td>
</tr>
<tr>
<td>Absent</td>
<td>14</td>
<td>43.4</td>
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</table>

*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.](image1)

![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.](image2)
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, VEGFA, 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 (Fig. 5).
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).
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 VEGFA 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.

### Table V. Indicators of shorter PFS after gefitinib treatment.

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

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


