Induction of PD-L1 Expression by the EML4–ALK Oncoprotein and Downstream Signaling Pathways in Non–Small Cell Lung Cancer

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Abstract

Purpose: Therapies targeted to the immune checkpoint mediated by PD-1 and PD-L1 show antitumor activity in a subset of patients with non–small cell lung cancer (NSCLC). We have now examined PD-L1 expression and its regulation in NSCLC positive for the EML4–ALK fusion gene.

Experimental Design: The expression of PD-L1 at the protein and mRNA levels in NSCLC cell lines was examined by flow cytometry and by reverse transcription and real-time PCR analysis, respectively. The expression of PD-L1 in 134 surgically resected NSCLC specimens was evaluated by immunohistochemical analysis.

Results: The PD-L1 expression level was higher in NSCLC cell lines positive for EML4–ALK than in those negative for the fusion gene. Forced expression of EML4–ALK in Ba/F3 cells markedly increased PD-L1 expression, whereas endogenous PD-L1 expression in EML4–ALK–positive NSCLC cells was attenuated by treatment with the specific ALK inhibitor alectinib or by RNAi with ALK siRNAs. Furthermore, expression of PD-L1 was downregulated by inhibitors of the MEK–ERK and PI3K–AKT signaling pathways in NSCLC cells positive for either EML4–ALK or activating mutations of the EGFR. Finally, the expression level of PD-L1 was positively associated with the presence of EML4–ALK in NSCLC specimens.

Conclusions: Our findings that both EML4–ALK and mutant EGFR upregulate PD-L1 by activating PI3K–AKT and MEK–ERK signaling pathways in NSCLC reveal a direct link between oncogenic drivers and PD-L1 expression. Clin Cancer Res; 21(17); 4014–21. ©2015 AACR.
Translational Relevance
Therapies targeted to the immune checkpoint mediated by PD-L1 and PD-L1 show promising efficacy for various tumors. A high PD-L1 expression level in tumors is thought to be associated with poor clinical prognosis as well as with the response to such targeted therapies. The regulation of PD-L1 expression in tumors has remained ill-defined, however. Activating mutations of the EGFR, which are responsible for a subset of non–small-cell lung cancer (NSCLC), have been found to induce PD-L1 expression. We now show that PD-L1 expression is induced by the EML4–ALK fusion gene, which defines another molecular subset of NSCLC. Furthermore, EML4–ALK and mutant EGFR modulate PD-L1 expression via common downstream signaling pathways mediated by PI3K–AKT and by MEK–ERK. Our findings suggest that oncogenic drivers induce immune escape in NSCLC by upregulating PD-L1, and they provide a basis for the development of therapies targeted to PD–1–PD-L1 for such oncogene-driven tumors.

Materials and Methods

Cell culture and reagents
PC9, 1-87, and LK87 cells were obtained as previously described (20, 28). HCC827, H1975, H1650, H2228, H322, A549, H23, H1212, H1437, H1573, H1944, H157, H460, and H1299 cells were obtained from the ATCC, and LL18 cells were kindly provided by Y. Maehara (Kyushu University). H596 cells were from Lonza. Isolated Ba/F3 cells were obtained as previously described (29). A Cell Line Nucleofector Kit V was obtained from Lonza. Isolated Ba/F3 cells (2 × 10⁶) were suspended in 100 μL of Nucleofector solution containing 2 μg of plasmid DNA and were then subjected to electroporation according to program X-001 with the use of a Nucleofector II Device (Amaxa Biosystems).

Plasmid transfection
An expression vector for EML4–ALK was established as previously described (29). A Cell Line Nucleofector Kit V was obtained from Lonza. Isolated Ba/F3 cells (2 × 10⁶) were suspended in 100 μL of Nucleofector solution containing 2 μg of plasmid DNA and were then subjected to electroporation according to program X-001 with the use of a Nucleofector II Device (Amaxa Biosystems).

RNA interference
Cells were plated at 50% to 60% confluence in 6-well plates or 25-cm² flasks, and then incubated for 24 hours before transient transfection for 48 hours with siRNAs mixed with the Lipofectamine reagent (Invitrogen). The siRNAs specific for ALK mRNA (ALK-1, 5'-ACACCCCAAAUUAIAICCAAC-3'; ALK-2, 5'-UCACCAAAIUIUCAACACCA-3') as well as a nonspecific siRNA (5'-GUIGAGAGAIAlUIUGACGIU-3') were obtained from Nippon EGT. All data presented were obtained with the ALK-1 siRNA, but similar results were obtained with the ALK-2 siRNA.

Flow cytometric analysis
Cells were stained with biotinylated monoclonal antibodies to human PD-L1 or to mouse PD-L1 (eBioscience) as well as with phycoerythrin-labeled streptavidin (BD Biosciences) for flow cytometric analysis with a FACS Calibur instrument equipped with CELLQuest software (BD Biosciences).

RT and real-time PCR analysis
Total RNA was extracted from the use of an RNaseasy Mini Kit (Qiagen) and was subjected to reverse transcription (RT) with the use of PrimeScript RT Master Mix (Takara). The resulting cDNA was then subjected to real-time PCR analysis with the use of SYBR Premix Ex Taq II (Takara) and a Thermal Cycler Dice Real Time System II (Takara). The PCR primers (forward and reverse, respectively) included those for PD-L1 (5'-CAATGTGACCATGGACTGAAA-3' and 5'-GGCATATGAATGGTGTCCCGAGA-3') and 18S rRNA (5'-ACTCAACACGGGAAACCTCA-3' and 5'-AACACAGACAAATCGCTCCAC-3'). The amount of PD-L1 mRNA was normalized by that of 18S rRNA.

Immunoblot analysis
Immunoblot analysis was performed as previously described (28). Rabbit polyclonal antibodies to human phosphorylated ALK (Y1604), to ALK, to phosphorylated EGFR (Y1068), to EGFR, to phosphorylated AKT, to AKT, to phosphorylated ERK, to ERK, to phosphorylated STAT3, to STAT3, and to β-actin were obtained from Cell Signaling Technology. All antibodies were used at a 1:1,000 dilution. Horseradish peroxidase–conjugated goat antibodies to rabbit immunoglobulin G were obtained from Abcam. Immune complexes were detected with the use of Pierce Western Blotting Substrate Plus (Thermo Scientific) and the ChemiDoc XRS system (Bio-Rad).

Patients and ALK fusion analysis
We screened consecutive patients who underwent surgical resection of their NSCLCs between January 1998 and December 2010 at Kumue University Hospital. In the present study, we included only patients who had not been treated with chemotherapy or radiotherapy before surgery and whose tumors were wild-type for EGFR and were evaluated for ALK status. ALK fusions were identified by fluorescence in situ hybridization. The present study conforms to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board. All patients provided written informed consent.

Immunohistochemical analysis of PD-L1 expression in tumor specimens
Surgical specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections were dehydrated in paraffin with xylene, rehydrated with a graded series of ethanol solutions, and treated with H₂O₂ in methanol to block endogenous peroxidase activity. Immunohistochemical analysis of PD-L1 expression in the human tumor specimens was performed as previously described (20). Each slide was incubated for 30 minutes with rabbit polyclonal antibodies to human PD-L1 (Lifespan Biosciences). The intensity of staining was evaluated according to the following scale: 0, no staining; 1, weak staining; 2, moderate...
staining; and 3, strong staining. The proportion of all tumor cells found to express PD-L1 was determined and then multiplied by the staining intensity score to obtain a final semiquantitative H-score (maximum value of 300 corresponding to 100% of tumor cells positive for PD-L1 with an overall staining intensity score of 3). All immunohistochemical images were evaluated by two experienced observers (A. Kawahara and M. Kage) who were unaware of the identity of the specimens, and the mean of the two determinations was used for further analysis.

Statistical analysis
For in vitro studies, quantitative data are presented as means ± SD (unless indicated otherwise). Data analyses were performed with GraphPad Prism for Windows (GraphPad Software). The relation between PD-L1 expression in tumor specimens and other patient characteristics, including ALK fusion status, was examined with the use of the Wilcoxon rank-sum test two-sided as performed with JMP 10 software (SAS Institute). A P value of <0.05 was considered statistically significant.

Results
PD-L1 mRNA and protein abundance in NSCLC cell lines according to EGFR and ALK status
We first measured the surface expression of PD-L1 and PD-L1 mRNA abundance in NSCLC cell lines whose EGFR mutation and ALK fusion status had been previously determined (Table 1). Five of the cell lines (HCC827, H1975, PC9, 11_18, and H1650) harbor EGFR mutations and two (H2228 and H3122) harbor the EML4–ALK fusion gene, whereas the other lines examined are

Table 1. Cell surface expression of PD-L1 and PD-L1 mRNA abundance in lung cancer cell lines classified according to oncogene status and histology

<table>
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<tr>
<th>Cell line</th>
<th>EGFR status</th>
<th>ALK status</th>
<th>Histology</th>
<th>PD-L1 mRNA*</th>
<th>Surface PD-L1†</th>
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<tr>
<td>HCC827</td>
<td>del(E746–A750)</td>
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<td>L858R + T790M</td>
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<td>PC9</td>
<td>del(E746–A750)</td>
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<td>Adeno</td>
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</tr>
<tr>
<td>11_18</td>
<td>L858R</td>
<td>Wild-type</td>
<td>Adeno</td>
<td>9.54</td>
<td>130.18</td>
</tr>
<tr>
<td>H1650</td>
<td>del(E746–A750)</td>
<td>Wild-type</td>
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<tr>
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<td>H3122</td>
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<td>H596</td>
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<td>Wild-type</td>
<td>Wild-type</td>
<td>Large cell</td>
<td>0.95</td>
<td>54.44</td>
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</table>

Abbreviation: Adeno, adenocarcinoma.

*PD-L1 mRNA abundance was determined by RT and real-time PCR analysis and was normalized by that of 18S rRNA.
†Surface expression of PD-L1 was determined by flow cytometry and is presented as MFI values.

Figure 1.
PD-L1 surface expression and mRNA abundance in NSCLC cell lines according to EGFR and ALK status. A, expression of PD-L1 at the cell surface in a panel of NSCLC cell lines, including five harboring EGFR mutations, two positive for EML4–ALK fusion, and 13 wild-type for EGFR and ALK was determined by flow cytometry. Each point represents the MFI for PD-L1 in one cell line. The mean value for PD-L1 expression in each group of cell lines is indicated by the horizontal bar. Each determination was repeated at least twice. B, PD-L1 mRNA abundance in the NSCLC cell lines was determined by quantitative RT-PCR analysis and normalized by that of 18S rRNA. Each point corresponds to the normalized value for PD-L1 mRNA level in one cell line, with the mean value for each group of cell lines being indicated by the horizontal bar.
wild-type for both *EGFR* and *ALK*. Consistent with our previous results (20), flow cytometric analysis revealed that the surface expression of PD-L1 in cell lines with *EGFR* mutations was higher than that in most lines that are wild-type for *EGFR* and *ALK*, with average mean fluorescence intensity (MFI) values of 265 versus 48 (Fig. 1A). In both *EML4–ALK*–positive cell lines, the surface expression of PD-L1 was also greater than that in the lines with wild-type *EGFR* and *ALK*, with average MFI values of 253 versus 48 (Fig. 1A; Supplementary Fig. S1). The results of quantitative RT-PCR analysis of PD-L1 mRNA were consistent with those of flow cytometric analysis. The abundance of PD-L1 mRNA was, thus, also greater in cells harboring *EML4–ALK* than in most of those that are wild-type for *EGFR* and *ALK*, with mean normalized values of 11 versus 2 (Fig. 1B). Whereas two wild-type cell lines (H157 and H596) with an adenosquamous histology showed higher levels of PD-L1 mRNA and surface protein compared with the other wild-type cell lines, these data overall suggested that increased expression of PD-L1 is associated with *ALK* fusion as well as with *EGFR* mutation.

**PD-L1 expression is induced by the EML4–ALK fusion protein**

To investigate the effect of *EML4–ALK* rearrangement on PD-L1 expression, we transfected Ba/F3 cells with an *EML4–ALK* expression vector. Immunoblot analysis with antibodies to phosphorylated or total forms of ALK confirmed the expression and phosphorylation of EML4–ALK in the transfected cells (Fig. 2A). Quantitative RT-PCR analysis revealed that *EML4–ALK* expression resulted in a marked increase in the abundance of PD-L1 mRNA in Ba/F3 cells. To exclude the possibility that this finding was the result of a nonspecific effect of transfection, we examined the action of alectinib, a potent and selective inhibitor of ALK. Alectinib blocked EML4–ALK phosphorylation, and it reduced

Figure 2.

Effect of EML4–ALK on PD-L1 expression. A, left, Ba/F3 cells were transfected with an expression plasmid for EML4–ALK, cultured for 6 hours, and then incubated in the presence of alectinib (60 nmol/L) or DMSO vehicle for 18 hours. The cells were then lysed and subjected to immunoblot analysis with antibodies to phosphorylated ALK (pALK), to total ALK, and to β-actin (loading control). Parental Ba/F3 cells were examined as a control. The bands detected by the antibodies to phosphorylated or total ALK correspond to the EML4–ALK fusion protein. Middle, RT and real-time PCR analysis of PD-L1 mRNA in cells treated as in the left. Normalized data are expressed relative to the value for parental Ba/F3 cells, are means ± SD of triplicates, and are representative of three independent experiments. Right, flow cytometric analysis of PD-L1 surface expression in cells treated as in the left. B, left, H2228 cells were incubated in complete medium with or without 60 nmol/L alectinib for 24 hours, after which cell lysates were subjected to immunoblot analysis as in A, Middle, RT and real-time PCR analysis of PD-L1 mRNA in cells treated as in the left. Data are representative of three independent experiments. Right, flow cytometric analysis of PD-L1 surface expression in cells treated as in the left.

C, left, H3122 and H2228 cells were transfected with a nontargeting (NT) siRNA or a siRNA specific for *ALK* mRNA for 48 hours, after which cell lysates were subjected to immunoblot analysis as in A, Middle, RT and real-time PCR analysis of PD-L1 mRNA in cells treated as in the left. Data are representative of three independent experiments. Right, flow cytometric analysis of PD-L1 surface expression in cells treated as in the left.
the amount of PD-L1 mRNA in Ba/F3 cells expressing EML4–ALK almost to the level of that in parental Ba/F3 cells. Flow cytometric analysis revealed that EML4–ALK also increased the level of PD-L1 expression at the surface of Ba/F3 cells and that this effect was markedly inhibited by alectinib. These results indicated that the EML4–ALK fusion protein upregulates PD-L1 expression at the mRNA and protein levels.

We next examined the effect of alectinib on endogenous PD-L1 expression in EML4–ALK–positive cell lines (Fig. 2B). Alectinib blocked EML4–ALK phosphorylation in H2228 cells, and this effect was accompanied by downregulation of the amounts of PD-L1 mRNA and surface protein. We further investigated the effect of EML4–ALK depletion by RNAi on PD-L1 expression in H3122 and H2228 cells (Fig. 2C). Immunoblot analysis confirmed that transfection of these cells with an ALK-targeted siRNA resulted in depletion of the fusion protein. Knockdown of EML4–ALK resulted in marked decreases in both the abundance of PD-L1 mRNA and PD-L1 surface expression in both H3122 and H2228 cells. We obtained similar results with a second siRNA targeted to a different sequence within EML4–ALK mRNA (data not shown). Together, these findings indicated that PD-L1 expression is increased as a result of the increased ALK tyrosine kinase activity in cells positive for EML4–ALK rearrangement.

Inhibition of PI3K–AKT or MEK–ERK signaling downregulates PD-L1 expression in NSCLC cells harboring EGFR mutations or EML4–ALK

We found that PD-L1 expression is induced by the EML4–ALK fusion protein in NSCLC cell lines. To identify the downstream signaling pathways that regulate PD-L1 expression in cells with either EGFR mutations or ALK fusions, we first examined the effects of specific inhibitors of PI3K (LY294002), the ERK kinase MEK (U0126), and STAT3 (S3I-201) on PD-L1 expression in HCC827 and H3122 cells, which harbor mutated EGFR and the EML4–ALK fusion gene, respectively. Alectinib inhibited EML4–ALK phosphorylation as well as phosphorylation of the downstream targets STAT3, AKT, and ERK in H3122 cells.
cells, whereas the EGFR–TKI erlotinib attenuated EGFR phosphorylation as well as phosphorylation of STAT3, AKT, and ERK in HCC827 cells (Fig. 3A). Erlotinib also downregulated PD-L1 expression at the mRNA and surface protein levels in HCC827 cells, similar to the action of alectinib in H3122 cells (Fig. 3B and C). The PI3K inhibitor LY294002 and the MEK inhibitor U0126 similarly downregulated PD-L1 expression as well as attenuated AKT and ERK phosphorylation, respectively, in both HCC827 and H3122 cells (Fig. 3). In contrast, the STAT3 inhibitor S3I-201 attenuated STAT3 phosphorylation, but had no effect on PD-L1 expression at the mRNA or surface protein level in HCC827 and H3122 cells (Fig. 3). We further investigated the effects of LY294002 and U0126 on PD-L1 expression in Ba/F3 cells transfected with the EML4–ALK expression vector. Treatment of the transfected cells with LY294002 or U0126 resulted in downregulation of PD-L1 mRNA abundance (Supplementary Fig. S2). To exclude the possibility that these results were due to nonspecific effects of LY294002 or U0126, we transfected H3122 cells with siRNAs specific for AKT or ERK mRNAs. Immunoblot analysis confirmed that transfection of the cells with each siRNA resulted in the marked and selective depletion of the targeted protein (Supplementary Fig. S3A). Consistent with the effects of LY294002 and U0126, the amount of PD-L1 mRNA was suppressed as a result of AKT or ERK depletion (Supplementary Fig. S3B). Overall, these data indicated that PD-L1 expression is regulated by the PI3K–AKT and MEK–ERK signaling pathways in both EGFR mutation–positive cells and ALK fusion–positive cells.

Immunohistochemical analysis of PD-L1 expression in tumor specimens

Finally, we performed immunohistochemical analysis to examine PD-L1 expression in ALK rearrangement–positive NSCLC specimens (Fig. 4A). A high-expression level of
PD-L1 has previously been associated with the presence of EGFR mutations (20), with such mutations and EML4–ALK fusions having been found to be mutually exclusive. We screened tumor specimens from 134 patients with NSCLC wild-type for EGFR, including 11 individuals harboring EML4–ALK (Supplementary Table S1). Consistent with previous observations (20), expression of PD-L1 was significantly higher in tumors from women than in those from men (P < 0.001), in those with an adenocarcinoma histology than in those with a squamous cell carcinoma histology (P < 0.001), and in those from never-smokers than in those from smokers (P < 0.001). PD-L1 expression was also significantly higher in tumors positive for ALK rearrangement than in those wild-type for ALK and EGFR (P < 0.001; Fig. 4B). These results, thus, revealed that a high PD-L1 expression level in resected NSCLC tumors was positively associated with the presence of EML4–ALK.

Discussion

Accumulating evidence suggests that PD-L1 is expressed in many types of human cancer, including NSCLC (2–5). The precise mechanisms by which PD-L1 expression is regulated have remained unclear, however. We have now found that PD-L1 is expressed at a higher level in NSCLC cells positive for EML4–ALK (Supplementary Table S1). Consistent with previous observations (20), expression of PD-L1 was significantly higher in tumors from women than in those from men (P < 0.001), in those with an adenocarcinoma histology than in those with a squamous cell carcinoma histology (P < 0.001), and in those from never-smokers than in those from smokers (P < 0.001). PD-L1 expression was also significantly higher in tumors positive for ALK rearrangement than in those wild-type for ALK and EGFR (P < 0.001; Fig. 4B). These results, thus, revealed that a high PD-L1 expression level in resected NSCLC tumors was positively associated with the presence of EML4–ALK.

Expression of PD-L1 was previously shown to be induced via the MEK–ERK or STAT3 signaling pathway in several hematologic neoplasms (30–33). PD-L1 expression in various solid tumors was also found to be increased as a result of activation of the PI3K–AKT pathway due to dysfunction of phosphatase and tensin homolog [PTEN; refs. 34–37]. Given that the EML4–ALK tyrosine kinase activates various downstream signaling pathways, including those mediated by STAT3, MEK–ERK, or PI3K–AKT (22, 38), we focused on the possible role of these three major pathways in the induction of PD-L1 expression in NSCLC positive for ALK rearrangement. With the use of specific inhibitors of MEK and PI3K, we found that PD-L1 expression is upregulated via MEK–ERK and PI3K–AKT signaling in EML4–ALK fusion–positive NSCLC cells. In contrast, a STAT3 inhibitor had no substantial effect on PD-L1 expression in such cells. This latter finding differs from the previous observation that PD-L1 expression was induced through MEK–ERK and STAT3 pathways in NPM–ALK fusion–positive anaplastic large cell lymphoma (32, 33), suggesting that PD-L1 expression in ALK fusion–positive tumors is modulated via different signaling pathways in a manner dependent on tumor cell type or the fusion partner of ALK. We and others recently revealed that mutant forms of EGFR also induce expression of PD-L1, although the downstream signaling pathways of EGFR responsible for this effect were not identified (19, 20). We now show that MEK–ERK and PI3K–AKT pathways regulate PD-L1 expression in EGFR mutation–positive NSCLC cells, indicating that two distinct oncogenes, EGFR and ALK, share common signaling pathways for regulation of PD-L1 expression in NSCLC.

In conclusion, with the use of cell lines and human tumor specimens, we have identified EML4–ALK as a key determinant of PD-L1 expression in NSCLC. We further showed that both EML4–ALK and mutant forms of EGFR modulate PD-L1 expression via common downstream pathways mediated by MEK–ERK and by PI3K–AKT. Our findings, thus, indicate that oncogenic drivers play a direct role in induction of PD-L1 expression and thereby contribute to immune escape in NSCLC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: K. Matsumoto, T. Hoshino, I. Okamoto
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Ota, K. Azuma, A. Kawahara, S. Takamori, I. Okamoto
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Ota, S. Hattori, E. Iwama, T. Harada, K. Matsumoto, I. Okamoto
Writing, review, and/or revision of the manuscript: K. Ota, E. Iwama, T. Hoshino, Y. Nakamichi, I. Okamoto
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Iwama, J. Tanizaki, M. Kake, Y. Nakamichi, I. Okamoto
Study supervision: T. Harada, K. Takayama, Y. Nakamishi, I. Okamoto

Acknowledgments

The authors thank A. Sato for technical assistance.

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Received January 6, 2015; revised May 11, 2015; accepted May 13, 2015; published OnlineFirst May 27, 2015.

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