Clinical Cancer Research

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

Keiichi Ota[1], Koichi Azuma[2], Akihiko Kawahara[3], Satoshi Hattori[4], Eiji Iwama[1,5], Junko Tanizaki[6], Taishi Harada[1], Koichiro Matsumoto[1], Koichi Takayama[1], Shinzo Takamori[7], Masayoshi Kage[3], Tomoaki Hoshino[2], Yoichi Nakanishi[1,8], and Isamu Okamoto[1,8]

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.*

Introduction

The interaction of programmed cell death–ligand 1 (PD-L1, also known as B7-H1 or CD274) expressed on antigen-presenting cells and parenchymal cells with its receptor programmed cell death–1 (PD-1, also known as CD279) on T cells is a physiologic mechanism underlying escape from immune activity (1). PD-L1 is also expressed by various human tumors, resulting in inhibition of the immune response to the cancer cells and consequent facilitation of tumor progression and metastasis (2–5). A high level of PD-L1 expression in several types of malignancy has been associated with a poor clinical outcome, although some findings remain controversial (6–9). Recent clinical trials have shown that inhibition of the PD-1–PD-L1 interaction with antibodies specific for these proteins is a promising approach to cancer treatment (10, 11). The expression level of PD-L1 at the tumor cell surface has been found to correlate with the likelihood of a response to PD-1– or PD-L1–targeted therapy (11). The molecular mechanisms responsible for the regulation of PD-L1 expression in tumors remain unclear, however.

Oncogenic drivers play central roles in tumorigenesis as well as in tumor cell survival and proliferation. Non–small cell lung cancer (NSCLC) is at the forefront of molecularly targeted therapy as a result of rapid advances in our understanding of its genetic drivers (12, 13). Mutations of the EGFR gene that result in constitutive activation of the receptor tyrosine kinase have, thus, been identified as oncogenic drivers in a subset of NSCLC tumors (14–16), and tyrosine kinase inhibitors (TKI) specific for EGFR have had a substantial impact on treatment outcome in patients with such tumors (13, 17, 18). We and others recently showed that PD-L1 expression in NSCLC is upregulated as a result of *EGFR* mutation, implicating oncogenic drivers in regulation of the expression of immunosuppressive molecules (19, 20). Chromosomal rearrangement involving the anaplastic lymphoma kinase (ALK) and echinoderm microtubule–associated protein–like 4 (EML4) genes also defines a distinct molecular subset of NSCLC, with the resulting fusion gene manifesting pronounced transforming activity (21, 22). Treatment with ALK-targeted TKIs has also been established for patients with *EML4–ALK* fusion–positive NSCLC (13, 23–27). We have now examined the role of *EML4–ALK* rearrangement in PD-L1 expression in NSCLC cells. In addition, we examined the signaling pathways that modulate PD-L1 expression in NSCLC cells harboring driver oncogenes, including *ALK* fusions and mutated *EGFR*.

[1]Research Institute for Diseases of the Chest, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan. [2]Division of Respirology, Neurology, and Rheumatology, Department of Internal Medicine, Kurume University School of Medicine, Fukuoka, Japan. [3]Department of Diagnostic Pathology, Kurume University Hospital, Fukuoka, Japan. [4]Biostatistics Center, Kurume University, Fukuoka, Japan. [5]Department of Comprehensive Clinical Oncology, Faculty of Medical Sciences, Kyushu University, Fukuoka, Japan. [6]Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts. [7]Department of Surgery, Kurume University School of Medicine, Fukuoka, Japan. [8]Center for Clinical and Translational Research, Kyushu University Hospital, Fukuoka, Japan.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Corresponding Author: Isamu Okamoto, Kyushu University Hospital, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Phone: 81-92-642-5378; Fax: 81-92-642-5390; E-mail: okamotoi@kokyu.med.kyushu-u.ac.jp

doi: 10.1158/1078-0432.CCR-15-0016

Translational Relevance

Therapies targeted to the immune checkpoint mediated by PD-1 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, H2122, H1437, H1573, H1944, H157, H460, and H1299 cells were obtained from the ATCC; and 11_18 cells were kindly provided by Y. Maehara (Kyushu University), H596 cells by T. Mitsudomi (Kinki University, Osaka, Japan), and H3122 cells by P. Jänne (Dana-Farber Cancer Institute, Boston, MA). These human NSCLC cell lines were cultured in RPMI-1640 medium (Gibco) or DMEM (Gibco), each supplemented with 10% FBS. Human cell lines were authenticated by short tandem repeat profile using the Cell ID System (Promega). Mouse Ba/F3 cells were obtained from RIKEN BioResource Center Cell Bank and were maintained in RPMI-1640 medium supplemented with 10% FBS and with 10% WEHI-conditioned medium as a source of murine IL3. All cells were maintained under a humidified atmosphere of 5% CO_2 at 37°C. For *in vitro* studies, alectinib (Selleckchem), erlotinib (Cell Signaling Technology), LY294002 (Cell Signaling Technology), U0126 (Cell Signaling Technology), and S3I-201 (Selleckchem) were each dissolved in DMSO (Wako) and stored at −20° or −80°C for a maximum of 2 weeks.

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 (2×10^6) 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-cm^2 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′-ACACCCAAAUUAAUACCAA-3′; ALK-2, 5′-UCAGCAAAUUCAACCACCA-3′) as well as a nonspecific siRNA (5′-GUUGAGAGAUAUUAGAGUU-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 cells with the use of an RNeasy 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′-CAATGTGACCAGCACACTGAGAA-3′ and 5′-GGCATAATAAGATGGCTCCCAGAA-3′) and 18S rRNA (5′-ACTCAACACGGGAAACCTCA-3′ and 5′-AACCAGACAAATCGCTCCAC-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 NSCLC between January 1998 and December 2010 at Kurume 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 depleted of paraffin with xylene, rehydrated with a graded series of ethanol solutions, and treated with H_2O_2 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

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

Cell line	EGFR status	ALK status	Histology	PD-L1 mRNA[a]	Surface PD-L1[b]
HCC827	del(E746–A750)	Wild-type	Adeno	30.93	442.74
H1975	L858R+T790M	Wild-type	Adeno	21.65	355.92
PC9	del(E746–A750)	Wild-type	Adeno	13.41	278.39
11_18	L858R	Wild-type	Adeno	9.54	130.18
H1650	del(E746–A750)	Wild-type	Adeno	3.04	111.44
H2228	Wild-type	EML4–ALKv3	Adeno	11.98	275.88
H3122	Wild-type	EML4–ALKv1	Adeno	9.86	231.03
H322	Wild-type	Wild-type	Adeno	0.75	45.23
A549	Wild-type	Wild-type	Adeno	0.75	28.17
1-87	Wild-type	Wild-type	Adeno	0.57	71.88
LK87	Wild-type	Wild-type	Adeno	0.46	26.89
H23	Wild-type	Wild-type	Adeno	0.42	14.95
H2122	Wild-type	Wild-type	Adeno	1.85	43.93
H1437	Wild-type	Wild-type	Adeno	1.82	26.10
H1573	Wild-type	Wild-type	Adeno	0.76	35.11
H1944	Wild-type	Wild-type	Adeno	2.24	48.13
H157	Wild-type	Wild-type	Adenosquamous	12.61	138.37
H596	Wild-type	Wild-type	Adenosquamous	5.39	80.87
H460	Wild-type	Wild-type	Large cell	1.67	22.53
H1299	Wild-type	Wild-type	Large cell	0.95	54.44

Abbreviation: Adeno, adenocarcinoma.
[a]PD-L1 mRNA abundance was determined by RT and real-time PCR analysis and was normalized by that of 18S rRNA.
[b]Surface expression of PD-L1 was determined by flow cytometry and is presented as MFI values.

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

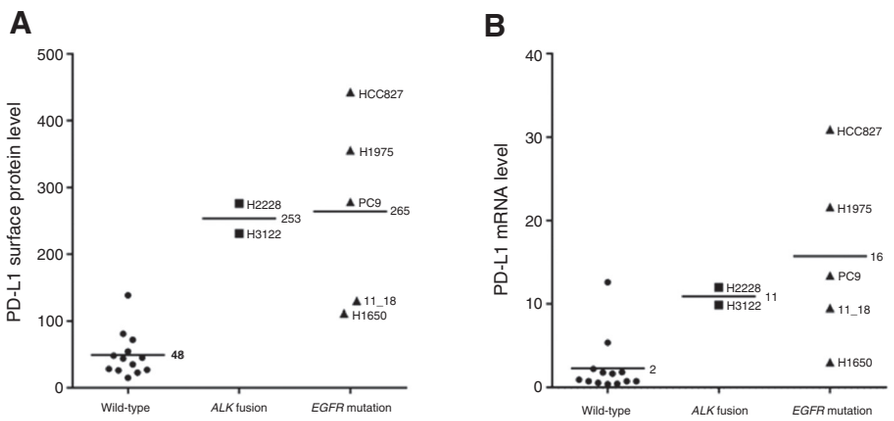

Figure 1.
PD-L1 surface expression and mRNA abundance in NSCLC cell lines according to *EGFR* mutation and *ALK* translocation status. A, expression of PD-L1 at the cell surface in a panel of NSCLC cell lines, including five harboring *EGFR* mutations, two positive for *EML4–ALK* fusion, and 13 wild-type 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

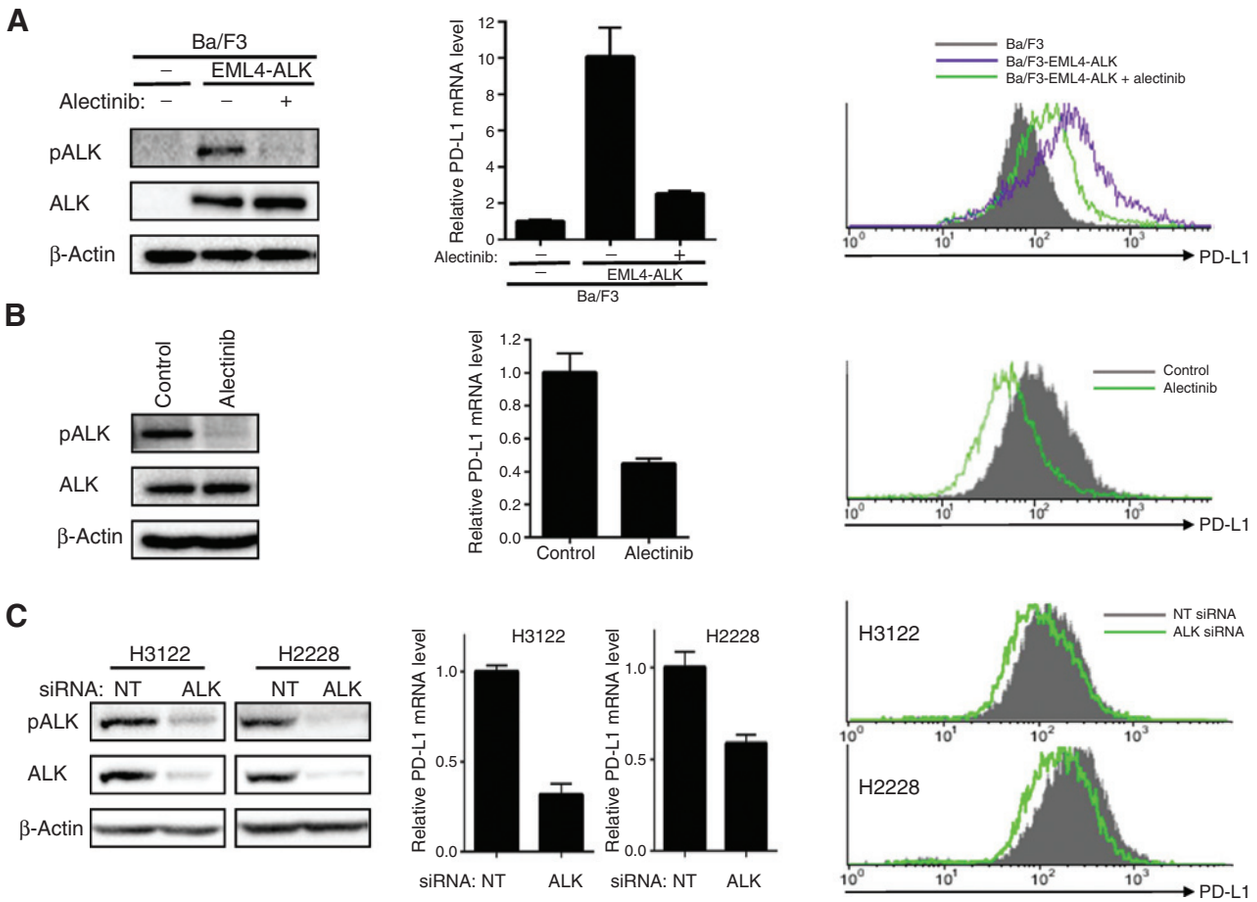

Figure 2.
Effect of EML4–ALK on PD-L1 expression. A, left, Ba/F3 cells were transfected with an expression plasmid for EML4–ALK, cultured for 6 hours, and then incubated in the presence of alectinib (60 nmol/L) or DMSO vehicle for 18 hours. The cells were then lysed and subjected to immunoblot analysis 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

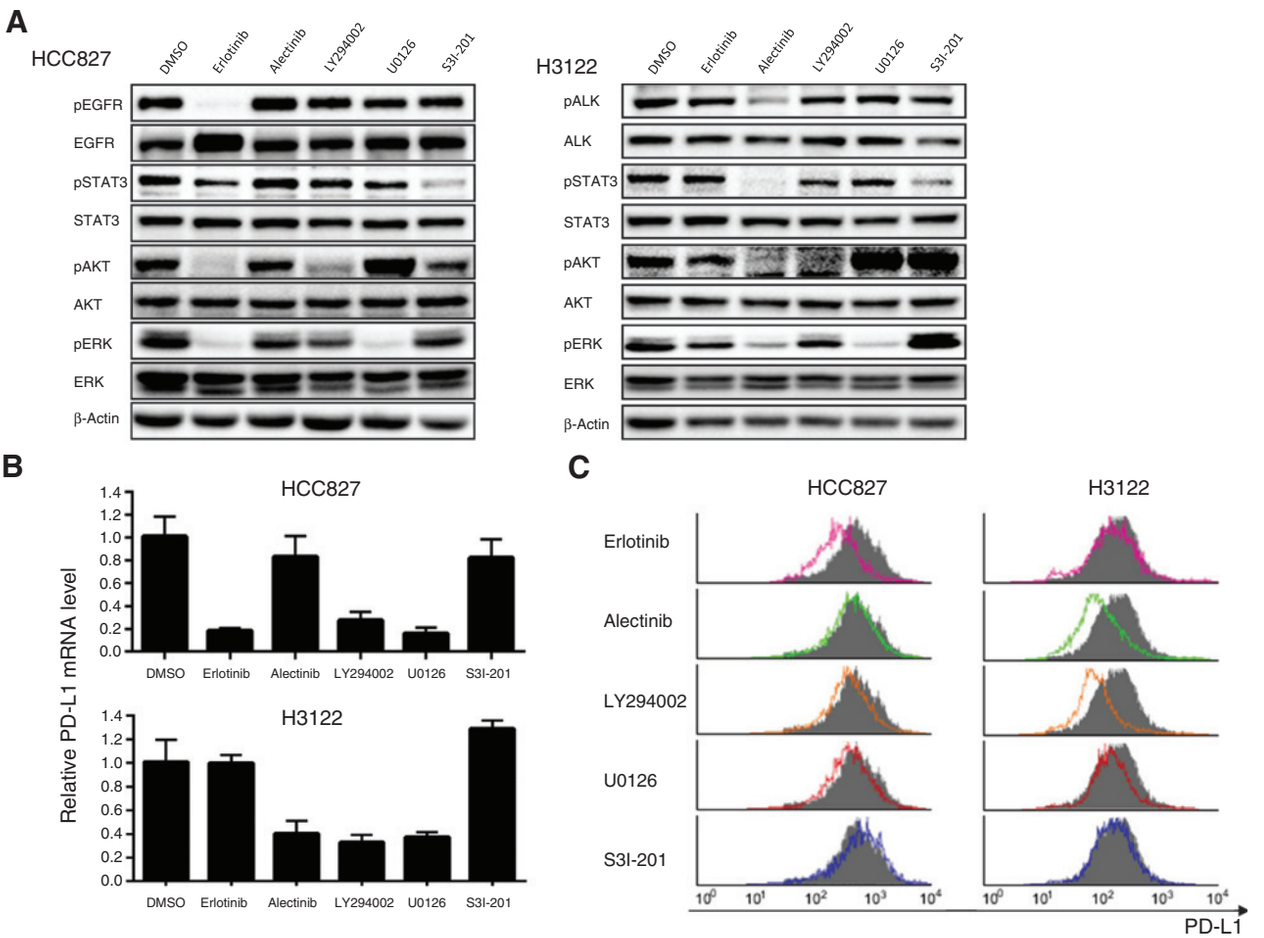

Figure 3.
Effects of inhibitors of PI3K–AKT, MEK–ERK, and STAT3 signaling on PD-L1 expression in *EGFR* mutation–positive (HCC827) and *EML4–ALK* fusion–positive (H3122) cells. A, HCC827 or H3122 cells were incubated in the presence of erlotinib (100 nmol/L), alectinib (60 nmol/L), LY294002 (10 μmol/L), U0126 (20 μmol/L), S3I-201 (100 μmol/L), or DMSO vehicle for 24 hours, after which cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated or total forms of EGFR, ALK, STAT3, AKT, or ERK as well as with those to β-actin (loading control). B, RT and real-time PCR analysis of PD-L1 mRNA in cells treated as in A. Normalized data are expressed relative to the value for DMSO-treated cells, are means ± SD of triplicates, and are representative of three independent experiments. C, flow cytometric analysis of PD-L1 surface expression in cells treated as in A. Colored and gray traces correspond to drug- and DMSO-treated cells, respectively.

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

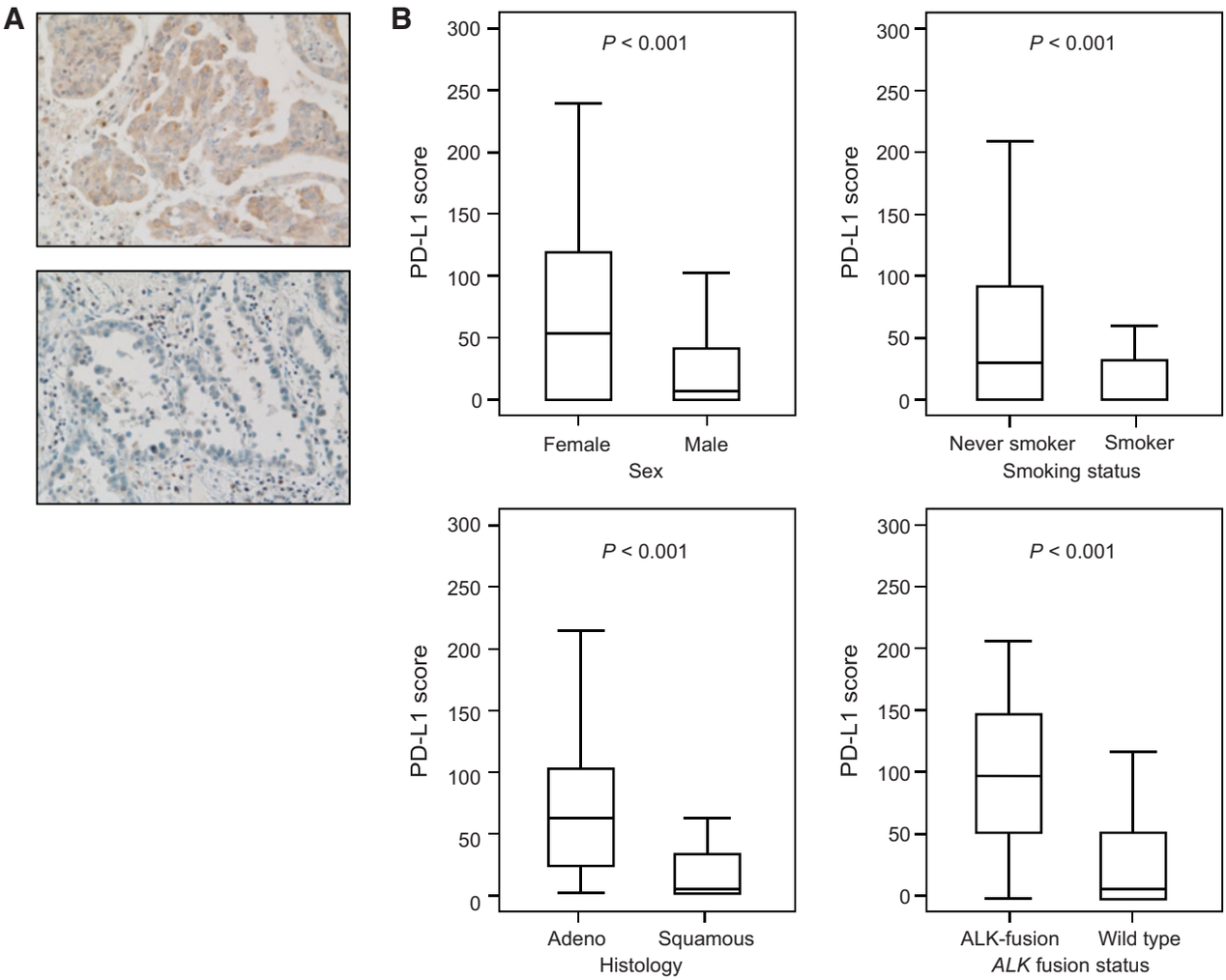

Figure 4.
Relation between immunohistochemical staining score for PD-L1 in NSCLC specimens and patient characteristics. A, immunohistochemical analysis of PD-L1 in two NSCLC tissue sections showing either strong (top) or weak (bottom) staining intensity. PD-L1 immunoreactivity was detected at the cell membrane or in the cytoplasm (or both) of tumor cells. Original magnification, ×400. B, significant association of overall PD-L1 staining *H*-score with sex, smoking status, tumor histology, or *ALK* fusion status. Data are presented as box-and-whisker plots, and *P* values were determined with the Wilcoxon rank-sum test.

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* than in those wild-type for both *EGFR* and *ALK*. We further showed that forced expression of EML4–ALK in Ba/F3 cells resulted in the upregulation of PD-L1 expression at the mRNA and protein levels. Conversely, the specific ALK inhibitor alectinib or RNAi with siRNAs specific for ALK mRNA suppressed expression of PD-L1 in NSCLC cells positive for *EML4–ALK*. Our results, thus, indicate that PD-L1 expression is induced in NSCLC cells by the EML4–ALK tyrosine kinase, with this induction being a key event in the pathogenesis of *ALK* fusion–positive NSCLC.

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 the present study, we also investigated whether expression of PD-L1 differs according to *ALK* fusion status in resected NSCLC tumors. Immunohistochemical analysis revealed that a high level of PD-L1 expression was positively associated with the presence of *ALK* rearrangement, consistent with our results indicating that EML4–ALK induces PD-L1 expression in NSCLC. A recent study also found that PD-L1 expression as detected by immunohistochemical analysis tended to be higher in NSCLC tumor specimens positive for *ALK* rearrangement than in those negative for *EGFR* mutation and *ALK* translocation (39). We previously found that a high-expression level of PD-L1 was associated with the presence of *EGFR* mutations in NSCLC (20). Given that PD-L1 expression in tumors is correlated with the likelihood of a response to therapies that target the PD-1–PD-L1 interaction (11), further studies are warranted to evaluate the efficacy of immunotherapies such as treatment with immune checkpoint inhibitors for such oncogene-driven 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
Development of methodology: K. Ota, E. Iwama, T. Harada, K. Matsumoto, I. Okamoto
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. Nakanishi, I. Okamoto
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Iwama, J. Tanizaki, M. Kage, Y. Nakanishi, I. Okamoto
Study supervision: T. Harada, K. Takayama, Y. Nakanishi, I. Okamoto

Acknowledgments

The authors thank A. Sato for technical assistance.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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

References

1. Nurieva RI, Liu X, Dong C. Yin-Yang of costimulation: crucial controls of immune tolerance and function. Immunol Rev 2009;229:88–100.
2. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 2012;12:252–64.

3. Lyford-Pike S, Peng S, Young GD, Taube JM, Westra WH, Akpeng B, et al. Evidence for a role of the PD-1:PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. Cancer Res 2013;73:1733–41.
4. Gatalica Z, Snyder C, Maney T, Ghazalpour A, Holterman DA, Xiao N, et al. Programmed cell death 1 (PD-1) and its ligand (PD-L1) in common cancers and their correlation with molecular cancer type. Cancer Epidemiol Biomarkers Prev 2014;23:2965–70.
5. Mittendorf EA, Philips AV, Meric-Bernstam F, Qiao N, Wu Y, Harrington S, et al. PD-L1 expression in triple-negative breast cancer. Cancer Immunol Res 2014;2:361–70.
6. Thompson RH, Kuntz SM, Leibovich BC, Dong H, Lohse CM, Webster WS, et al. Tumor B7-H1 is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up. Cancer Res 2006;66: 3381–5.
7. Nomi T, Sho M, Akahori T, Hamada K, Kubo A, Kanehiro H, et al. Clinical significance and therapeutic potential of the programmed death-1 ligand/ programmed death-1 pathway in human pancreatic cancer. Clin Cancer Res 2007;13:2151–7.
8. Hino R, Kabashima K, Kato Y, Yagi H, Nakamura M, Honjo T, et al. Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. Cancer 2010;116:1757–66.
9. Sznol M, Chen L. Antagonist antibodies to PD-1 and B7-H1 (PD-L1) in the treatment of advanced human cancer. Clin Cancer Res 2013;19: 1021–34.
10. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 2012;366:2455–65.
11. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 2012;366:2443–54.
12. Oxnard GR, Binder A, Janne PA. New targetable oncogenes in non–small cell lung cancer. J Clin Oncol 2013;31:1097–104.
13. Kaneda H, Yoshida T, Okamoto I. Molecularly targeted approaches herald a new era of non–small cell lung cancer treatment. Cancer Manag Res 2013;5:91–101.
14. Lynch TJ, Bell DW, Sordella R, Gurubhagavatula S, Okimoto RA, Brannigan BW, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non–small cell lung cancer to gefitinib. N Engl J Med 2004;350:2129–39.
15. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science 2004;304:1497–500.
16. Pao W, Miller V, Zakowski M, Doherty J, Politi K, Sarkaria I, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci USA 2004;101:13306–11.
17. Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, et al. Gefitinib or chemotherapy for non–small cell lung cancer with mutated EGFR. N Engl J Med 2010;362:2380–8.
18. Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, et al. 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 2010;11:121–8.
19. Akbay EA, Koyama S, Carretero J, Altabef A, Tchaicha JH, Christensen CL, et al. Activation of the PD-1 pathway contributes to immune escape in EGFR-driven lung tumors. Cancer Discov 2013;3:1355–63.
20. Azuma K, Ota K, Kawahara A, Hattori S, Iwama E, Harada T, et al. Association of PD-L1 overexpression with activating EGFR mutations in surgically resected non–smal cell lung cancer. Annal Oncol 2014;25:1935–40.
21. Soda M, Choi YL, Enomoto M, Takada S, Yamashita Y, Ishikawa S, et al. Identification of the transforming EML4-ALK fusion gene in non–small cell lung cancer. Nature 2007;448:561–6.
22. Koivunen JP, Mermel C, Zejnullahu K, Murphy C, Lifshits E, Holmes AJ, et al. EML4-ALK fusion gene and efficacy of an ALK kinase inhibitor in lung cancer. Clin Cancer Res 2008;14:4275–83.
23. Kwak EL, Bang YJ, Camidge DR, Shaw AT, Solomon B, Maki RG, et al. Anaplastic lymphoma kinase inhibition in non–small cell lung cancer. N Engl J Med 2010;363:1693–703.
24. Okamoto I, Nakagawa K. Echinoderm microtubule-associated protein-like 4-anaplastic lymphoma kinase-targeted therapy for advanced non–small cell lung cancer: molecular and clinical aspects. Cancer science 2012;103: 1391–6.
25. Shaw AT, Kim DW, Nakagawa K, Seto T, Crino L, Ahn MJ, et al. Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. N Engl J Med 2013;368:2385–94.
26. Iwama E, Okamoto I, Harada T, Takayama K, Nakanishi Y. Development of anaplastic lymphoma kinase (ALK) inhibitors and molecular diagnosis in ALK rearrangement-positive lung cancer. OncoTargets and Ther 2014;7: 375–85.
27. Solomon BJ, Mok T, Kim DW, Wu YL, Nakagawa K, Mekhail T, et al. First-line crizotinib versus chemotherapy in ALK-positive lung cancer. N Engl J Med 2014;371:2167–77.
28. Wang S, Takayama K, Tanaka K, Takeshita M, Nakagaki N, Ijichi K, et al. Nicotine induces resistance to epidermal growth factor receptor tyrosine kinase inhibitor by alpha1 nicotinic acetylcholine receptor-mediated activation in PC9 cells. J Thorac Oncol 2013;8:719–25.
29. Sasaki T, Okuda K, Zheng W, Butrynski J, Capelletti M, Wang L, et al. The neuroblastoma-associated F1174L ALK mutation causes resistance to an ALK kinase inhibitor in ALK-translocated cancers. Cancer Res 2010;70: 10038–43.
30. Berthon C, Driss V, Liu J, Kuranda K, Leleu X, Jouy N, et al. In acute myeloid leukemia, B7-H1 (PD-L1) protection of blasts from cytotoxic T cells is induced by TLR ligands and interferon-gamma and can be reversed using MEK inhibitors. Cancer Immunol Immunother 2010;59:1839–49.
31. Liu J, Hamrouni A, Wolowiec D, Coiteux V, Kuliczkowski K, Hetuin D, et al. Plasma cells from multiple myeloma patients express B7-H1 (PD-L1) and increase expression after stimulation with IFN-g and TLR ligands via a MyD88-, TRAF6-, and MEK-dependent pathway. Blood 2007;110:296–304.
32. Marzec M, Zhang Q, Goradia A, Raghunath PN, Liu X, Paessler M, et al. Oncogenic kinase NPM/ALK induces through STAT3 expression of immunosuppressive protein CD274 (PD-L1, B7-H1). Proc Natl Acad Sci USA 2008;105:20852–7.
33. Yamamoto R, Nishikori M, Tashima M, Sakai T, Ichinohe T, Takaori-Kondo A, et al. B7-H1 expression is regulated by MEK/ERK signaling pathway in anaplastic large cell lymphoma and Hodgkin lymphoma. Cancer science 2009;100:2093–100.
34. Crane CA, Panner A, Murray JC, Wilson SP, Xu H, Chen L, et al. PI(3) kinase is associated with a mechanism of immunoresistance in breast and prostate cancer. Oncogene 2009;28:306–12.
35. Jiang X, Zhou J, Giobbie-Hurder A, Wargo J, Hodi FS. The activation of MAPK in melanoma cells resistant to BRAF inhibition promotes PD-L1 expression that is reversible by MEK and PI3K inhibition. Clin Cancer Res 2013;19:598–609.
36. Parsa AT, Waldron JS, Panner A, Crane CA, Parney IF, Barry JJ, et al. Loss of tumor suppressor PTEN function increases B7-H1 expression and immunoresistance in glioma. Nat Med 2007;13:84–8.
37. Xu C, Fillmore CM, Koyama S, Wu H, Zhao Y, Chen Z, et al. Loss of Lkb1 and Pten leads to lung squamous cell carcinoma with elevated PD-L1 expression. Cancer cell 2014;25:590–604.
38. Takezawa K, Okamoto I, Nishio K, Janne PA, Nakagawa K. Role of ERK-BIM and STAT3-survivin signaling pathways in ALK inhibitor-induced apoptosis in EML4-ALK-positive lung cancer. Clin Cancer Res 2011; 17:2140–8.
39. D'Incecco A, Andreozzi M, Ludovini V, Rossi E, Capodanno A, Landi L, et al. PD-1 and PD-L1 expression in molecularly selected non–small cell lung cancer patients. Br J Cancer 2015;112:95–102.