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ORIGINAL RESEARCH

ROS1-fusion protein induces PD-L1 expression via MEK-ERK activation in non-small cell lung cancer

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ABSTRACT

Introduction: Despite some of the oncogenic driver mutations that have been associated with increased expression of programmed death-ligand 1 (PD-L1), the correlation between PD-L1 expression and ROS1 fusion in NSCLC cells, especially for those with Crizotinib resistance has not been fully addressed.
Materials and Methods: The expression of PD-L1 in 30 primary NSCLC tumors with/without ROS1-fusion protein was evaluated by immunohistochemical (IHC) analysis. To assess the correlation between ROS1 fusion and PD-L1 expression, we down-regulated ROS1 with RNA interference or specific inhibitor (Crizotinib) in ROS1-fusion positive NSCLC cell line HCC78; or up-regulate ROS1-fusion gene in an immortalized human bronchial epithelial cell line (HBE). Mouse xenograft models were also used to determine the effect of ROS1 expression on PD-L1 expression *in vivo*. Crizotinib-resistant cell line was generated for measuring the association between Crizotinib resistance and PD-L1 expression.
Results: ROS1-rearrangement in primary NSCLC tumor was significantly associated with up-regulated PD-L1 expression. PD-L1 expression was significantly up-regulated in bronchial epithelial cells after forced expression of ROS1 fusion and was eliminated when HCC78 xenograft mouse models were treated with Crizotinib. We found PD-L1 expression was modulated by MEK-ERK pathway signaling in both parental and Crizotinib-resistant NSCLC cells with ROS1 fusion.
Conclusions: The correlation between ROS1-fusion and PD-L1 overexpression suggested that PD-L1/PD-1 blockade could be the second-line treatment option for the Crizotinib-resistant NSCLC with *ROS1* rearrangement.

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PD-L1; ROS1 fusion; non-small cell lung cancer

Introduction

Blocking immune checkpoint signaling with antibodies against programmed cell death-1 (PD-1) or programmed cell death-ligand 1 (PD-L1) has been used to treat a wide spectrum of cancers, such as metastatic melanoma, renal cell carcinoma, NSCLC and other tumors.[1,2] The PD-L1 expression in non-squamous subtype of NSCLC has been correlated with response to Nivolumab, an anti-programmed cell death-1 (PD-1) antibody,[3,4] and PD-L1 expression remains the major tool for the selection of patients who might be beneficial from immune checkpoint inhibitor (ICI) treatment.[5,6] Owning to the heterogeneity of PD-L1 expression levels, it is important to understand the mechanisms that regulate PD-L1 expression in tumor cells.

Accumulating evidences have been suggested that PD-L1 expression is regulated by oncogenic driver mutations in NSCLC.[7–12] ROS1-rearrangements, a driver mutation in NSCLC, accelerate NSCLC cell proliferation through the activation of the receptor tyrosine kinase.[10–12] The ROS1-fusion protein has also been found in many other cancers, including gastric cancer, ovarian cancer, cholangiocarcinoma, colorectal cancer and angiosarcoma.[13,14] It has been observed that up-regulated PD-L1 expression in NSCLC is correlated with oncogenic RAS signaling, while the tyrosine kinase ROS1 is involved in RAS signaling.[12,15] Most recently, high-level expression of PD-L1 has been associated with *ROS1* rearrangement in NCSLC,[16,17] which is in line with other studies that PD-L1 expression in NSCLC is driven by aberrant activation of oncogenes (such as EGFR and ALK).[18–21] In this study, we found that PD-L1 expression was up-regulated by *ROS1* rearrangement through the MEK-ERK pathway signaling in NSCLC with Crizotinib resistance. Our finding suggested that PD-L1/PD-1 blockade as a new strategy for treating Crizotinib-resistant NSCLC with ROS1 rearrangement.

Material & methods

Bio-specimens selection

Thirty NSCLC specimens were collected from NSCLC patients who had not been treated with chemotherapy or

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radiotherapy prior to surgery between December 2015 and December 2017 at West China Hospital. None of these tumors had *EGFR* mutation or *ALK*-rearrangement. *ROS1* fusions were detected by Immunohistochemistry (IHC) and further confirmed by fluorescence *in situ* hybridization (FISH). Break-apart FISH approach was performed using BAC clones corresponding to the 5′ (RP11-835I21) and 3′ (RP11-1036C2) sequences flanking the ROS1 gene labeled by nick translation in green and red. Positive cases were defined as tumors harboring with more than 15% of cells with split signals. *EGFR* mutation status was detected by EGFR RGQ PCR Kit (QIAGEN, # 870121). *ALK*- rearrangement status was tested by IHC using D5F3 (CST, #3633). All samples were collected from patients with informed consent, and all related procedures were performed with the approval of the internal review and ethic boards of West China hospital (#2018-596).

Cell culture and reagents

HCC78 was purchased from the DSMZ. H3122 was kindly provided by Prof. Yong Peng (Sichuan University). PC-9 was purchased from the European Collection of Authenticated Cell Cultures (ECACC). Other cell lines including A549, H1299, HBE, HCC827 and H1975 cells were purchased from the American Type Culture Collection (ATCC). PC-9 and A549 were cultured in DMEM medium with 10% fetal bovine serum (FBS). Other cells were cultured in RPMI-1640 medium in the presence with 10% FBS fetal bovine serum. To establish the Crizotinib-resistant cells, HCC78 cells were maintained in complete medium with Crizotinib from a starting concentration of 100 nM to a final concentration of 1 μM over 6 months. The Crizotinib-resistant cells were maintained in complete medium with 0.5 μM Crizotinib. All cells were maintained under a humidified atmosphere of 5% CO2 at 37°C. Crizotinib (Sigma, #PZ0191), U0126 (Selleck, #S1102 chem), PD0325901 (Selleck, #S1036chem) and Cisplatin (Selleck, # S1166) were dissolved in dimethyl sulfoxide (DMSO) and stored at −20° or −80°C.

Immunohistochemistry

Surgical specimens were fixed overnight for 24 h in 10% buffered formalin. The paraffin-embedded tissue was sectioned at 5 μm thick slices. Each sections slide was incubated for 2 h with human PD-L1 antibody (Proteintech, #66248-1-Ig) at 1:500 dilutions. All stains were quantified in 10 tumor-containing fields. The percentage of PD-L1 positive tumor cell surface staining was scored as 0 (<5%), 1 (≥5–20%), 2 (≥20–50%) or 3 (≥50%). All immunohistochemical images were evaluated by two experienced pathologists who were unaware of the identity of the specimens. The average of the two determinations was used in subsequent analyses.

Plasmid transfection

The plasmids expressing SLC34A2-ROS1 and FIG-ROS1 fusion proteins as well as the negative control plasmid were generously provided by Prof. Druker's laboratory (Knight Cancer Institute). HBE cells were plated in 6-well plates with 60% confluence. We used 2 μg of plasmid DNA, 4 μl P3000 reagent and 6μ Lipofectamine 3000 reagent (Invitrogen, #L3000001) for transfection. The culture medium was changed 12 h after transfection.

RNA interference

Cells were plated in 6-well plates with 70% confluence. We used 7 μl siRNA (20 μM) and 7 μl Lipofectamine 3000 reagent for the transfection, and the culture medium was changed 24 h after transfection. The ROS1 mRNA-specific siRNAs were obtained from Shanghai GenePharma Co., including ROS1-1 sense (5′-CAGAGUAGUAGCUGCAAAUTT-3′); ROS1-1 antisense (5′-AUUUGCAGCUACUACUCUGTT-3′); ROS1-2 sense (5′-GGAUCUGGCAGCUAGAAAUTT-3′); and ROS1-2 antisense (5′-AUUUCUAGCUGCCAGAUCCTT-3′).

Antibodies for immunoblot and flow cytometry

Cells were washed once with ice-cold PBS and lysed in 100 μl RIPA lysis buffer (Solarbio, #R0010) with PMSF and Cocktail. Lysate was extracted with SDS-PAGE loading buffer (Solarbio, #P1040) for 5 min at 100°C. Proteins were transferred to Immobilon-FL 0.45 μm PVDF membranes (Millipore) and subjected to immunoblot analysis with following antibodies:PD-L1 (1:1000, CST, #13684 S), phospho-ROS1 (1:1000, CST, #3078 S), ROS1 (1:1000, CST, #3266 S), *p*-ERK (1:1000, CST, #9106 S), ERK (1:1000, CST, #4695 S), *p*-STAT3(1:1000, CST, #9145 S), STAT3 (1:1000, CST, #9139 S), *p*-AKT (1:1000, CST, #4060 S) and AKT (1:1000, CST, #4685), β-actin (1:5000, ZSGB-BIO, #TA-09). Bio-Rad ChemiDoc™ MP imaging system or LI-COR Odyssey Platform was used for western blot detection with either HRP-conjugated or IR dye secondary antibodies, respectively. Flow cytometry antibodies for PD-L1 were purchased from BD Biosciences, conjugated with PE-CY7 (BD, #558017) or APC (BD, #563741).

Quantitative real time-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, #10296010). Reverse transcription was done using the PrimeScript™ RT Master Mix (Takara, #RR036A) using 1 μg of total RNA, followed by real-time PCR analysis with TB Green Premix Ex Taq (Takara, #RR420A). The PCR primers (forward and reverse, respectively) included those for PD-L1 (5′-TGGCATTTGCTGAACGCATTT-3′ and 5′-TGCAGCCAGG TCTAATTGTTTT-3′) and 18 s-rRNA (5′- GATGGGCGGCG GAAAATAG-3′ and 5′-GCGTGGATTCTGCATAATGGT-3′). The amount of PD-L1 mRNA was normalized by that of 18 s-rRNA.

Mouse xenograft models

Human HCC78 NSCLC cell line was used to establish a subcutaneous xenograft mouse model in 6-week-old nude mice (BALB/c nude; GemPharmatech, Nanjing, China). A total of 1×10^7 HCC78 cells were resuspended

in 80 µl Matrigel and were inoculated subcutaneously to the left dorsal flanks of the nude mice. Twelve nude mice were randomly divided into control and Crizotinib treatment groups (six mice per group). Tumors with a diameter of 4–6 mm were treated by oral gavage with vehicle or Crizotinib (25 mg/kg) once per day for 14 consecutive d. Tumor size was measured by electronic caliper and recorded every 3 d. Tumor volumes were calculated by taking length to be the longest diameter across the tumor and width to be the corresponding perpendicular diameter, using the following formula: 0.5× (length×width^2) mm^2. The study was approved by the Animal Care Committee of West China Hospital (#2018217A).

Statistical analysis

Pairwise comparison for RT-PCR results was performed using T-test. Other logistic regression analyses were conducted using non-parametric Mann–Whitney U test. Analysis of variance in conjugation with Bonferroni's correction was used to compare multiple groups of data. The statistical testing results were determined by the SPSS 22.0 or GraphPad Prism 6 software (GraphPad, San Diego, CA) and R Project for Statistical Computing (Augasse, Austria). $p < .05$ was considered statistically significant and denoted as follows: *p < .05, **p < .01, ***p < .001.

Results

PD-L1 expression is correlated with ROS1 fusion in NSCLC

To determine the correlation between ROS1 fusion and PD-L1 expression, we measured PD-L1 protein levels in 30 NSCLC tumors without *EGFR* mutation and *ALK*-rearrangement, including 10 ROS1-fusion positive and 20 ROS1-fusion negative cases (Figure 1a and Supplementary Table 1). We found that PD-L1 expression was significantly upregulated in tumors with ROS1 fusion, compared with those without *ROS1* rearrangement (Figure 1b,c).

To further characterize the association between ROS1 fusion and PD-L1 expression, we measured PD-L1 expression in a series of NSCLC cell lines with/without ROS fusion. HCC78 is known as an NSCLC cell line with *SLC34A2-ROS1* rearrangement but without *EGFR* mutation and *ALK* fusion. We confirmed the *SLC34A2-ROS1* fusion in HCC78 cell line using short tandem repeat (STR) experiment (Supplementary Figure 1a). Since PD-L1 expression was correlated *EGFR* mutation and *ALK* fusion,[18,22] for the comparison, NSCLC cell lines with EGFR mutation (HCC827, H1975 and PC9) and NSCLC cell line with ALK fusion (H3122) were selected in this analysis. As control, an immortalized human bronchial epithelial cell line (HBE) and NSCLC cell lines (A549 and H1299) without EGFR mutation, ALK fusion and ROS1 fusion were also included in this study. As expected, NSCLC cell lines with EGFR mutation and ALK fusion were correlated with a higher level of PD-L1 expression (Figure 1d,e). We found that PD-L1 expression was significantly up-regulated in HCC78 cells with *SLC34A2-ROS1* fusion than those without EGFR mutation, ALK fusion and ROS1 fusion (Figure 1d,e). Since HCC78 cell is negative for EGFR mutation and ALK fusion, ROS1 fusion might be an independent factor that modulates PD-L1 expression in NSCLC cells.

ROS1 fusion drives PD-L1 expression

To assess whether ROS1 fusion is a driver of PD-L1 expression, we depleted ROS1-fusion protein in HCC78 cells by a ROS1-targeted siRNA (Supplementary Figure 2a) and found that both protein (Figure 2a,b) and mRNA levels of PD-L1 were down-regulated after knockdown ROS1 (Figure 2c). Furthermore, we treated HCC78 cells with Crizotinib, a potent ROS1 inhibitor, and measured PD-L1 expression using flow cytometry, western blot and qPCR analyses. We found that Crizotinib blocked ROS1 phosphorylation (Supplementary Figure 2b) and significantly reduced PD-L1 expression in HCC78 cells (Figure 2d–f).

Furthermore, both mRNA and protein expression of PD-L1 was significantly increased after transfected *SLC34A2-ROS1* and *FIG-ROS1* fusion into HBE cells (Figure 2g–i, Supplementary Figure 2c). When *SLC34A2-ROS1* or *FIG-ROS1* transfected HBE cells were treated with Crizotinib, the PD-L1 expression was down-regulated (Figure 2j,k).

Targeting ROS1 fusion leads to the elimination of PD-L1 expression *in vivo*

We next sought to determine the effect of targeting ROS1 fusion on PD-L1 expression *in vivo*, using a *ROS1*-rearrangement HCC78 xenograft mouse model. Twelve nude mice were randomly divided into two groups with/without Crizotinib treatment and were subcutaneously inoculated with 1×10^7 HCC78 cells. Tumors with a diameter of 4–6 mm were treated by oral gavage with vehicle or Crizotinib (25 mg/kg) once per day for 14 consecutive d. We found that the elimination of ROS1 expression in HCC78 xenograft model by Crizotinib inhibited tumor growth, and was correlated with down-regulated expression of PD-L1 (Figure 2l-n), which was in line with our *in vitro* results that ROS1 fusion modulated PD-L1 expression.

ROS1 fusion modulates PD-L1 expression through MEK-ERK pathway signaling

To characterize the signaling pathways that are involved in the modulation of PD-L1 expression by ROS1 fusion, we measured downstream signaling of ROS1 in response to Crizotinib treatment, including MEK-ERK, JAK-STAT and PI3K-AKT.[13,23] Increasing concentrations of Crizotinib were significantly correlated with downregulation of ERK phosphorylation in HCC78 cells, whereas the phosphorylation of STAT3 and AKT was barely affected by Crizotinib treatment (Figure 3a). Similarly, when ROS1-fusion protein in HCC78 cells was depleted by ROS1 siRNAs, only ERK phosphorylation was deactivated (Figure 3b).

To further assess the correlation between ERK activation and ROS1 fusion-induced PD-L1 expression, we measured the effects of MEK-ERK specific inhibitor (PD0325901 and U0126) on PD-L1 expression in HCC78 cells. Both ERK

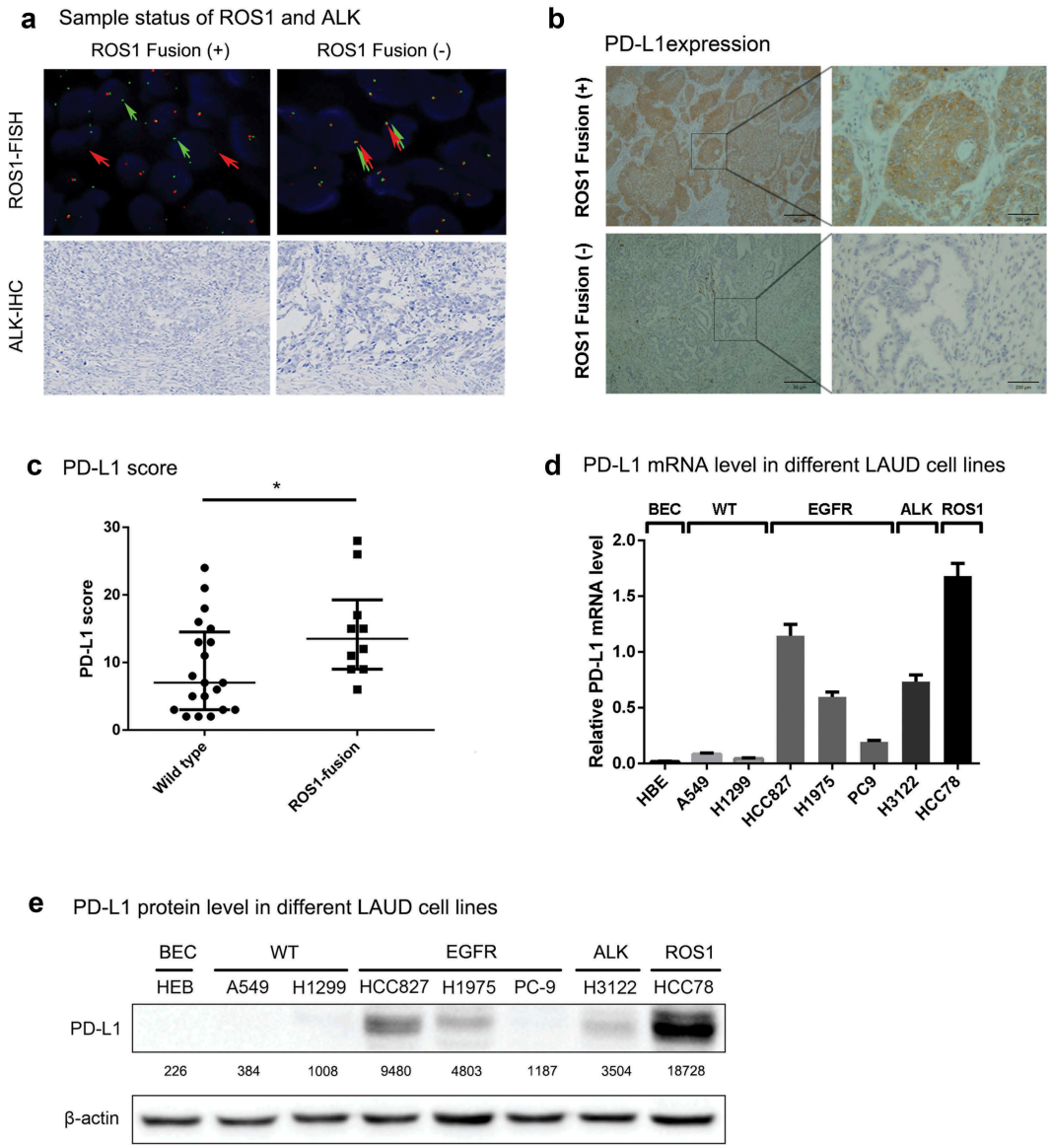

Figure 1. ROS1 fusion is associated with upregulated expression PD-L1 in NSCLC.

(a) ROS1-rearrangement and ALK fusion status in primary NSCLC sample. ROS1 fusion was measured using FISH using 5′ ROS1 probe (*green*) and a 3′ ROS1 probe (*red*). The normal ROS1 locus is shown as unsplit red and green pairs of probes (arrowheads). ALK fusion was measured using IHC. FISH and IHC analyses were performed on 30 NSCLC samples, and representative images are shown. (b) PD-L1 expression in paraffin-embedded primary NSCLC samples with or without *ROS1* fusion. Measured by IHC. IHC analyses were performed on 30 NSCLC samples, and representative images are shown. (c) Quantification of IHC results of the correlation between PD-L1 expression and ROS1 fusion in primary NSCLC samples. Logistic regression analysis was performed using non-parametric Mann–Whitney U test, *lines* represents median and interquartile range. *$p < .05$. (d) Relative PD-L1 mRNA expression in different cell lines was determined by qPCR. Triplicate experiments are presented with error bar as mean±SD, and logistic regression analysis was performed using T-test. (e) PD-L1 protein expression in different cell lines was detected by Western blot and the gray value is marked below. BEC, bronchial epithelial cell. HBE, a bronchial epithelial cell line. A549 and H1299, wild type for EGFR, ALK and ROS1 mutation. HCC827, H1975 and PC9, with EGFR mutation. H3122 with ALK fusion. HCC78 with ROS1 fusion.

phosphorylation and PD-L1 expression were down-regulated after treating HCC78 cells with PD0325901 or U0126 (Figure 3c–f), suggesting that MEK-ERK signaling facilitated PD-L1 expression in ROS1-fusion positive cells in NSCLC, rather than JAK-STAT or PI3 K-AKT pathways.

PD-L1 expression is upregulated in Crizotinib-resistant NSCLC Cells

Since both ROS1 and PD-L1 expressions in Crizotinib-sensitive HCC78 cells were downregulated by ROS1 inhibitor, we further determined the correlation between ROS1-fusion and PDL-1 expression in Crizotinib-resistant NSCLC cells. We established a Crizotinib-resistant HCC78 (HCC78CR) NSCLC cell line using the method as Song et al. described previously[24] (Supplementary Figure 3). Comparing with parental HCC78 cells, HCC78CR cells were resistant to Crizotinib with over eightfold higher IC50, and significantly less apoptosis ($p < .001$, Fisher's exact test) (Figure 4a, b). Though PD-L1 expression in HCC78CR cells was not affected by Crizotinib (Figure 4c,d), the overexpression of PD-L1 in Crizotinib-resistant HCC78CR cells was also specifically correlated with the activation ERK pathway signaling (Figure 4e).

Since platinum-based therapy is widely used as the second-line treatment for patients who have received

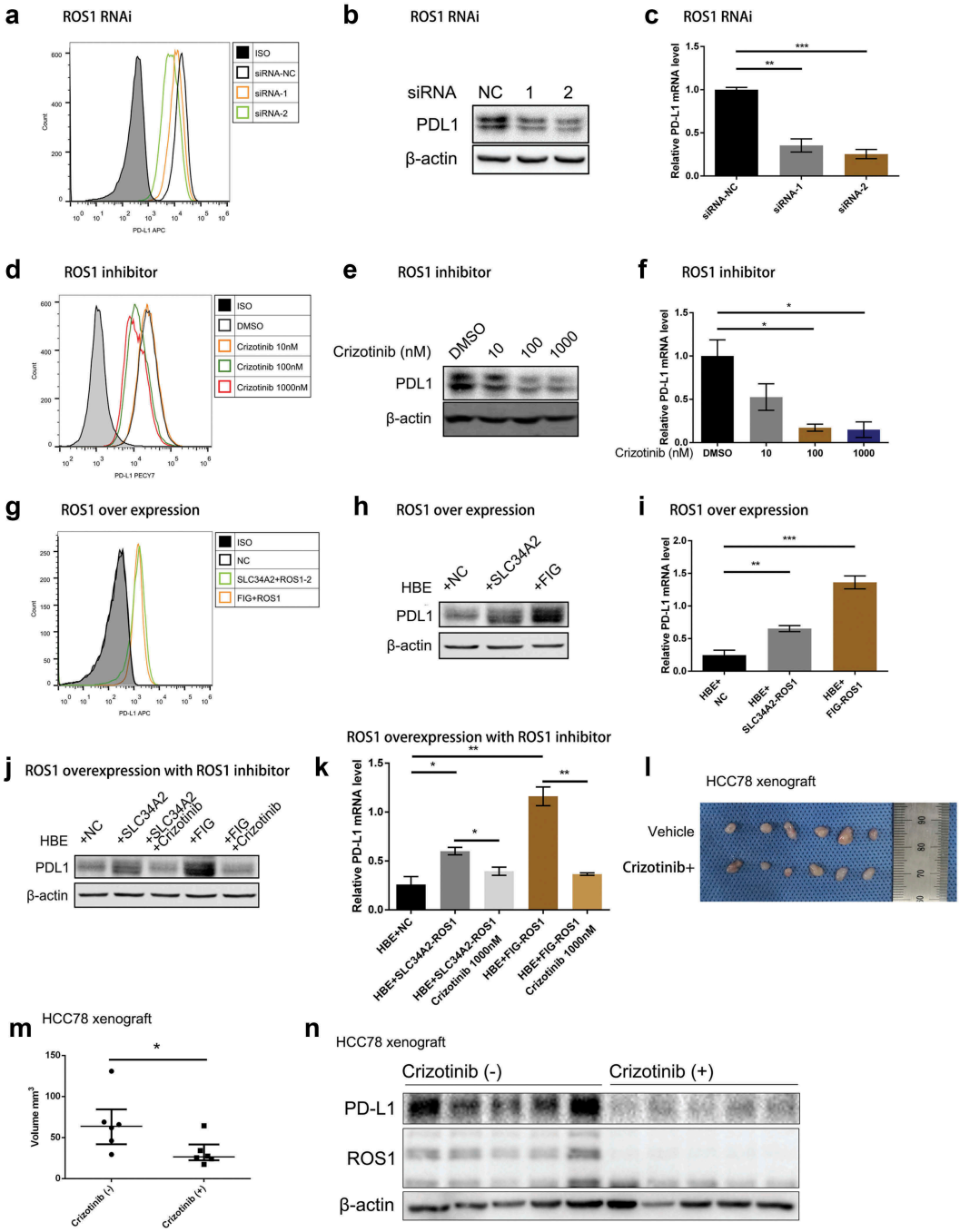

Figure 2. ROS1 fusion drives PD-L1 expression in vitro and in vivo.

(a–c) Downregulation of PD-L1 expression in HCC78 cells was induced by ROS1-specific siRNAs. HCC78 cells were transfected with negative control or ROS1-specific siRNAs, PD-L1 expression was measured 48-h post-transfection by FACS (a), Western blot (b) and qPCR (c). Logistic regression analysis was performed using T-test. (d–f) Down-regulation of PD-L1 expression in HCC78 cells after 48-h treatment with Crizotinib. PD-L1 expression was detected by FACS (d) Western blot (e) and qPCR (f). Logistic regression analysis was performed using T-test. (g–i) Overexpression of PD-L1 expression in HBE cells with ROS1 fusion encoding (*SLC34A2-ROS1* or *FIG-ROS1*) plasmid. PD-L1 expression was detected by FACS (g) Western blot (h) and qPCR (i). (j–k) PD-L1 expression was decreased by Crizotinib in HBE cells with transfected ROS1-fusion protein. PD-L1 expression was detected by Western blot (j) and qPCR (k). Logistic regression analysis was performed using T-test. (l–m) Tumor growth Image (l) and tumor size quantification (m) HCC78 xenograft model treated with Crizotinib by oral gavage for 14 d. Logistic regression analysis was performed using non-parametric Mann–Whitney U test, lines represent median and interquartile range. (n) Western blot analysis of ROS1 and PD-L1 expression in tumor cells harvested from HCC78 xenograft model 14 d after Crizotinib treatment (25 mg/kg/d). *p < .05, **p < .01, ***p < .001.

prior Crizotinib,[25] we performed cisplatin IC50 test on Crizotinib-resistant and sensitive strains. Crizotinib-resistant strains (HCC78CR) showed higher IC50 levels than sensitive strains (HCC78), with cisplatin IC50 25.86 uM and 10.08 uM, respectively (Figure 4f). These results suggest that Crizotinib-resistant NSCLC cells with ROS1-rearrangement are also resistant to traditional chemotherapy, and PD-L1/PD-1 blockade may be a new strategy to overcome Crizotinib resistance in ROS1-rearranged NSCLC.

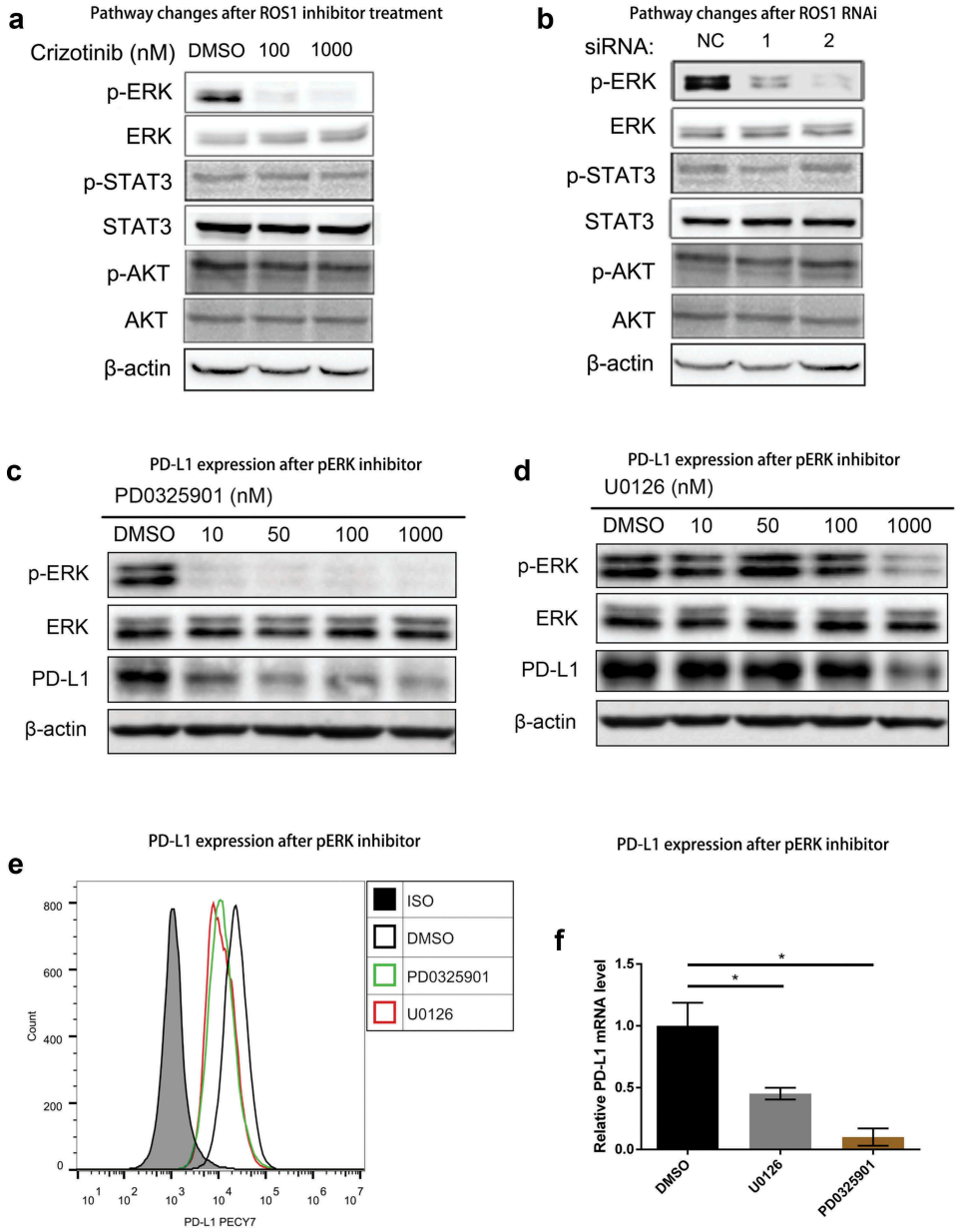

Figure 3. ERK mediates PD-L1 expression in NSCLC cells with ROS1 fusion.

(a) Immunoblot analysis of ROS1 downstream signaling in HCC78 cells after treated with Crizotinib for 48 h. (b) Immunoblot analysis of ROS1 downstream signaling in HCC78 cells after 48 h after ROS1 knockdown. (c–d) PD-L1 expression was downregulated in HCC78 cells after treated with MEK-ERK specific inhibitor PD0325901 (c) and U0126 (d), measured by Western blot. (e) PD-L1 expression was down-regulated in HCC78 cells after treated with MEK-ERK specific inhibitor PD0325901 and U0126 for 48 h, measured by FACS. (f) PD-L1 expression was down-regulated in HCC78 cells after treated with MEK-ERK specific inhibitor PD0325901 and U0126 for 48 h, measured by qPCR. Logistic regression analysis was performed using T-test. *$p < .05$.

Discussion

In this study, we demonstrated that ROS1 rearrangement plays a critical role in modulating PD-L1 expression through activation of ERK signaling, which contributes to immune escape of Crizotinib-resistance NSCLC cells with ROS1-rearrangement.

PD-L1 expression has been approved as a biomarker for PD-1/PD-L1 inhibitor therapy by the US Food and Drug Administration for patients with advanced NSCLC treated with pembrolizumab.[26] Several oncogenic alterations, such as mutations in *MET, KRAS* and *ALK*, have been associated with upregulated expression of PD-L1 in NSCLC.[7,22,27] However, discordant correlation between these common oncogenic alterations and PD-L1 expression was also observed in a subset of NSCLC patients,[5,16] suggesting additional oncogenic alterations is needed to defined subpopulation of patients who might be beneficial from PD-1/PD-L1 inhibitors. Here, we reported that ROS1-fusion protein was correlated with up-regulated PD-L1 expression in primary NSCLC without *EGFR* and *ALK* alterations. Forced expression of SLC34A2-ROS1 or FIG-ROS1 in human bronchial epithelial cells dramatically increased the expression of PD-L1; while attenuated PD-L1 expression was observed in ROS1-fusion positive NSCLC cell line HCC78 after depletion of

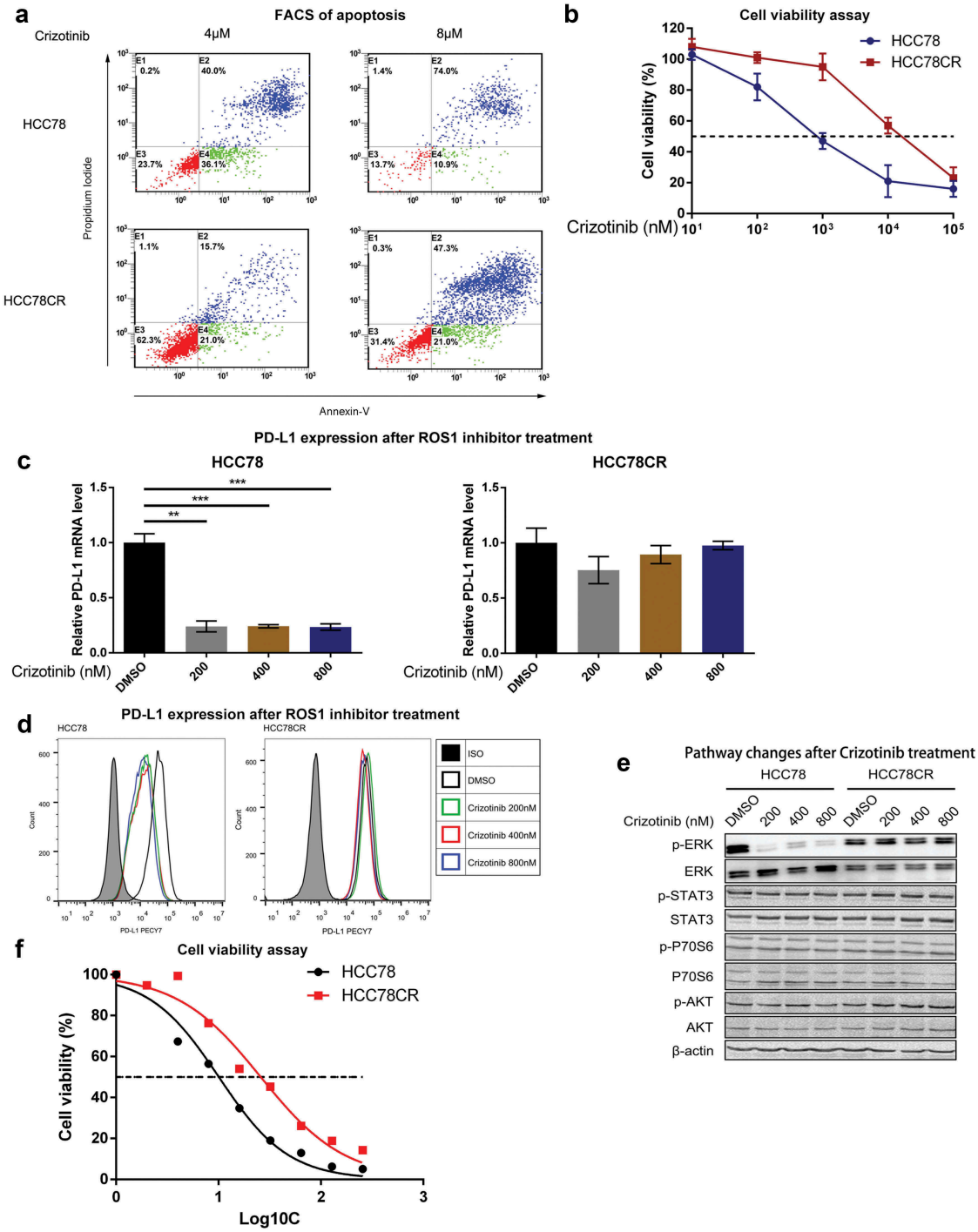

Figure 4. PD-L1 is overexpressed in Crizotinib-resistant HCC78CR cells.

(a) Crizotinib-resistant HCC78CR cells showed less apoptosis in response to Crizotinib treatment. Apoptosis was measured by FACS using Annexin V-FITC and Propidium Iodide. In comparing with parental HCC78, proportion of apoptotic cells in HCC78CR was assessed using 2-tails Fisher's exact test, with $p = .0001$ at 4 μM Crizotinib, and $p = .0002$ at 8 μM Crizotinib. (b) Cell viability of HCC78 and HCC78CR cells treated with different concentrations of Crizotinib for 72 h, and IC50 was provided as mean ± standard deviation (SD). (c) Quantitative PD-L1 mRNA expression in HCC78 and HCC78CR cells after treated with different concentrations of Crizotinib for 48 h, measured by qPCR. Logistic regression analysis was performed using T-test. $^{**}p < .01$, $^{***}p < .001$. (d) PD-L1 protein expression in HCC78 and HCC78CR cells after treated with different concentrations of Crizotinib for 48 h, measured by FACS. (e) Major ROS1 down-stream signaling pathways were detected in HCC78 and HCC78CR cells after 48-h Crizotinib treatment at different concentrations. (f) Cisplatin IC50 test on Crizotinib-resistant (HCC78CR) and sensitive (HCC78) cell lines. IC50 25.86 μM for HCC78CR and 10.08 μM for HCC78 cells.

ROS1-fusion protein by RNA interference or ROS1 inhibitor. Our finding suggested that ROS1 fusion is not only an oncogenic driver of NSCLC cell proliferation and survival[28] but is also involved in remodeling the immune microenvironment. This finding is consistent with a recent report that high PD-L1 expression was significantly associated with ROS1 rearrangement in lung adenocarcinoma cohort.[16]

Analyzing downstream signaling of ROS1 in response to Crizotinib (ROS1 inhibitor), we found depletion of PD-L1 expression was associated with the downregulation of MEK-

ERK pathway signaling when HCC78 cells were treated with Crizotinib or ROS1 siRNAs. Our finding is in line with previous reported that PD-L1 expression is regulated through PI3K-AKT and RAS signaling in various solid tumors, as well as STAT3 or MEK-ERK pathway in some hematologic neoplasms.[15,29,30] Collectively, our results highlighted the molecular basis of ROS1 rearrangement in regulating PD-L1 expression in NSCLC.

Although *ROS1*- or *ALK*-rearranged non-small cell lung cancer (NSCLC) is sensitive to Crizotinib, a subset of *ROS1* fusion-positive patients may acquire drug resistance following Crizotinib treatment.[14,24] EGFR pathway activation and secondary mutations in *ROS1*, such as G2032R and L2155S, have been recognized as the mechanisms of the Crizotinib resistance.[24,31] Although efforts have been made to develop more potent inhibitors to overcome Crizotinib resistance in *ROS1*-rearranged NSCLC, these clinical trials on unselected populations have been discontinued due to the less significant results.[32] While the National Comprehensive Cancer Network (NCCN) guideline of NSCLC recommended that Crizotinib-resistance patients with positive ROS1-rearrangement might be treated with PD-1/PD-L1 inhibitor,[32] the genetic basis of this approach has not been addressed.

American Society of Clinical Oncology Clinical Practice Guideline recommends that platinum-based therapy in the second line with or without bevacizumab for patients who have received prior Crizotinib.[25] However, the response rate is not very encouraging.[33] It has been reported that NSCLC with EMT-positive tumors were less sensitive to chemotherapy or radiotherapy.[34] Since PD1/PD-L1 blockade therapy for NSCLC gives superior treatment responses than chemotherapy for those PD-L1 positive patients,[34–38] effort has been made to define the NSCLC patient with Crizotinib resistance who might be beneficial from PD-L1 inhibitors. For instance, it has been suggested that EGFR/PD-L1 positive NSCLC with EGFR-TKI resistance response to PD-L1 inhibitors.[39]

In our study, we found that IC50 of cisplatin in Crizotinib-resistant line of HCC78CR cells was also significantly higher than parental line HCC78. The highly expressed PD-L1 in HCC78CR cells with relatively lower response to cisplatin indicating that immunotherapy may be a promising second-line therapeutic strategy to overcome Crizotinib resistance, especially for the NSCLC patients with ROS1 fusion. Our results warrant further *in vivo* studies and clinical trials to evaluate the efficacy of immunotherapies for the NSCLC with *ROS1* rearrangement.

Acknowledgments

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Disclosure of potential conflicts of interest

The authors declare no potential conflicts of interest.

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Author Contributions

L.L. and Y.P. conceived the project. Z.L., K.Z. and S.W. performed all experiments. X.W. performed pathological analysis. C.L., J.Z., Q.G., Z. Y. and Y.Y. assisted with some experiments. L.L. and Y.P. supervised the study. Z.L., K.Z., Q.C. and L.L. wrote the manuscript. All authors discussed the results and revised the manuscript.

Competing interests

The authors declare no conflict of interest.

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