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Increased PD-L1 Expression in Acquired Cisplatin-Resistant Lung Cancer Cells via Mir-181a

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Cancer immunotherapy has dramatically improved the prognosis of non-small cell lung cancer (NSCLC). In tumor cells, programmed death ligand-1 (PD-L1), also known as cluster of differentiation 274 (CD274), is a key target for cancer immunotherapy. Cisplatin (CDDP), a first-class NSCLC treatment drug, reportedly induces PD-L1 expression, and regulates cancer immunity. Herein, the regulatory mechanism of PD-L1 was investigated in CDDP-treated NSCLC and acquired CDDP-resistant NSCLC. Two types of NSCLC cell lines, A549 and H69, and their CDDP-resistant cell lines, A549R and H68R, were used to investigate PD-L1 expression and microRNA mir-181a expression. Murine lung cancer LL/2 cells were injected to mice for *in vivo* study. Although CDDP induced PD-L1 expression in A549 and H69 cells, A549R and H69R cells expressed extremely higher levels of PD-L1. CDDP-induced mir-181a was detected in A549 and H69 cells, but not A549R and H69R cells. Moreover, the CDDP-induced ATM-mir-181a-c-FOS pathway repressed PD-L1 expression in A549 cells, while A549R cells blocked this negative regulatory mechanism to further increase PD-L1 expression. Exogenous mir-181a in LL/2 cells could repress the intratumoral exhausted T cells, and increase the T cells function, and repress the tumor growth. Increased PD-L1 expression in acquired cisplatin-resistant lung cancer cells is dependent on mir-181a in NSCLC.

Keywords: c-FOS; cisplatin; lung adenocarcinoma; mir-181a; PD-L1
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Introduction

Cancer immunotherapy has been employed for the treatment of non-small cell lung cancer (NSCLC) for more than 10 years. Immune checkpoint inhibitors, particularly those preventing interaction of programmed death ligand-1 (PD-L1) with PD-L1 receptor, have dramatically improved the survival of some patients with lung cancer (Doroshow et al. 2019). PD-L1, also known as cluster of differentiation 274 (CD274), has emerged as a contributor that induces T cell exhaustion and tumor growth (Thommen et al. 2015). Therefore, investigation of the PD-L1 regulatory mechanism in NSCLC is important for tumor treatment.

Cisplatin (cis-diaminedichloroplatinum II, CDDP) is a first-class NSCLC drug that decreases the 5-year lung cancer-associated death rate by 6.9% (Pignon et al. 2008). CDDP was reported to induce PD-L1 expression in cancer cells, including head and neck squamous cell carcinoma and NSCLC (Tran et al. 2017; Fournel et al 2019). In NSCLC patients, CDDP treatment can significantly increase PD-L1 expression, and combination of PD-L1 blockage with CDDP can improve the therapeutic effect (Wakita et al. 2019). Moreover, elevation of PD-L1 expression contributes to acquired resistance to CDDP in NSCLC (Yan et al. 2016). Herein, the regulatory mechanism of PD-L1 in NSCLC and CDDP-resistant NSCLC was investigated.

PD-L1 expression is mainly regulated by interferon receptor signaling pathways in cancer cells, and interferon-gamma (INF-γ) can active the JAK1/JAK2-STAT1/STAT2/STAT3-IRF1 axis to regulate PD-L1 expression (Garcia-Diaz et al. 2019). Meanwhile, activator protein-1 (AP-1) also contributes to regulate PD-L1 expression in response to INF-γ stimulation (Atsaves et al. 2019).

MicroRNAs (miRNAs) are small, non-coding RNA

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molecules that inhibit protein translation by directly binding to the 3'-untranslated region (UTR) of their target genes, and they are important regulators of almost all cellular signaling pathways, including immune checkpoints and various cellular processes in cancer (Ambros 2001; Wang et al. 2017). Interferon receptor signaling pathways are also regulated by miRNAs. For example, mir-181a binds to the 3'-UTR of INF-γ mRNA and regulates INF-γ expression (Sang et al. 2015). c-Fos, an AP-1 subunit, regulates interferon receptor signaling pathways, and is a target gene of mir-181a (Wu et al. 2012). Furthermore, CDDP reportedly regulates miRNA expression in cancer cells, which contributes to regulating cancer cell survival, migration and acquiring drug resistance (Yang et al. 2016). For example, mir-181a, miR-519a and miR-374a are regulated by CDDP in head and neck squamous cell carcinoma (Huang et al. 2011). Thus, the relationship between miRNA- and CDDP-induced PD-L1 expression in NSCLC is worthy of study.

Herein, we compared expression of PD-L1 in CDDP-treated NSCLC and acquired CDDP-resistant NSCLC. The role of mir-181a in regulating PD-L1 expression and anti-tumor response were also investigated in acquired CDDP-resistant NSCLC *in vitro* and *in vivo*.

Materials and Methods

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies (Tveden-Nyborg et al. 2021).

Cell culture and treatment

The human NSCLC cell line A549, H69 and murine lung cancer LL/2 obtained from American Type Culture Collection (Manassas, VA, USA) was cultured in DMEM (Gibco, Waltham, MA, USA) or RPMI-1640 (Gibco) supplemented with 10% fetal calf serum, 2 mM glutamine (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml), and maintained at 37˚C with CO_2 in a humidified atmosphere.

CDDP-resistant NSCLC named A549R and H69R cells were established by stepwise protocol as reports (Barr et al. 2013). Briefly, A549 and H69 cells were treated with CDDP (Sigma, St. Louis, MO, USA) at IC50 for 72 h and allowed to recover for another 72 h. Through this cycle, A549 and H69 cells were continuously cultured in escalating concentrations of CDDP over half year to get the CDDP-resistant A549R and H69R cells. A549R and H69R cells were continuously cultured in DMEM complete medium with 10 μg/ml CDDP.

For drug treatment, A549, A549R, H69 and H69R cells were seeded and cultured in CDDP-free medium overnight. Then cells were treated with or without indicated amount CDDP for 12 and 24 h. The cells were pretreated with 10 μm Ku-55933 (an ATM kinase inhibitor) (Sigma) or dimethyl sulfoxide (DMSO; vehicle control) for 2 h.

Mice and tumor injection

C57BL6/J male mice were used to inject LL/2 cells at 8 weeks. LL/2 cells (5×10^5) were injected subcutaneously to the flank of mice. Tumor sizes were followed every 2-3 days from day 7 after tumor injection. Mice were scarified at day 15 after tumor injection. The size of tumor was calculated by length × width^2/2. Experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of China Medical University.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

RNA was purified by using the Trizol reagent (Invitrogen, Waltham, MA, USA) and DNase I treatment in A549 and A549R cells. cDNA was generated from 500 ng total RNA by PrimeScript RT reagent (Takara, Tokyo, Japan), and then cDNA was subjected to qRT-PCR using SYBR green real-time PCR Master Mix (TOYOBO, Tokyo, Japan). Small RNA RNU6B (U6 snRNA) or 18S rRNA were used as internal control. The primers used are described in Table 1.

Table 1. Sequences of oligonucleotides and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) primers.

Names	Sequence (5′-3′)
mir-181a-RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGC ACTGGATACGACACTCACC
mir-181a-Fw	GGGCAAAGTGCTTACAGTG
mir-181a-Rv	CAGTGCGTGTCGTGGAGT
RNU6B-RT	CGCTTCACGAATTTGCGTGTCAT
RNU6B-Fw	GCTTCGGCAGCACATATACTAAAAT
RNU6B-Rv	CGCTTCACGAATTTGCGTGTCAT
Cd274-Fw	TGCCGACTACAAGCGAATTACTG
Cd274-Rv	CTGCTTGTCCAGATGACTTCGG
18S rRNA-Fw	CTTAGAGGGACAAGTGGCG
18S rRNA-Rv	ACGCTGAGCCAGTCAGTGTA

RT, reverse transcription; Fw, forward; Rv, reverse.

Transfection

A549, A549R or LL/2 cells were seed in 10-cm dishes and transfected with negative control oligonucleotide, mir-181a or antisense oligonucleotide targeting mir-181a by lentivirus (Genepharma, Shanghai, China). The transfected cells were maintained for 48 h before treatment.

A549 cells were seed in 6-well plate and transfected with 3 μg empty vector or vector containing c-FOS by PEI Max (Polysciences, Warrington, PA, USA) as manufacturer guide. c-FOS cDNA was picked up form a cDNA library and cloned to pCR3 vector. 48 hours later, cells were used for drug treatment.

Western blot analysis

Cells were lysed in sodium dodecyl sulfate (SDS) sample buffer. Equal amounts of protein samples were separated in SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by trans-bolting to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, Burlington, MA, USA). Antibodies against PD-L1, ATM, p-ATM, c-FOS, β-actin (Santa Cruz Biotechnology, Dallas, TX, USA; 1:2,000 dilution), and horseradish peroxidase (HRP)-conjugated secondary antibody were used, and the specific protein level was visualized by using ECL as the HRP substrate. The density of each band was measured by Image J.

Flow cytometry (FACS)

A549 and A549R cells were stained with fluorescein isothiocyanate (FITC) anti-human CD274 (B7-H1, PD-L1) antibody (Biolegend, San Diego, CA, USA) for 30 min on ice.

Tumor-infiltrating lymphocytes (TIL) were purified by using collagenase D, DNase I digestion and Percoll density gradient. After FcR blocking, antibodies against, BV650-CD8, FITC-CD45, PerCP/Cyanine5.5-PD-1, and BV605-Tim-3 (Biolegend) were diluted at 1:400 in phosphate-buffered saline (PBS) with bovine serum albumin (BSA) for staining on ice for 30 min. For cytokine expression analysis, TIL were stimulated with cell activation cocktail (with Brefeldin A) (Biolegend) for 4 h at 37°C. APC-TNF-α and APC-GZMB (Biolegend) were used to stain the Intracellular proteins following the guide of BD Fixation/permeabilization kit (BD, Franklin Lakes, NJ, USA).

After wash by FACS buffer (PBS with 0.5% BSA, 1 μm EDTA), the cells were acquired on BD FACSCanto™ Cell Analyzer (BD) and analyzed with FlowJo v.10 software.

MTT assay

Cells were seeded into 96-well plates overnight for drug treatment. 0.5 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT, Sigma) solution were added to the cells, which were incubated for 2 h at 37°C. The residual cells were dissolved in DMSO and the optical density was measured at 490 nm wavelength using a microplate reader (Thermo Fisher Scientific, Middletown, VA, USA). Inhibition was calculated by the following equation: Inhibition ratio (%) = (A492 control − A492 sample) / (A492 control − A492 blank) × 100.

Statistical analysis

Results are presented as the mean ± standard deviation (SD). Differences between groups were examined for statistical significance using Student's t-test. Differences between groups in cell inhibition curves and tumor growth curves were examined using two-way ANOVA.

Results

PD-L1 is upregulated in CDDP-resistant NSCLC

To study the expression of PD-L1 in CDDP-resistant NSCLC, the A549 NSCLC cell line was exposed to increasing concentrations of CDDP to generate CDDP-resistant sub-colony A549R cells. Firstly, CDDP-induced cell death was measured by MTT assay in both A549 and A549R cells. A 10 μg/ml concentration of CDDP induced 37.1% A549 cell death at 24 h, while only 6.9% A549R cell death was observed under the same conditions. Moreover, CDDP-induced A549 cell inhibition curves at 12 h and 24 h were significantly different to those of A549R cells (Fig. 1A). This indicates that A549R cells are resistant to CDDP.

Transcription of *Cd274*, encoding PD-L1, was quantified by qRT-PCR in A549 and A549R cells after CDDP addition. Treatment with 10 μg/ml CDDP increased the expression of *Cd274* 2.3-fold at 24 h in A549 cells, but *Cd274* expression was not further elevated when the CDDP concentration was increased to 20 μg/ml (Fig. 1B). Consistently, the increased PD-L1 expression after CDDP addition was observed in another NSCLC cell lines H69 (Fig. 1C). In A549R cells, expression of *Cd274* was further increased 2-fold compared with CDDP-treated A549 cells, but further CDDP addition did not significantly alter expression of *Cd274* in A549R cells (Fig. 1D). This may be because A549R cells were cultured in 10 μg/ml CDDP to maintain drug resistance.

Moreover, expression of PD-L1 was quantified by FACS and western blot assay analyses in A549 and A549R cells after 10 μg/ml CDDP treatment for 24 h. FACS analysis indicated that PD-L1-positive cells were increased in A549 cells after CDDP addition, and PD-L1-positive cells were further increased in A549R cells (Fig. 1E). Consistently, western blot analysis indicated that A549R cells had contained more PD-L1 than CDDP-treated A549 cells (Fig. 1F). H69R, another CDDP-resistant NSCLC cells, were used to confirm the expression of *Cd274*. Increased *Cd274* expressions were observed in H69R cells compared to CDDP-treated H69 cells (Fig. 1G).

These results showed that CDDP-induced upregulation of PD-L1 could be further increased in CDDP-resistant NSCLC cells. Therefore, a negative regulatory mechanism of PD-L1 expression may occur after CDDP addition in NSCLC, while this system may be blocked in CDDP-

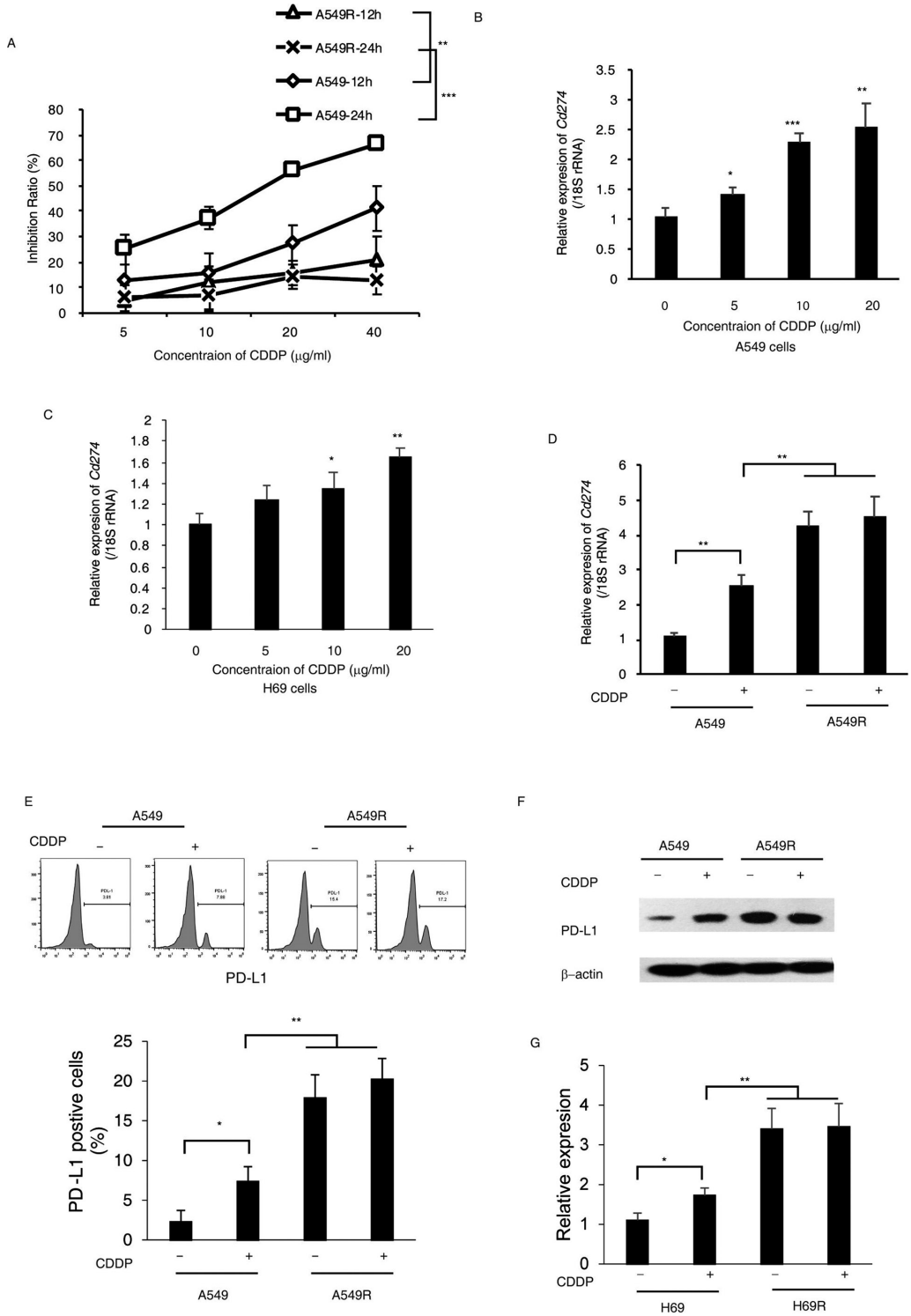

Fig. 1. PD-L1 is upregulated in CDDP-resistant NSCLC.
(A) The cell inhibition ratio was measured by MTT assay in A549 and A549R cells. A549 and A549R cells were treated with CDDP at the indicated concentrations for 12 and 24 h. Results are expressed as mean ± standard deviation (SD; n = 3). Two-way analysis of variance (ANOVA) was used to test differences (**$p < 0.01$, ***$p < 0.001$). qRT-PCR was used to measure expression of *Cd274* in A549 cells (B) and H69 cells (C) treated with the indicated doses of CDDP for 24 h. (D) Expression of *Cd274* was measured by qRT-PCR in A549 and A549R cells treated with or without 10 μg/ml CDDP for 24 h. (E) PD-L1-positive cells were measured by FACS analysis in A549 and A549R cells treated with or without 10 μg/ml CDDP for 24 h. Results are expressed as mean ± SD (n = 3; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (F) Western blot analysis of the expression of PD-L1 in A549 and A549R cells treated with or without 10 μg/ml CDDP for 24 h. (G) Expression of *Cd274* was measured by qRT-PCR in H69 and H69R cells treated with or without 10 μg/ml CDDP for 24 h.

resistant A549 cells.

Mir-181a expression is repressed in CDDP-resistant NSCLC

Mir-181a reportedly negatively regulates the INF-γ pathway, and thereby, contributes to regulate PD-L1 expression (Sang et al. 2015; Garcia-Diaz et al. 2019). Mir-181a was therefore quantified in A549 and A549R cells after CDDP addition by qRT-PCR. Expression of mir-181a was significantly increased by CDDP in A549 cells (Fig. 2A) and H69 cells (Fig. 2B) in dose-dependent manner. Mir-181a expression in A549R cells was significantly lower than in CDDP-treated A549 cells (Fig. 2C). To understand the relationship between mir-181a and PD-L1 expression in CDDP-treated A549R cells, mir-181a was transfected into A549R cells and *Cd274* expression was measured by qRT-PCR. Overexpression of mir-181a in A549R cells significantly reduced the expression of *Cd274* (Fig. 2D). Consistently, PD-L1-positive cells were significantly decreased in mir-181a-overexpressing A549R cells (Fig. 2E). CDDP-induced increase of mir-181a was not observed in H69R cells (Fig. 2F).

This indicates that CDDP-induced mir-181a is a negative regulator of PD-L1 expression in NSCLC, and increased PD-L1 expression in CDDP-resistant NSCLC cells might be associated with a reduction in mir-181a.

CDDP regulates PD-L1 expression via the ATM-mir-181a-c-FOS pathway in NSCLC

Since CDDP is reported to regulate mir-181a expression via phosphorylation of ATM in head and neck squamous cell carcinoma (Huang et al. 2011), Ku-55933, an ATM kinase inhibitor, was used to analyze the role of the ATM pathway and mir-181a expression in NSCLC. The CDDP-induced increase in mir-181a expression was significantly repressed after Ku-55933 addition in A549 cells (Fig. 3A), whereas expression of *Cd274* was further significantly increased after Ku-55933 in CDDP-treated A549 cells (Fig. 3B). This indicates that expression of PD-L1 and mir-181a is dependent on the ATM pathway in CDDP-treated A549 cells.

Next, we transiently expressed mir-181a in A549 cells, and expression of *Cd274* and the ATM pathway were analyzed after CDDP addition. The extremely high levels of *Cd274* induced by Ku-55933 and CDDP were significantly reduced after additional mir-181a in A549 cells (Fig. 3C). This confirmed that *Cd274* expression is regulated by the ATM pathway via mir-181a in CDDP-treated A549 cells.

Furthermore, mir-181a is reported to bind c-FOS mRNA and thereby negatively regulate c-FOS expression (Wu et al. 2012). The relationship between mir-181a and c-FOS expression was investigated using an mir-181a antisense oligonucleotide to block the function of mir-181a in CDDP-treated A549 cells. In control cells, CDDP obviously reduced the expression of c-FOS, and expression of c-FOS was derepressed after mir-181a antisense oligonucleotide addition in A549 cells (Fig. 3D). This indicates that CDDP repressed c-FOS expression via mir-181a in A549 cells.

Finally, the relationship between c-FOS and PD-L1 was explored by overexpressing c-FOS in CDDP-treated A549 cells. CDDP-induced *Cd274* expression was further significantly increased in c-FOS-overexpressing A549 cells (Fig. 3E). This indicates that CDDP regulates PD-L1 expression via c-FOS in A549 cells.

Overall, the results indicate a negative regulatory mechanism for PD-L1 in CDDP-treated NSCLC via the ATM-mir-181a-c-FOS pathway.

CDDP-resistant NSCLC represses the ATM-mir-181a-c-FOS pathway

The ATM-mir-181a-c-FOS pathway status was then checked in CDDP-resistant NSCLC by western blot assay. CDDP increased the ratio of phosphorylated ATM to ATM in A549 cells, and the ratio of phosphorylated ATM to ATM in A549R cells was lower than that in CDDP-treated A549 cells (Fig. 4A). CDDP-induced downregulation of c-FOS in A549 cells was not observed in A549R cells (Fig. 4B). This suggests that the ATM-mir-181a-c-FOS pathway was repressed in CDDP-resistant NSCLC, which further derepresses PD-L1 expression.

Mir-181a in tumor enhance the anti-tumor response in vivo

Since tumoral PD-L1 could repress the cytotoxic $CD8^+$ T cells function, induce T cell exhaustion and promote tumor growth, LL/2, an immunogenic lung cancer, was injected to mice, and the tumor growth curve and T cell function were analysis. The growth of LL/2 tumor cells with exogenous mir-181a was significantly slower compared to the LL/2 without mir-181a (Fig. 5A). Decreased PD-L1 expression was observed in LL/2 with mir-181a expression (Fig. 5B).

Further, the function of cytotoxic $CD8^+$ T cells was analyzed by using tumor-infiltrating lymphocytes (TIL). Exhausted T cells indicating as $PD\text{-}1^+$ and $Tim\text{-}3^+$ $PD\text{-}1^+$ $CD8^+$ T cells were reduced (Fig. 5C-E) in TIL of mir-181a-expressed LL/2, and the cytotoxicity function indicating $TNF\text{-}\alpha^+$ (Fig. 5F) and $GZMB^+$ (Fig. 5G) $CD8^+$ T cells were significantly increased in the TIL of mir-181a-expressed LL/2. This suggested that lung cancer with mir-181a could enhance the anti-tumor response and repress the T cell exhaustion via downregulation of PD-L1.

Discussion

Herein, CDDP was found to induce PD-L1 expression in NSCLC, especially in CDDP-acquired resistant NSCLC. High levels of PD-L1 expression in tumor cells will likely repress the antitumor response by inducing T cell exhaustion, which induces overexpression of inhibitory receptors and decreases effector cytokine production and cytolytic activity, leading to failure to eliminate cancer (Jiang et al. 2015). Consistently, multiple chemotherapeutic treatments

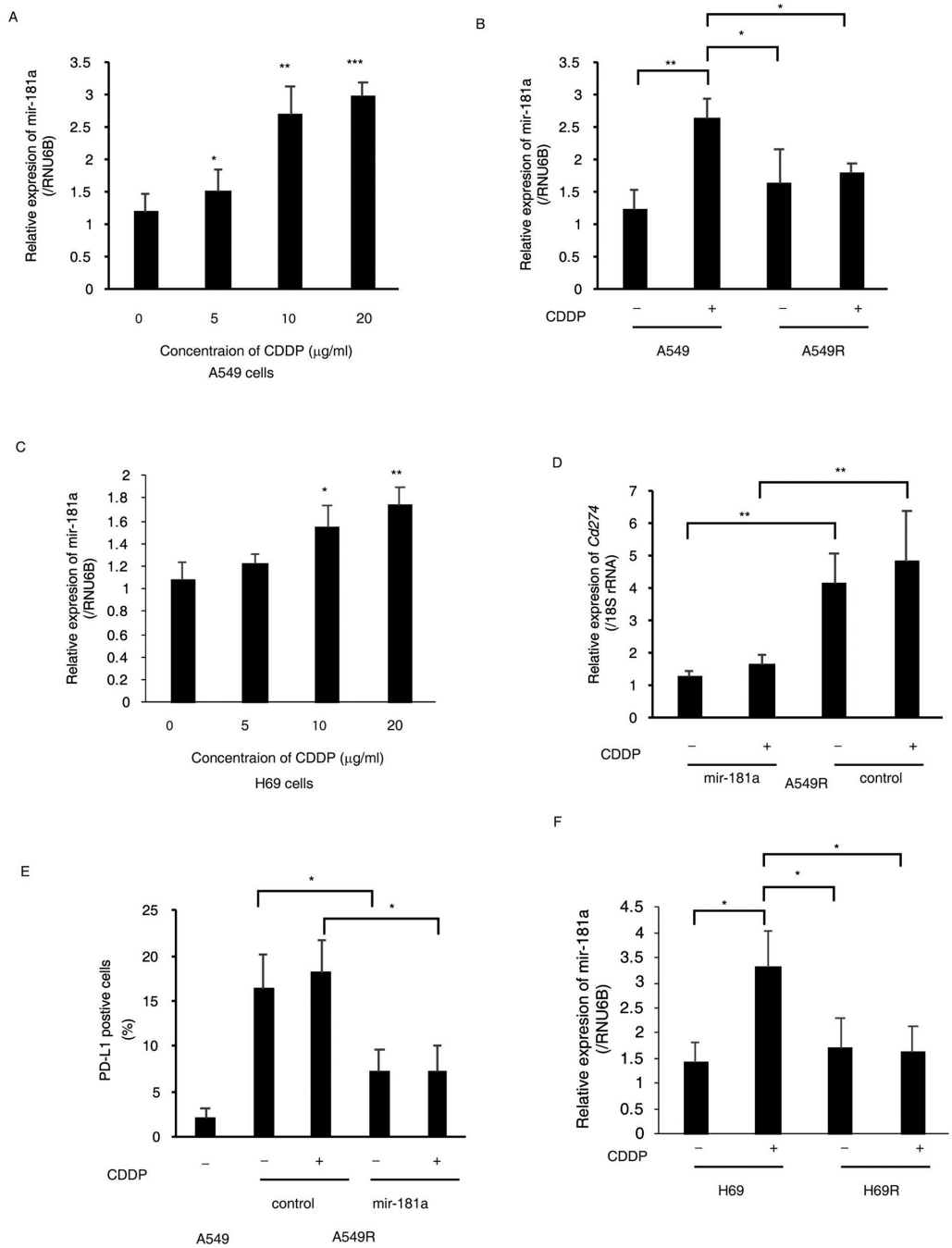

Fig. 2. Mir-181a expression is repressed in CDDP-resistant NSCLC.
(A) Expression of mir-181a was analyzed by qRT-PCR in A549 cells treated with CDDP at the indicated concentrations for 24 h. (B) Expression of mir-181a was measured by qRT-PCR in A549 and A549R cells treated with or without 10 μg/ml CDDP for 24 h. (C) Expression of mir-181a was analyzed by qRT-PCR in H69 cells treated with CDDP at the indicated concentrations for 24 h. (D) Expression of *Cd274* was measured by qRT-PCR in A549R cells transfected with mir-181a or negative control oligonucleotide (control). (E) PD-L1-positive cells were measured by FACS analysis in A549R cells. A549R cells were transiently transfected with mir-181a or negative control oligonucleotide (control), and then treated with or without 10 μg/ml CDDP for 24 h. (F) Expression of mir-181a was measured by qRT-PCR in H69 and H69R cells treated with or without 10 μg/ml CDDP for 24 h. Results are expressed mean ± SD (n = 3; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

involving gemcitabine, paclitaxel and fluorouracil can induce PD-L1 expression in cancer cells (Peng et al. 2015; Ng et al. 2018). However, CDDP is known to induce antitumor immunomodulation by increasing MHC class I expression and recruiting effector cells (de Biasi et al. 2014). This indicates that CDDP may enhance the antitumor response, and may also contribute to T cell exhaustion.

In acquired CDDP-resistant NSCLC, PD-L1 expres-

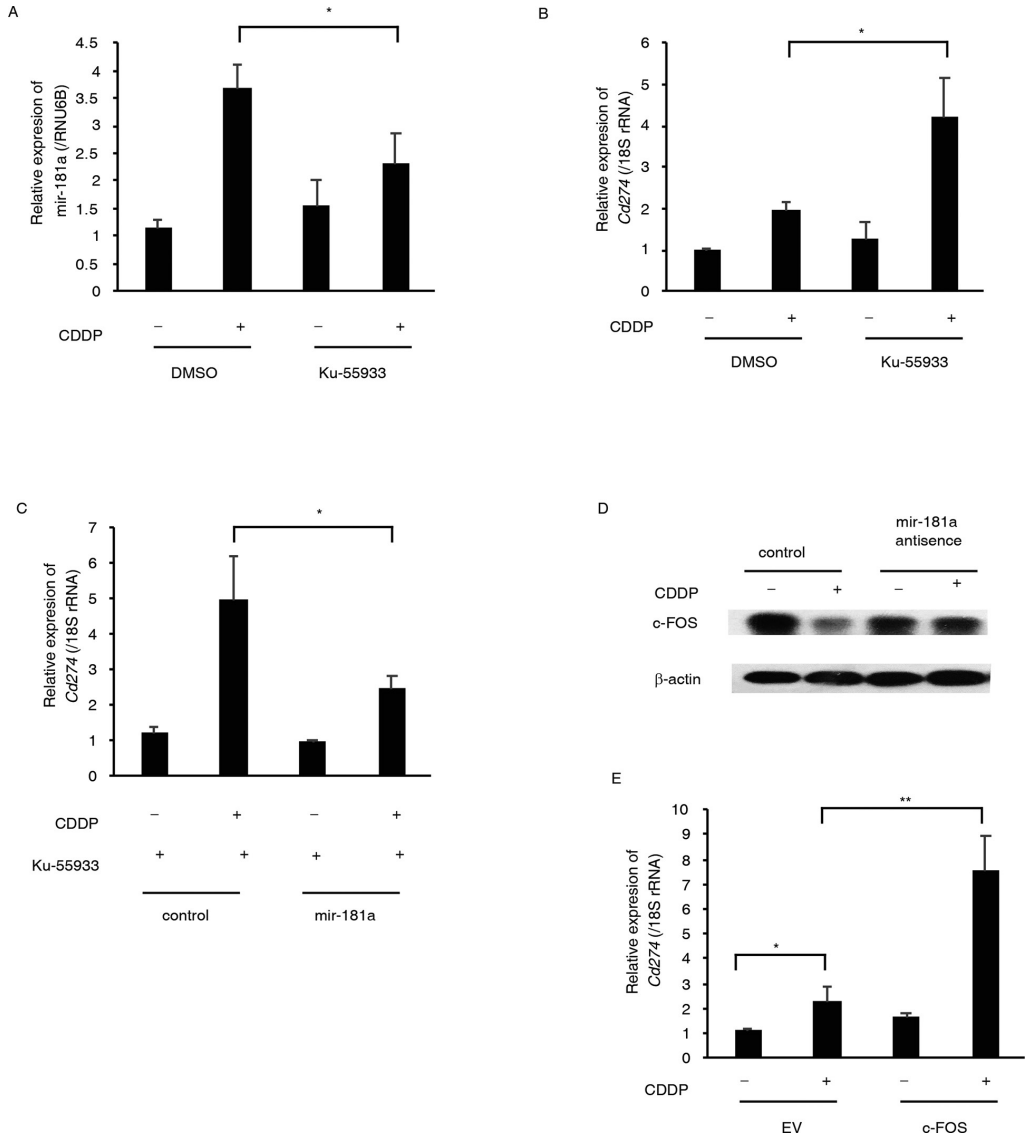

Fig. 3. CDDP regulates PD-L1 expression via the ATM-mir-181a-c-FOS pathway in NSCLC. Expression of mir-181a (A) and *Cd274* (B) was analyzed by qRT-PCR in A549 cells treated with or without 10 μg/ml CDDP for 24 h. Ku-55933 (10 μm) was pretreated for 2 h prior to CDDP addition. Dimethyl sulfoxide (DMSO) was used as vehicle control. (C) Expression of *Cd274* was analyzed by qRT-PCR in A549 cells transfected with mir-181a or negative control oligonucleotide (control). Cells were then treated with CDDP and Ku-55933 as above. Results are expressed as mean ± SD (n = 3; *$p < 0.05$). (D) Expression of c-FOS was measured by western blot assay in A549 cells transfected with negative control oligonucleotide or mir-181a antisense oligonucleotide. Cells were then treated with or without 10 μg/ml CDDP for 24 h. (E) Expression of *Cd274* was measured by qRT-PCR in A549 cells transfected with vector harboring c-FOS or empty vector (EV). Cells were then treated with or without 10 μg/ml CDDP for 24 h. Results are expressed as mean ± SD (n = 3; *$p < 0.05$, **$p < 0.01$).

sion was further enhanced. The tumor microenvironment has been demonstrated to play a key role in the development of cisplatin resistance, and CDDP-resistant tumor cells can modify cytokines and chemokine expression in the tumor microenvironment (Chen and Chang 2019). Additionally, combination of CDDP and PD-L1 blockage can improve the prognosis of NSCLC patients (Wu et al. 2019). This indicates that increased PD-L1 expression may contribute to acquired CDDP resistance and alter the tumor microenvironment in NSCLC.

The miRNA mir-181a is reportedly induced in response to CDDP addition, and it regulates cell death in NSCLC and cervical cancer cells (Galluzzi et al. 2010; Xu et al. 2019). Herein, increased mir-181a was observed to regulate c-FOS expression in CDDP-treated NSCLC. c-FOS is a subunit of transcription factor AP-1, which can directly bind the first intron of the PD-L1 gene and promote PD-L1 transcription in Hodgkin's lymphoma cells (Green et al. 2012). Consistently, exogenous c-FOS in NSCLC could further elevate CDDP-induced PD-L1 expression in NSCLC. Thus, CDDP may regulate PD-L1 expression via c-FOS and mir-181a in NSCLC.

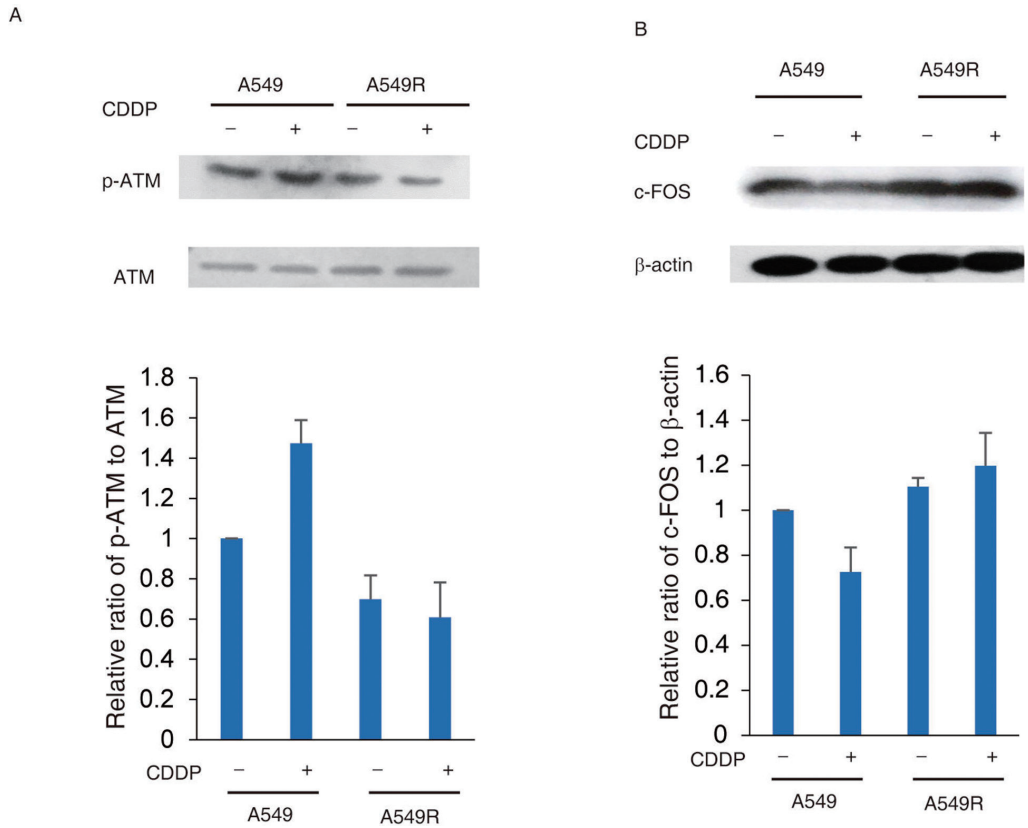

Fig. 4. CDDP-resistant NSCLC represses the ATM-mir-181a-c-FOS pathway.
Western blot analysis was used to measure the abundance of p-ATM and ATM (A) and c-FOS (B) in A549 and A549R cells treated with or without 10 μg/ml CDDP for 24 h. All the experiment was repeated 3 times. The relative density was shown in the lower panels. Results are expressed as mean ± SD.

CDDP binds to the N7 position of purines to form intra- and inter-strand crosslinks that cause DNA damage (Mantri et al. 2007). Meanwhile, CDDP-induced DNA damage can recruit and active ATM to mediate DNA damage response signaling pathways. ATM activates a second wave of phosphorylation of downstream factors regulating expression of target genes involved in DNA repair, apoptosis, and other key pathways (Maréchal and Zou 2013). Herein, mir-181a was found to be regulated by the ATM pathway in response to CDDP addition. Therefore, CDDP did not induce mir-181a expression because it is unresponsiveness to the ATM pathway in NSCLC.

In vivo study indicates that mir-181 in tumor cells could repress the PD-L1 expression, and repress the T cell exhaustion and promote cytotoxicity T cell function. Interestingly, PD-L1 is expressed in both tumor cells and stroma cells in microenvironment of lung cancer, lymphoid malignancies and others (Miyoshi et al. 2016; Wu et al. 2018). Regulatory T cells (Tregs) were found to express PD-L1 to inhibit the functions of CD8 T cells (Liu et al. 2014). During the development of Tregs, mir-181a deficiency impaired *de novo* generation of thymic regulatory T cells (Lyszkiewicz et al. 2019). Besides, mir-181 controls T cell activation, and regulates immunosenescent (Kim et al. 2021). Therefore, understanding whether CDDP-induced PD-L1 expression in Tregs and whether mir-181a plays a key role in regulating PD-L1 expression in Tregs could be another interesting research points in the future.

In vivo study found that exhausted T cells (Tex) were reduced, and the cytotoxicity functions of CD8 T cells were increased in the microenvironments of mir-181-expressed tumor cells. PD-1 and Tim-3 are typical immune inhibitory receptors. Tim-3$^+$PD-1$^+$ TILs exhibit the most severe exhausted phenotype as defined by failure to proliferate and produce IL-2, TNF, and IFN-γ (Sakuishi et al. 2010). Therefore, the decreased PD-1$^+$ and Tim-3$^+$PD-1$^+$ TIL may promote the proliferation of TIL and increase the number of cytotoxicity T cells in the host bearing mir-181-expressed tumor cells. Thus, tumor bearing mir-181 may repress T cell exhaustion and promote T cell function via repressing PD-L1 expression.

In conclusion, CDDP was found to induce negative feedback regulation of PD-L1 expression in NSCLC via ATM phosphorylation-mediated mir-181a expression. Additionally, CDDP-resistant NSCLC blocked this negative regulation system to further induce PD-L1 expression. Increased mir-181a in lung cancer could enhance the anti-tumor response *in vivo* via downregulation of PD-L1 (Fig. 6).

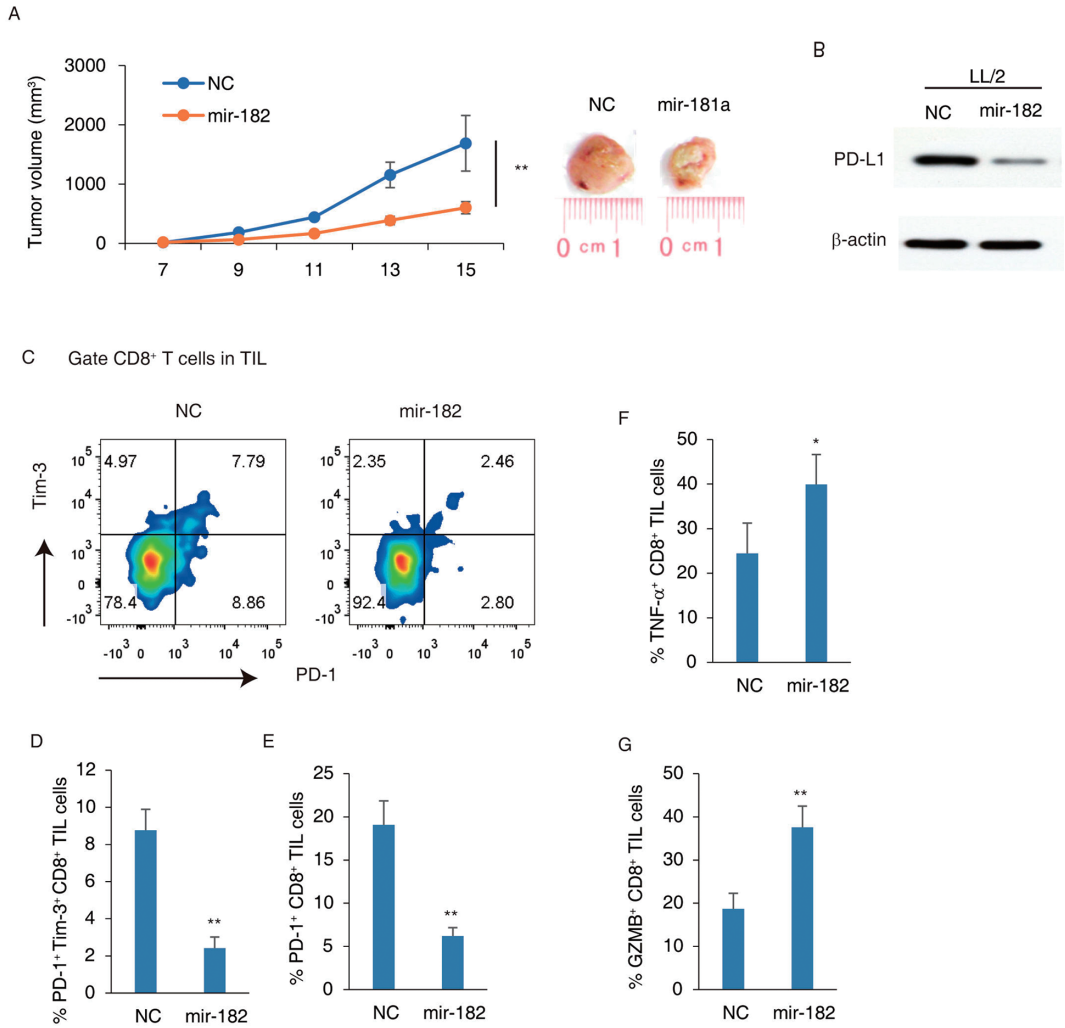

Fig. 5. Mir-181a in lung cancer enhance the anti-tumor response *in vivo*.
(A) Tumor growth curve was measured by using LL/2 cell with or without exogenous mir-181a. Results are expressed as mean ± SD (n = 5, $**p < 0.01$). The typical photos of tumor were shown. (B) Western blot analysis was used to measure the abundance of PD-L1 from LL/2 tumor at day 15 after tumor injection. (C-E) PD-1$^+$ and Tim-3$^+$ CD8$^+$ T cells from TIL were measured by FACS at day 15 after tumor injection. TNF-α^+ (F) and GZMB$^+$ (G) CD8$^+$ T cells from TIL were measure by FACS at day 15 after tumor injection. Results are expressed mean ± SD (n = 3; $*p < 0.05$, $**p < 0.01$).

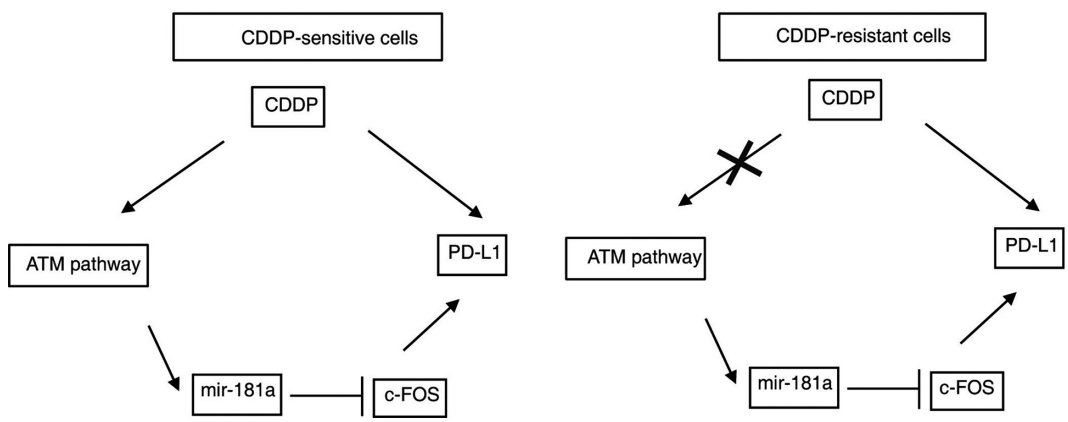

Fig. 6. Proposed regulatory mechanism of PD-L1 in acquired CDDP-resistant NSCLC.

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Conflict of Interest

The authors declare no conflict of interest.

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