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Original Article

POU2F1 induces the immune escape in lung cancer by up-regulating PD-L1

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Abstract: Purpose: The aim was to research the POU2F1 related genes and mechanism during the progress of immune escape of lung cancer. Methods: Lung cancer cell lines (H1993, HCC827, A549, H2228, H3122 and H1975) and Human normal lung epithelial cell line (BEAS-2B) were involved in this study. Overexpression or knockdown of POU2F1 was processed in lung cancer cells. POU2F1, PD-L1 and CRK expression in cells were detected by WB and RT-PCR. Flow cytometry and immunofluorescence was used to detect PD-L1 expression on the cell surface. Luciferase reporter detected the promoter activity of CRK. C57BL/6 mice models with knocked down of of POU2F1 were constructed. After tumor formation, anti-PD-1 was administered to detect tumor suppressing ability. IHC assay showed the number of intratumoral CD3+, CD8+, GranzB+ T cells. Results: POU2F1 and PD-L1 were positively correlated in lung cancer cell lines. Overexpression of POU2F1 promoted the expression level of PD-L1 in lung cancer cells. POU2F1 transcription activated the expression of CRK, and further promoted the expression of PD-L1. Knockdown of POU2F1 promoted the efficacy of Anti-PD-1. In addition, tumor growth ability decreased after POU2F1 was knocked down. Cytotoxic effector cytokines levels, tumor suppressive chemokines and interleukin increased, while IL17a level decreased when POU2F1 was knocked down. Conclusion: POU2F1 activates the expression of CRK, further promotes the expression of PD-L1, and finally improves the immune escape in lung cancer.

Keywords: Lung cancer, POU2F1, PD-L1, immune escape

Introduction

Lung cancer is the most common malignant tumor with extensive morbidity and mortality [1]. Most patients are diagnosed at an advanced stage because of the poor prognosis [2]. With the rapid development of medical technology, immunotherapy has become a hot spot in the treatment of lung cancer [3]. It is worth noting that the Chinese Society of Clinical Oncology lung cancer treatment guidelines focus on tumor immunotherapy. At present, immunotherapeutic such as PD-1/PD-L1 immunosuppressive agents account for the majority [4]. The antibodies for blocking the PD-1/PD-L1 pathway enhances the autoimmune response, improves the body's own immune system to detect and attack cancer cells, breaks the tumor immune escape mechanism, thereby inhibits tumors and achieving cancer treatment [5].

In 2014, Opdivo (BRISTOL MYERS SQUIBB) and Keytruda (MERCK SHARP DOHME) were successively approved for marketing. Then, PD-1 inhibitors were approved by FDA as first-line drugs for the treatment of non-small cell lung cancer, which is an important milestone in immunotherapy for non-small cell lung cancer [6]. As shown in a phase III randomized controlled trial, after radical concurrent chemoradiotherapy for patients with unresectable locally advanced non small-cell lung cancer (NSCLC), PD-L1 inhibitor (Durvalumab) could effectively improve Progression-Free-Survival (PFS), disease remission rate and duration of remission, distant metastasis or death [7]. Based on the National Comprehensive Cancer Network (NCCN) guidelines for lung cancer, Durvalumab has been recommended as a consolidation therapy for locally advanced NSCLC after concurrent chemoradiotherapy [8]. However, for EGFR or ALK-positive patients, first-line

immunotherapy is not recommended. Besides, for first-line treatment of advanced lung cancer, the use of immunotherapy is limited to patients with higher PD-L1 expression. Although immunotherapy has brought new hope to patients, the effectiveness of immunotherapeutic inhibitors is not satisfactory due to the consistency of tumors and individualized differences in patients [9]. Thereby, more detail molecular mechanism of immunotherapeutics in lung cancer, need to be further explored for auxiliary and supplementary.

In previous studies, POU2F1 has been confirmed to participate in various cancers. In hepatic cell carcinoma, the gene was associated with NRP1, which further regulated the regulate metastasis progress [10]. As reported by Feng et al. [11], NRP1 was an independent factor for overall survival time and chemotherapy response in advanced non-small cell lung cancer. Moreover, it was a critical gene for lung branching and angiogenesis [12]. Besides, it was closely related with radio-resistance of NSCLC cells by participating the VEGF-PI3K-NF-κB pathway [13]. Moreover, down-regulated NRP1 could inhibit invasion, migration and tumor genesis via participating in VEGF, PI3K, and Akt pathways in lung cancer [12]. Apart from this, down-regulated NRP1 could not only decrease the clonogenic and self-renewal potential processes, but also be closely related with tumor-initiating properties [14]. Thereby, we inferred that POU2F1 was also a critical gene for lung cancer.

Interestingly, POU2F1 was found to be closely related with PD-L1 in lung cancer by a large number of pre-studies. It was worth noting that c-Crk affected p120-catenin transcriptional repression and then promoted metastasis in NSCLC [15]. Recent study confirmed that Crk proteins could be regarded as important signal molecules in immune functions [16]. Thereby, POU2F1 might participate in the pathogenesis of lung cancer by activating the expression of CRK. Interestingly, our findings are consistent with the above evidence. This study might provide new targets or potential adjuvant treatment mechanisms for immunotherapy of lung cancer.

Methods

Cell culture

Lung cancer cell lines (H1993, HCC827, A549, H2228, H3122 and H1975) and Human normal lung epithelial cell line (BEAS-2B) were purchased from American Type Culture Collection (ATCC, USA). Lung cancer cells were routinely cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin, placed in a 37°C, 5% CO_2 incubator, digested and subcultured by 0.02% EDTA.

Overexpression plasmid construction and transfection

Primers were designed to amplify the POU2F1 coding region based on the POU2F1 sequence. PCR amplification was carried out using PyrostarTM DNA Polymerase (TaKaRa) using 2 μl of cDNA as a template. After electrophoresis, the PCR product was recovered and verified by sequencing. The recovered product and the pcDNA3.1 vector were double digested with EcoRI and KpnI. Electrophoresis and gel recovery were performed after Celsius digestion at 37°C. The recovered POU2F1 fragment and the pcDNA3.1 vector fragment were ligated with T4 DNA Ligase (TaKaRa) at 16°C. The ligation product was transformed with DH5 competent cells, plated on the surface of an LB plate containing 100 mg/L ampicillin, and cultured in a 37°C biochemical incubator overnight. The single colony was selected for expanding cultivation. The plasmid was extracted and identified by double enzyme digestion, and the positive clone was verified by sequencing. The cells were seeded into 6-well plates. Transfection was performed when the cell confluence reached about 70%. The plasmid transfection amount was 4 μg/well, and transfection process was according to the instruction manual of lipofectamine 2000 (Invitrogen). The culture media were changed after 4-6 hours of transfection.

Knockdown of POU2F1 or CRK

The interference sequence shRNA was designed based on the cDNA sequence of POU2F1 and CRK. The lentiviral vector system (pGC-FU) was purchased from GENEcHEM Co., ltd (Shanghai, China). Competent cells were prepared by the calcium chloride method. The pGC-FU-POU2F1 ligated product was used to transform competent bacteria, and the shRNA-POU2F1 interference vector was identified. The universal primer insert of pGC-FU vector was used for sequencing. The sequenced pGC. FU-sh plasmid and two auxiliary packaging elements were transfected into 293T cells with

Lipofetamine 2000. The expression of virus green fluorescent protein after packaging was observed under a fluorescent microscope, the virus titer was detected and calculated for using. The shRNA sequence was synthesized by Shanghai Yingjun Biotechnology Co., Ltd. The sequence of shRNA was 5'-GCAGCGAGTCAAGATGAGAGT-3'. The cells were planted in a 24-well plate, transfection was undertaken when the cell confluence was about 30%, and the total amount of the whole medium before transfection was 0.45 ml. Lentivirus with 1 ug (50 pmol) of shRNA was taken, a certain amount of serum-free dilution was added and mix well to make RNA dilution solution. The final volume was 25 μl. 1.5 ul of Entranster-R4000 was added, and then 24 ul of serum-free diluted liquid was added and mixed well to make an Entranster-R4000 dilution to a final volume of 25 μl. The Entranster-R4000 dilution and the shRNA dilution were thoroughly mixed and allowed to stay at room temperature for 15 minutes. The transfection complex is prepared successfully. 50 μl of the transfection complex was added dropwise to cells and mixed well. The culture media were changed after 4-6 hours of transfection.

RT-PCR assay

Trizol reagent was added to the sample following the instructions for RNA extraction, and total RNA were obtained. The purity and concentration of RNA were detected. Then, 1 μg total RNA was reverse transcribed with M-MLV reverse transcriptase. PCR amplification was processed. Simultaneous amplification of β-actin was undertaken as an internal reference. The primers were as follows: POU2F1 (forward) GAGCAGCGAGTCAAGATGAGA, (reverse) GGGCTGCTTCTCAAAGTCCA; PDL1 (forward) TTTCAATGTGACCAGCAC, (reverse) GGCATAATAAGATGGCTC; CRK (forward) ACTATGTGCTCAGCGTCTCA, (reverse) ATTCCACCACTGCTCTTCA. Besides, Cytotoxic effector cytokines (granzyme B, TNF-a, IFN-g), tumor suppressive chemokines (CCL5, CXCL9, CXCL10), interleukin (IL1b, IL12b, IL15 and IL17a) were also detected as previous study reported [17]. The relative expression software tool was used to detect the amount of above mRNAs.

Western blot

Total protein of tumor tissue was extracted, centrifuged for 20 min at 4°C, 12000 r/min, supernatant was taken and stored at -20°C. The BCA protein quantification kit was used to detect the protein concentration. SDS-PAGE electrophoresis was performed at a volume of 50 μg per well. The separated protein was transferred to a nitrocellulose membrane, blocked by 5% skim milk for 2 h, incubated with the primary antibody (POU2F1, Anti-POU2F1 rabbit polyclonal antibody, Cat. No. ml221070, mlbio co, ltd; PD-L1, Rabbit monoclonal to PD-L1, Cat. No. ab228415, Abcam; β-actin, Rabbit monoclonal [EPR16769] to Actin, Cat. No. ab179467, Abcam) at 4°C overnight and then incubated with secondary antibody (1:10000, Goat Anti-Rabbit IgG H&L (HRP), Cat. No. ab6721, Abcam). The relative value of protein was calculated.

Flow cytometry

The prepared cells were taken, the cell concentration was adjusted to 5 × 10^6/100 μl and the cells were added to the flow cytometry test tube. 5 μl of PDL1-FITC were added, and mixed protecting from light. The cells were incubated at 4°C for 30 min, washed, centrifuged at 1200 rpm for 10 min, and then washed twice. The supernatant was removed, the sample was dried, and 0.5 ml of the fixative was added and mixed. Finally, the sample was machine detected by flow cytometry (Becton Dickinson, Heidelberg, Germany). CELLQuestk software was used to analysis the FACS data.

Immunofluorescence assay

The slides which had climbed the cells were immersed 3 times with PBS in the culture plate. The slides were fixed with 4% paraformaldehyde for 15 min, immersed in PBS for 3 times, and then permeabilized in 0.5% Triton X-100 at room temperature for 20 min. Normal goat serum was added to the slide and blocked at room temperature for 30 min. The blocking solution was removed. Primary antibody (PD-L1, Rabbit monoclonal to PD-L1, Cat. No. ab228415, Abcam) was added to each slide and incubated at 4°C overnight. The samples were incubated with fluorescent secondary antibody (1:1000, Goat Anti-Rabbit IgG H&L (HRP), Cat. No. ab6721, Abcam) at room temperature for 1 h, and immersed in PBST for 3 times. Then, it was incubated with DAPI in the dark for 5 min to stain the nucleus. The liquid on the climbing plate was blotted, the sample was sealed with a sealing liquid containing an

anti-fluorescence quencher, and the image was observed under a fluorescence microscope.

Dual luciferase assay

POU2F1 knockdown was undertaken in HCC-827 cells. The luciferase reporter detected the promoter activity of CRK. The brief steps were as follows. Cells were harvested for 48 hours after transfection, and lysed with 1 × passive lysis buffer and shaken for 15 minutes at room temperature on a shaker. Turner TD20/20 luminometer was used for detection, 50 μl of luciferase assay reagent was used to detect the background value of the reagent, and 20 μl of PLB lysate was applied to detect the fluorescein signal.

According to the study of Xu et al. [18], POU2F1 was predicted to combine with the promoter of CRK. The primers were designed according to this site, the desired target promoter fragment was cloned by PCR, and this fragment was inserted into the luciferase reporter plasmid. Positive clones were screened, sequenced, and amplified and the plasmid was purified. The above steps were designed and completed by Shanghai Yingjun Biotechnology Co., Ltd. 293 cells were cultured and seeded in 12-well plates. After 24 hours, the reporter plasmid and the transcription factor expression plasmid were co-transfected into the cells. The extracted protein was used for the detection of luciferase activity. The luciferase activity was measured by a fluorescence counter to calculate the fluorescence intensity.

CHIP PCR assay

One plate of cells was removed, 243 ul of 37% formaldehyde was added to reach a final concentration of formaldehyde of 1%, and incubated at 37°C for 10 min. 450 ul 2.5 M glycine were added in cells, mixed, and stayed at room temperature for 5 min for termination of cross-linking. The cells were collected, SDS Lysis Buffer and protease inhibitor complex were added, and ultrasonic crushing was processed. In 100 ul of sonicated product, 900 μl of ChIP Dilution Buffer and 20 μl of 50 × PIC were added. Total 60 μl Protein Agarose/Salmon Sperm DNA were added to mix at 4°C for 1 h. After that, the pellet was allowed to stand at 4°C for 10 min and centrifuged at 700 rpm for 1 min. The supernatant was taken and antibody (POU2F1, Anti-POU2F1 rabbit polyclonal antibody, Cat. No. ml221070, mlbio co, ltd) was added. The precipitation of immune complexes were washed and eluted. DNA samples were decrosslinked and recycled. PCR assay was then undertaken.

Detecting tumor growth ability and mouse body weight

Total 120 C57BL/6 mice were purchased from Chengdu Dashuo Experimental Animal Co., Ltd. The study was approved by the Ethics Committee of China Medical University and followed the guidelines of the management and use of laboratory animals. The experimental animals underwent all operations under anesthesia, and we made every effort to minimize their pain, suffering and death. C57BL/6 mice are strains commonly used in oncology, physiology, immunology, and genetics [19]. Inbred mice have high precision, good comparability, and uniform stress response [20]. Cellular immunity decreases with age, and this animal model is more likely to induce immune tolerance [21]. In previous studies, C57BL/6 mice have been widely used as animal model for immune escape system with good results [22]. Thereby, this animal model was used in this study. The mice were fed with free diet and drinking water. POU2F1 was knocked down in HCC827 cells, and 0.2 ml of cell suspension (2 × 10^6/ml) were injected into C57BL/6 mice subcutaneously. After antitumor formation, anti-PD-1 was administered to detect tumor suppressing ability. Based on the transfection and Anti-PD-1 administration, the mice were divided into four groups, including sh-NC+IgG, sh-POU2F1+IgG, sh-NC+Anti-PD-1 and sh-POU2F1+Anti-PD-1 groups. Anti-PD-1 and IgG were purchased from Bioss Biotechnology Co., Ltd (Beijing, China). Anti-PD-1 or IgG was intraperitoneal injected according to the dose of 100 μl/mice, 3 times/d for 3 days. The tumor volume, tumor weight and body were detected per 5 days. And the experiment was finished at 30th day.

IHC detects the number of CD3+, CD8+, GranzB+ T cells in the tumor tissue

4 μm thick paraffin-embedded tumor tissues were dewaxed to water, washed with PBS for antigen heat repair. The sections were immersed in EDTA buffer and heated, then washed with PBS, and incubated with 3% H_2O_2 methanol for 15 min to eliminate endogenous peroxi-

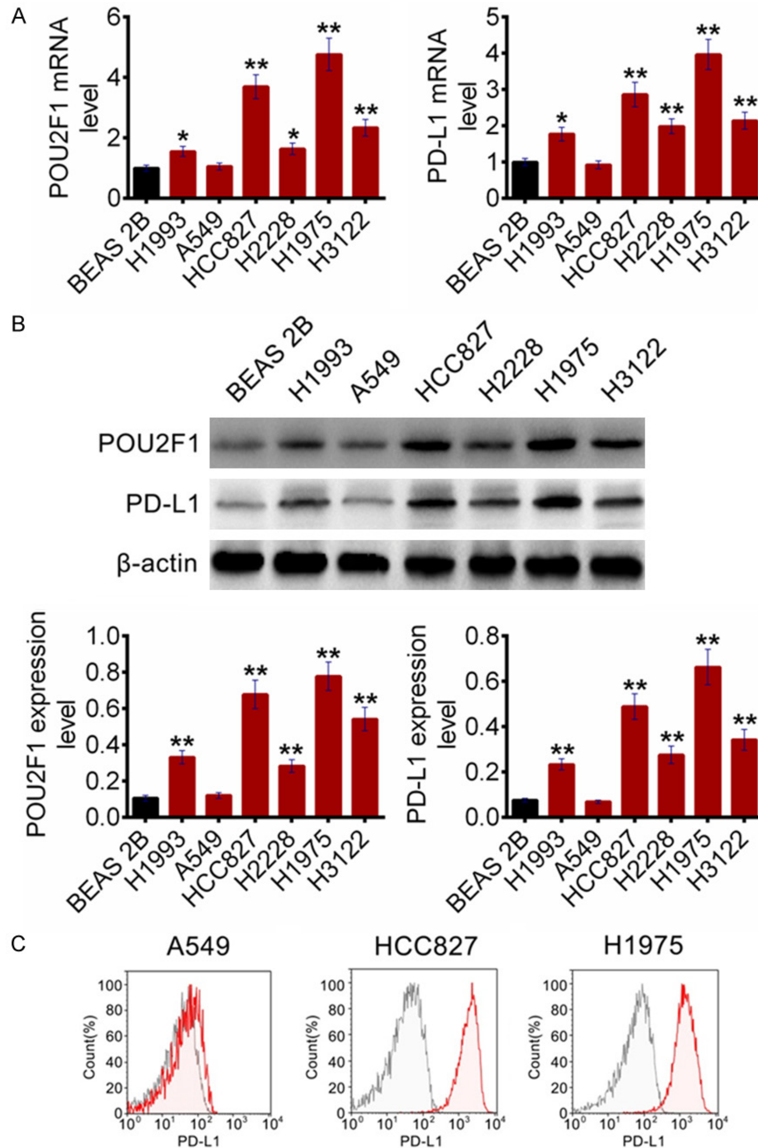

Figure 1. The levels of POU2F1 and PD-L1 were positively correlated in lung cancer cell lines. A. The expression of POU2F1 and PD-L1 were significant higher in most lung cancer cell lines (H1993, HCC827, H2228, H3122, H1975) than that in BEAS-2B cells. B. The protein levels of POU2F1 and PD-L1 were significant higher in most lung cancer cell lines (H1993, HCC827, H2228, H3122, H1975) than that in BEAS-2B cells. C. PD-L1 was higher expressed in the surface of HCC827 and H1975 cells, while they were low expressed on the surface of A549 cells. *P < 0.05, **P < 0.01.

dase activity. After washing with PBS, the primary antibody (PD-L1, Rabbit monoclonal to PD-L1, Cat. No. ab228415, Abcam; β-actin, Rabbit monoclonal [EPR16769] to Actin, Cat. No. ab179467, Abcam) was added at 4°C overnight, and horseradish peroxidase secondary antibody (1:1000, Goat Anti-Rabbit IgG H&L (HRP), Cat. No. ab6721, Abcam) was added, and the mixture was incubated for 30 min at 37°C in a water bath. After washing with PBS, The samples were visualized by DAB, counterstained with hematoxylin, and sealed with neutral gum.

Statistical analysis

All data were shown as mean ± SEM. SPSS 22.0 was applied for statistical analysis. Unpaired Student's t test was used to compare the different between two groups. $P < 0.05$ was regarded significant.

Results

The levels of POU2F1 and PD-L1 are positively correlated in lung cancer cell lines

The miRNA and protein levels of POU2F1 and PD-L1 in all lung cancer and control cell lines were detected by RT-PCR and western blot, respectively. As shown in **Figure 1A** and **1B**, the expression of POU2F1 and PD-L1 were significant higher in in most lung cancer cell lines (H1993, HCC827, H2228, H3122, H1975) than that in BEAS-2B cells. Thereinto, both genes were highest expressed in H1975 cells, and with middle levels in H2228 cells. However, the expressions of POU2F1 and PD-L1 were lower in A549 cells than those in BEAS 2B cell line, but the different was without significance. The expression level of PD-L1 was higher in cells with high expression level of POU2F1, showing a positive correlation. Flow cytometry was used to detect the expression of PD-L1 on the surface of A549, HCC827 and H1975 cells. Consistent with the results of western blot, PD-L1 was higher expressed in the surface of HCC827 and H1975 cells, while they were low expressed on the surface of A549 cells (**Figure 1C**).

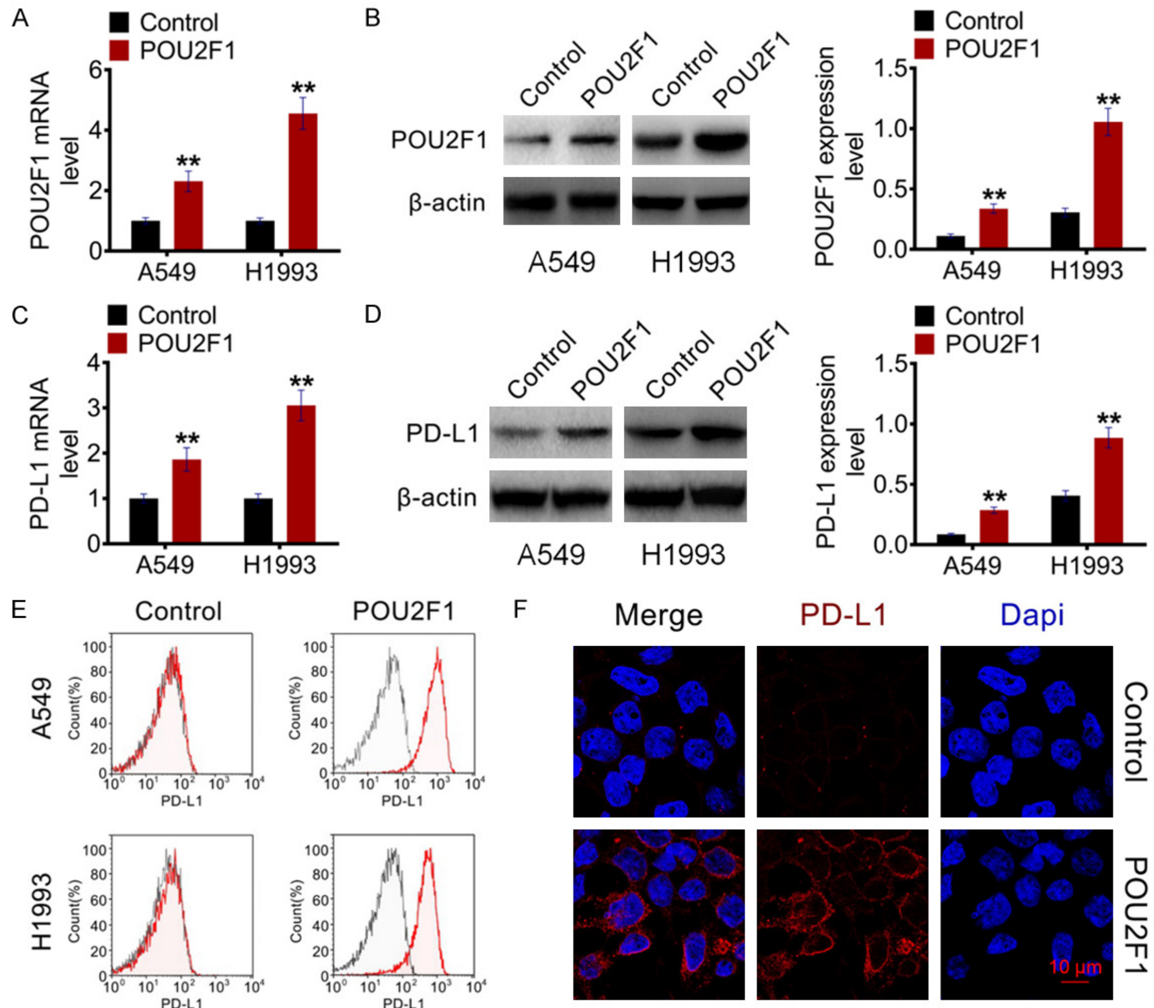

Figure 2. Overexpression of POU2F1 promoted the expression level of PD-L1 in lung cancer cell lines. A. POU2F1 was overexpressed in lung cancer cells (A549 and H1993). B. The protein level of POU2F1 was higher in lung cancer cells (A549 and H1993). C. PD-L1 expressions significantly rise in mRNA levels. D. PD-L1 expressions significantly rise in protein levels. E. After POU2F1 was overexpressed, the POU2F1 level was up-regulated in both A549 and H1993 cells. F. Immunofluorescence assay was used to detect PD-L1 levels. The POU2F1 level was up-regulated in both cells **P < 0.01.

Overexpression of POU2F1 promotesthe expression level of PD-L1 in lung cancer cell lines

POU2F1 was overexpressed in lung cancer cells (A549 and H1993), and POU2F1 levels were detected by WB and RT-PCR experiments to verify the overexpression efficiency. As shown in **Figure 2A** and **2B**, the transfection was successful in A549 and H1993 cell lines. After transfection, the expression of POU2F1 was overexpressed significantly. At the same time, PD-L1 expressions significantly rise in protein and mRNA levels after overexpression of POU2F1 (**Figure 2C** and **2D**). Flow cytometry was used to detect PD-L1 levels on the cell surface. After POU2F1 was overexpressed, the POU2F1 level was up-regulated in both A549 and H1993 cells (**Figure 2E**). Besides, immunofluorescence assay was used to detect PD-L1 levels. The results were similarly with Flow cytometry (**Figure 2F**).

Knockdown of POU2F1 inhibites the expression level of PD-L1 in lung cancer cell lines

POU2F1 was knockdown in lung cancer cells (HCC827 and H1975), and POU2F1 levels were detected by WB and RT-PCR experiments to verify the knockdown efficiency. As shown in **Figure 3A** and **3B**, the transfection was suc-

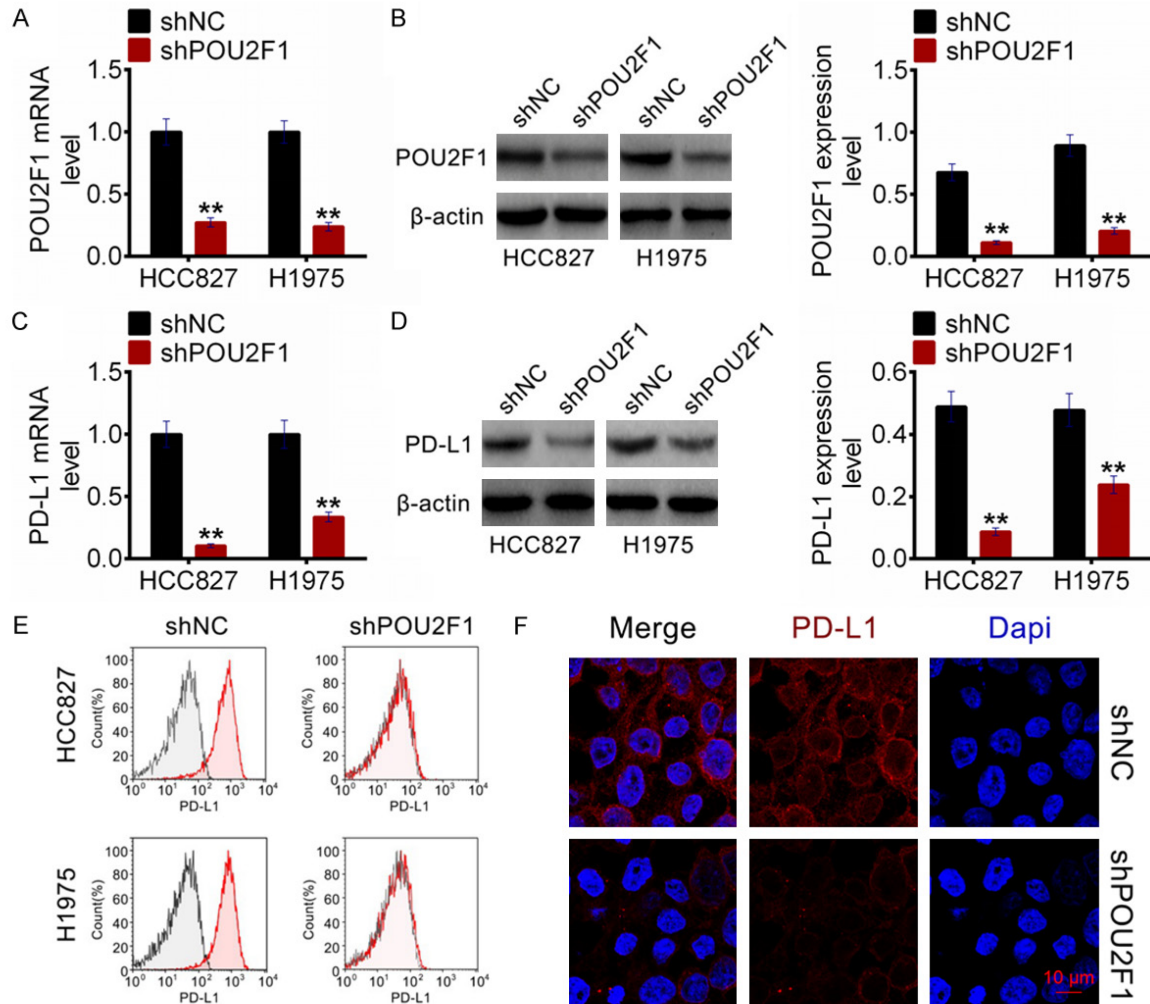

Figure 3. Knockdown of POU2F1 inhibited the expression level of PD-L1 in lung cancer cell lines. A. POU2F1 level was decreased after shPOU2F1 transfection. B. POU2F1 protein level was decreased after shPOU2F1 transfection. C. PD-L1 expressions were significantly decreased in mRNA levels after knockdown of POU2F1. D. PD-L1 expressions were significantly decreased in protein levels after knockdown of POU2F1. E. The PD-L1 level was decreased in both HCC827 and H1975 cells after POU2F1 was knockdown. F. Immunofluorescence assay confirmed that POU2F1 positively regulated the expression of PD-L1. $^{**}P < 0.01$.

cessful in HCC827 and H1975 cell lines. After transfection, the expression of POU2F1 was downexpressed significantly. At the same time, PD-L1 expressions were significantly decreased in protein and mRNA levels after knockdown of POU2F1 (**Figure 3C** and **3D**). Flow cytometry was used to detect PD-L1 levels on the cell surface. After POU2F1 was knockdown, the PD-L1 level was decreased in both HCC827 and H1975 cells (**Figure 3E**). Besides, immunofluorescence assay was used to detect PD-L1 levels. The results were similarly with Flow cytometry (**Figure 3F**). This result further confirmed that POU2F1 positively regulated the expression of PD-L1.

POU2F1 transcription activates the expression of CRK

POU2F1 was overexpressed or knocked down in lung cancer cells (A549, H1993, HCC827 and H1975), and then the levels of CRK were examined using WB and RT-PCR. As shown in **Figure 4A** and **4B**, overexpressed POU2F1 significantly increased the expression of CRK, while knockdown of POU2F1 obviously inhibited the level of CRK. According to the study of Xu et al. [14], POU2F1 could directly bind CRK promoter to regulate its transcription (**Figure 4C**). Luciferase reporter Assay detected the promoter activity of CRK, and confirmed that CRK pro-

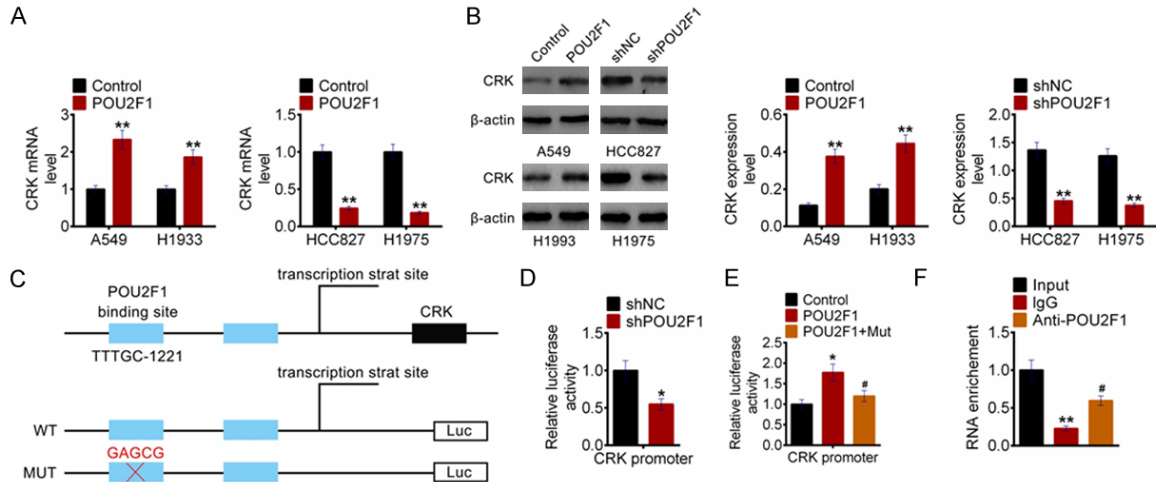

Figure 4. POU2F1 transcription activated the expression of CRK. A. POU2F1 positive regulated CRK expression. B. Western blot assay also confirmed that POU2F1 positive regulated CRK expression. C. POU2F1 was predicted to combine with the promoter of CRK. D. CRK promoter activity declined after POU2F1 knockdown. E. POU2F1 was overexpressed in lung cancer cells, and luciferase reporter was used to detect the promoter activity of CRK. F. The promoter fragment content increased in the anti-POU2F1 sample. *P < 0.05, **P < 0.01.

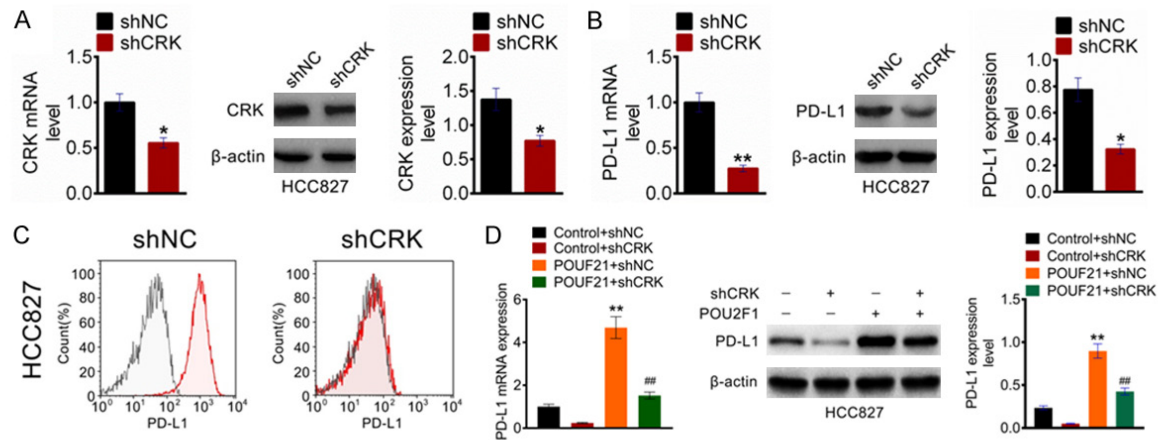

Figure 5. POU2F1 promoted the expression of PD-L1 by promoting the expression of CRK. A. CRK was knocked down in lung cancer cells. B. shCRK down-regulated the expression of PD-L1. C. Flow cytometry also confirmed that PD-L1 positively regulated PD-L1 Level. D. Rescue assay. **Compared with control+shNC group, P < 0.05; ##Compared with POUF21+shNC group, P < 0.01.

moter activity declined after POU2F1 knockdown (**Figure 4D**). A549 cells were overexpressed with POU2F1, and luciferase reporter was used to detect the promoter activity of CRK. After overexpression of POU2F1, the activity of the CRK promoter increased, but the activity of the mutated CRK promoter did not change. CHIP qPCR detected whether the predicted promoter region was associated with POU2F1 (**Figure 4E**). As the results, in the anti-POU2F1 sample, the promoter fragment content increased, indicating that POU2F1 binded to the CRK promoter (**Figure 4F**).

POU2F1 promotes the expression of PD-L1 by promoting the expression of CRK

CRK was knocked down in lung cancer cells (HCC827), and mRNA and protein levels of CRK were detected by RT-PCR and WB experiments to verify knockdown efficiency. As shown in **Figure 5A**, the expression of CRK was significantly down-regulated, which confirmed that the knockdown was successful. mRNA and protein levels of PD-L1 were also detected by RT-PCR and WB assays after knocking down of CRK. As the results, PD-L1 levels were declined,

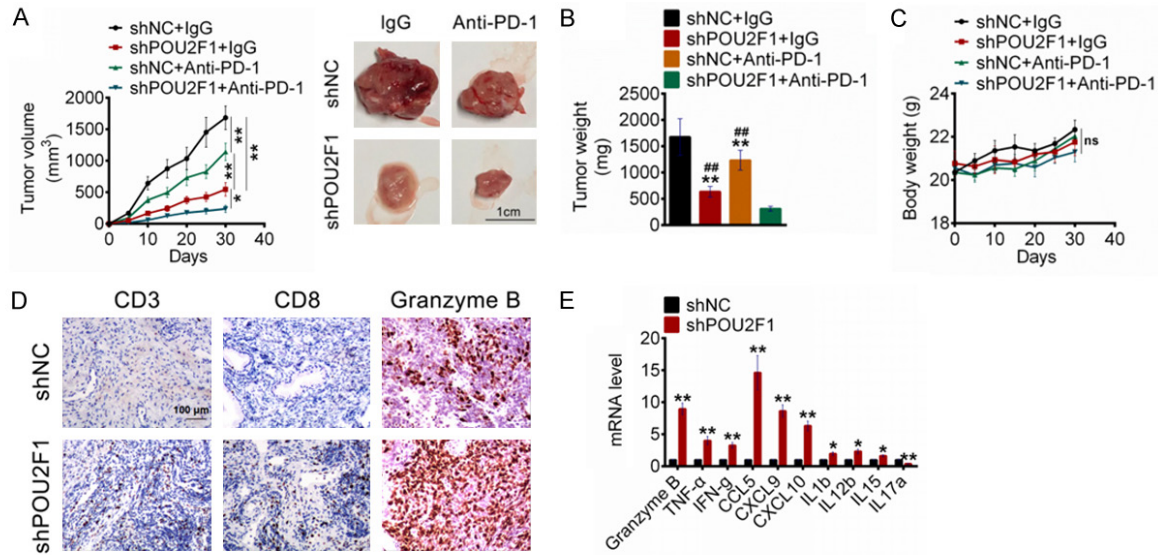

Figure 6. POU2F1 knockdown promoted the efficacy of Anti-PD-1. A. Knockdown of POU2F1 and/ or anti-PD-1 treatment was processed in C57BL/6 mice. The mice were divided into four groups, including shNC+IgG, shPOU2F1+IgG, shNC+Anti-PD-1 and shPOU2F1+Anti-PD-1 groups. The tumor volume decreased in shPOU2F1 group and shPOU2F1+Anti-PD-1 groups. B. The tumor weight decreased in shPOU2F1 group and shPOU2F1+Anti-PD-1 groups. C. Body weight was lower decreased in shPOU2F1 group and shPOU2F1+Anti-PD-1 groups. D. the number of CD3+, CD8+, GranzB+ T cells in the tumor increased, indicating that T cell infiltration was promoted in shPOU2F1 group and shPOU2F1+Anti-PD-1 groups. E. Cytotoxic effector cytokines (granzyme B, TNF-a, IFN-g) levels increased, and tumor suppressive chemokines (CCL5, CXCL9, CXCL10) levels increased. The level of interleukin (IL1b, IL12b, IL15) increased, while IL17a level decreased. *P < 0.05, **P < 0.01, ns, the different with no significance.

which showed that PD-L1 positively regulate the expression of PD-L1 (**Figure 5B**). The level of PD-L1 was also detected by flow cytometry and the results were consistent with RT-PCR and western blot assay (**Figure 5C**). POU2F1 was overexpressed in lung cancer cells, followed by knockdown of CRK, and then mRNA and protein levels of PD-L1 were detected by RT-PCR and WB experiments. For the rescue experiment, overexpression of POU2F1 caused PD-L1 to rise, but after CRK knockdown, POU2F1 could not upregulate PD-L1 levels. Moreover, shCRK could down regulate the expression of PD-L1 without POU2F1 overexpression (**Figure 5D**).

POU2F1 knockdown promotes the efficacy of Anti-PD-1

POU2F1 was knocked down in lung cancer cells and these cells were injected subcutaneously into C57BL/6 mice. After tumor formation, anti-PD-1 was administered to detect tumor suppressing ability. After POU2F1 knockdown, tumor growth ability decreased. The tumor volume and weight were significantly decreased (**Figure 6A** and **6B**). Anti-PD-1 treatment further promoted the inhibition. However, POU2F1 knockdown and Anti-PD-1 treatment did not affect the growth of body weight (**Figure 6C**). After POU2F1 knockdown, the number of CD3+, CD8+, GranzB+ T cells in the tumor increased, indicating that T cell infiltration was promoted (**Figure 6D**). Besides, RT-PCR detected the amount of mRNA in tumorigenic intratumoral immunity. After POU2F1 knockdown, Cytotoxic effector cytokines (granzyme B, TNF-a, IFN-g) levels increased, and tumor suppressive chemokines (CCL5, CXCL9, CXCL10) levels increased. The level of interleukin (IL1b, IL12b, IL15) increased. IL17a level decreased (**Figure 6E**). Thereby, POU2F1 knockdown promotes the efficacy of Anti-PD-1.

Discussion

Lung cancer was a common malignant tumor of the respiratory system [23]. In recent years, the immune escape problem of lung cancer has become a research hotspot in the field of cancer [24]. As reported in previous studies, the main pathogenesis of lung cancer was that cancer cells achieved immune escape via changing in surface antigens, abnormal expres-

sion of MHC-I molecules and costimulatory molecules, defects in dendritic cell function, and high expression of FasL in lung cancer cells [25, 26]. However, effective biomarker of target has not been screened. EGFR-TKI drugs have greatly improved the quality of life of some patients with genetic variants of NSCLC, and the development and application of PD-1/PD-L1 inhibitors has given new options to these patients [27]. There are currently three PD-1/PD-L1 inhibitors approved by the FDA for lung cancer, including Nivolumab, Pembrolizumab and Atezolizumab [28]. However, immunological drugs are only clinically used for advanced lung cancer, or for patients who have been ineffective after one or several other treatments are considered for immunotherapy. So far, more and more research data confirm that early lung cancer patients can also benefit from immunotherapy [29].

In this study, POU2F1 transcription was confirmed to activate the expression of CRK. CRK was a common adaptor protein, which participated in various oncogenic signal transduction pathways, and further affected several biological progresses, including differentiation, proliferation and migration [30]. Previous studies have shown that NRP1 could promote the migration of tumor cells, that is, promoted the metastasis of distant tumors [31, 32]. Besides, POU2F1 was confirmed to be positively correlated with PD-L1 in lung cancer cell lines in this study. The results were similarly with previous studies. A meta-analysis showed that positive PD-L1 expression of NSCLC patients was always companying with poor overall survival [33]. Importantly, PD-1 positive was closely related with EGFR mutations, and it was with higher sensitivity to erlotinib or gefitinib in lung cancer patients [34]. In 2011, Mu et al. [35] referred that high expressed PD-L1 could suppress tumor infiltrating dendritic cells and then induce poor prognosis and immune escape. The good news was that immune therapy for PD1 and PD-L1 has obtained antitumor activity in non-small cell lung cancer patients [36]. Based on above, POU2F1 induced the immune escape in lung cancer by upregulation of PD-L1.

In general, gene silencing refers to an important means of regulating gene expression in vivo, which mainly manifests as down-regulation or suppression of expression of specific genes [37]. There are two main types of gene silencing, including transcriptional and post-transcriptional gene silencing [38]. Gene silencing at the transcription level mainly includes gene methylation, position effects, trans-inactivation of homologous genes, subsequent modification, and gene silencing caused by repeated sequences, while post-transcriptional gene silencing mainly contains gene suppression caused by co-suppression and RNA interference [39]. Therefore, it is necessary to study the competition between POU2F1 and CRK to form a regulatory network. However, the regulatory network research is only at the animal research stage, and a large amount of research foundation is needed for clinical trials at present. Encouragingly, the good news is that significant progress has been made in research in therapy of the receptor PD-1 [40]. A non-blind, phase III experiment from the German Lung Cancer Research Center showed that pembrolizumab treatment can achieve longer PFS and OS, higher objective response rate, longer remission time, and relatively few adverse reactions for PD-L1 overexpressed lung cancer patients [41]. Another study on Pembrolizumab enrolled 495 newly diagnosed and relapsed NSCLC patients [42]. After receiving Pembrolizumab, ORR was 19.4% (18.0% in relapsed patients while 24.8% in newly diagnosed patients), and median PFS was 6.3 months (6.1 months for relapsed patients, 12.5 months for newly diagnosed patients). Therefore, studying the processes in the POU2F1-related ceRNA network and PD-1 inhibitors contributes to the treatment of clinical lung cancer patients.

Though the molecular mechanism of POU2F1 was researched in this study, there are some limitations in this study. Due to limitation of experimental conditions, we only use end-point RT-PCR to detect the level of transcripts. More accurate and reproducible results will be obtained using quantitative RT-PCR in the futher studies. Moreover, more related genes and pathways will be researched to provide more targets for lung cancer. Besides, the regulatory network research is only at the animal research stage, therapy of targeting POU2F1 was also need more research foundation in future research. These issues will be the focus of our next purpose. In conclusion, POU2F1 could activate the expression of CRK, further promote expression of PD-L1, and finally improve the immune escape in lung cancer. It might be

a new critical gene for diagnose and treatment of lung cancer.

Disclosure of conflict of interest

None.

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