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Splicing factor proline- and glutamine-rich regulates cytotoxic T lymphocytes-mediated cytotoxicity on non-small cell lung cancer by directly binding to PD-L1 3'UTR

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Abstract

Splicing factor proline- and glutamine-rich (SFPQ) can interact with RNAs to regulate gene expression. The function of SFPQ in the immunotherapy of non-small cell lung cancer (NSCLC) is investigated in this study. H1299 and A549 cells were transfected with shSFPQ plasmid. Cell counting kit-8 (CCK-8) and cell clone formation were utilized to detect survival and proliferation. Programmed death-ligand 1 (PD-L1) and SFPQ were detected in NSCLC patients treated with anti-PD-L1 antibody. Dual-luciferase assays, RNA immunoblotting, RNA pull-down, and mRNA stability assay were applied to verify the regulation of PD-L1 with SFPQ. Human peripheral blood mononuclear cells (PBMC)-derived dendritic cells were loaded with irradiated A549 and H1299 cells, which were cultured with autologous $CD8^+$T cells and tumor cells to perform in vitro tumor-specific cytotoxic T lymphocytes (CTL) cytotoxicity analysis. SFPQ silencing inhibited the survival and proliferation of H1299 and A549 cells with down-regulated PD-L1 expression. PD-L1 and SFPQ expression were markedly higher in anti-PD-L1 antibody treatment responders compared to non-responders, which showed a positive Pearson correlation ($R = 0.76$, $P < .001$). SFPQ up-regulated the relative mRNA and protein expression of PD-L1 by binding to the PD-L1 3'UTR to slow the decay of PD-L1 mRNA. SFPQ silencing promoted the killing effect of CTL on A549 and H1299 cells. SFPQ up-regulates PD-L1 expression by binding with PD-L1 3'UTR to slow the decay of PD-L1 mRNA, and SFPQ silencing promotes CTL-mediated cytotoxicity on NSCLC cells.

Abbreviations: CCK-8 = cell counting kit-8, CTL = cytotoxic T lymphocytes, DMEM = Dulbecco Minimum Essential Medium, FBS = fetal bovine serum, NSCLC = non-small cell lung cancer, PBMC = peripheral blood mononuclear cells, PD-L1 = programmed death-ligand 1, SFPQ = splicing factor proline- and glutamine-rich.

Keywords: lung, NSCLC, PD-L1, SFPQ

1. Introduction

With the aggressive and rapidly growing characteristics, non-small cell lung cancer (NSCLC) is estimated as the leading cause of cancer death in women and men, accounting for eighty percent of lung cancer patients, which may not be applicable for curative surgery.[1–3] Despite numerous advanced efforts to improve survival outcomes, NSCLC remains an incurable disease.[4,5] Most patients with NSCLC will relapse and become resistant to the first-line platinum-based therapy with unsatisfied efficacy.[6]

In recent years, neoadjuvant therapy, such as immune checkpoint blocking, has emerged as an essential treatment option in NSCLC[7] and improved survival in advanced NSCLC.[8,9] Unlike traditional chemotherapeutic agents, immune checkpoint blocking therapy boosts the instinct cytotoxic T lymphocytes (CTL) or natural killer cells mediated cytotoxicity.[10] Anti-programmed death-ligand 1 (PD-L1) antibodies, atezolizumab and avelumab, have been approved by the US Food and Drug Administration and/or European Medicines Agency to treat NSCLC.[11,12] Although anti-PD-L1 antibodies show tremendous clinical application prospects, the effective rate is only about 20% in the treatment of advanced NSCLC,[13,14] which seriously limits further utilization.

In recent years, it has been found that the combination of targeted therapy and immunotherapy can better benefit NSCLC

The authors have no funding and conflicts of interest to disclose.

The datasets generated during and/or analyzed during the current study are not publicly available, but are available from the corresponding author on reasonable request.

The study was approved by the ethics committee of Mudanjiang Medical University, The study was performed in strict accordance with the Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects.

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patients, reduce the occurrence of drug resistance and improve the survival time of patients. Nowadays, several companion diagnostic assays for PD-L1 expression have been introduced for identifying patients who may benefit from the anti-PD-L1 treatment.[15] Therefore, it is of great theoretical and clinical value to find new biomarkers, which may predict treatment efficiency and immunotherapy resistance.

Due to the interaction with coding and non-coding RNAs, splicing factor proline- and glutamine-rich (SFPQ) can act as a transcriptional repressor to dampen proto-oncogene expression in numerous cancers.[16] At the same time, no relevant investigation has been performed in NSCLC. Our investigation finds that SFPQ can promote PD-L1 expression in H1299 and A549 cells by directly binding to PD-L1 3'UTR with a post-transcriptional regulatory mechanism. In the meantime, SFPQ silencing in H1299 and A549 cells can promote cytotoxicity mediated by CTL.

2. Methods & materials

2.1. NSCLC patients

Advanced NSCLC patients treated with platinum-based chemotherapy and anti-PD-L1 antibody (atezolizumab or durvalumab), who had sufficient paraffin-embedded tumor samples for immunohistochemistry analysis and signed informed consent, were included in this study. Six months after anti-PD-L1 treatment, NSCLC patients were categorized as responders (decreased tumor size) and non-responders (stable or progressive disease), as indicated by previous research.[17] Balanced clinical variables and baseline demographics were obtained from the electronic medical record with the permission of patients, and Mudanjiang Medical University approved all the protocols. The detailed clinical characteristics of the patients were shown in Table S1, http://links.lww.com/MD/K490.

2.2. Immunohistochemistry

After microwave antigen retrieval, 2-micrometer sections were incubated with PD-L1 or SFPQ antibody (1:1000; Santa Cruz). Biotinylated secondary antibody, avidin: biotinylated enzyme complex, and 3,3'-diaminobenzidine substrate were sequentially added, and hematoxylin was utilized to counterstain the nuclear. Images were taken with a Nikon 80*i* microscope, and the relative expression of PD-L1 and SFPQ was analyzed. Pearson correlation was applied to indicate the significant relationship between PD-L1 and SFPQ expression.

2.3. Cell culture and transfection

A549 and H1299 cells ordered from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) were cultured in Dulbecco Minimum Essential Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37 °C with 5% CO_2.

Two short hairpin RNA (shRNA)-targeted SFPQ designed by GenePharma (Shanghai, China) were cloned into a pRNAT-U6.1/Neo plasmid (Biovector, Beijing, China) to construct pRNAT-U6.1/Neo-shSFPQ plasmids (shSFPQ-1 and shSFPQ-2), which were further transfected into A549 and H1299 cells (1×10^6 cells) with Lipofectamine 3000 (Invitrogen).

2.4. Western blotting

Lung cancer cells were lysed with Cell Lysis Buffer for Western and IP (Beyotime, Shanghai, China), and 20 μg soluble supernatant was separated with 10% SDS-PAGE and transferred to PVDF membranes (Amersham Biosciences), which were incubated with primary antibodies against SFPQ, PD-L1, and β-actin (Santa Cruz) and peroxidase-conjugated secondary antibody (Sigma-Aldrich). The relative densitometry of the bands was developed with a Cytiva Lifescience Amersham ECL Prime Western Blotting Detection Reagent and calculated by correcting for β-actin with NIH-Image J1.51p 22.

2.5. Cell-counting Kit 8 (CCK-8) assay

A549 or H1299 cells transfected with shSFPQ or shCtrl were plated into 6-well plates for 72 hours. Then, 10 μL Cell-Counting Kit-8 (CCK-8, Dojindo Laboratories) was added to each well and incubated for 2 hours at 37°C. The absorbance was assayed with the SpectraMax Plus 384 Microplate Reader (Molecular Devices) at 450 nm.

2.6. Colony formation assay

Transfected or un-transfected A549 or H1299 cells were cultured in 6-well plates (1×10^3 cells per well) for 2 weeks, which were further fixed with 4% paraformaldehyde. Crystal violet was utilized to stain the colon, and the number of colonies was counted.

2.7. Quantitative real-time PCR

Total RNAs extracted from A549 or H1299 cells with TRIzol (Invitrogen) were reverse transcribed into cDNA with the PrimeScript™ RT Kit (Takara) and amplificated on an Applied Biosystems 7500 Real-Time PCR system with SYBR Green Master Mix (Roche Ltd.), followed by the procedure: 95°C for 10 minutes, 40 cycles of 95 °C for 15 seconds, and 60 °C for 1 minute. The relative gene expression was quantified with the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was utilized as endogenous control. The primers were listed as follows: PD-L1, 5'- TGCCGACTACAAGCGAATTACTG-3' (forward) and 5'- CTGCTTGTCCAGATGACTTCGG-3' (reverse); GAPDH, 5'-GGGAGCCAAAAGGGTCAT-3' (forward) and 5'-GAGTCCTTCCACGATACCAA-3' (reverse).

2.8. PD-L1 mRNA stability assay

PD-L1 mRNA stability was measured with actinomycin D (Sigma-Aldrich) assay. Briefly, 5 μg/mL actinomycin D was added to the cell medium. After 2, 4, 6, 8 hours, RNA was isolated, and the relative PD-L1 expression was detected with RT-PCR.

2.9. Luciferase reporter assays

A549 or H1299 cells in 24-well plates were transfected with psiCHECK2-PD-L1-3'UTR or negative control (Ambion) with Lipofectamine 3000 (Thermo Fisher Scientific). *Renilla* luciferase activity was detected with the dual-luciferase reporter (Promega) 48 hours later, normalized to firefly activity, and presented as relative luciferase activity.

2.10. RNA immunoprecipitation

Cells were lysed with lysis buffer (2 mM EDTA,150 mM KCl, 0.5% NP-40, 25 mM Tris-HCl, pH 7.4) supplemented with Sodium Fluoride (20 mM) and RNaseOUT Recombinant Ribonuclease Inhibitor (100 U/mL). SFPQ-associated RNAs were immunoprecipitated with the dynabeads protein G (Life Technologies)-SFPQ antibody incubation complexes (5 μg of antibody per 500 μg of proteins) overnight at 4°C, which was further purified with TriPURE (Roche), treated with DNase I (Thermal Fisher Scientific), and analyzed by RT-PCR.

2.11. RNA pull-down assays

A549 or H1299 cells transfected with 50 pmol biotin-labeled PD-L1 3'UTR for 48 hours were lysed with lysis buffer, which was subjected to pre-saturated streptavidin magnetic beads in RNA-binding buffer for 30 minutes at 4°C. Laemmli lysis buffer was applied to dissociate protein binding with magnetic beads.

2.12. Tumor-specific CTL cytotoxicity assay

The Ficoll-Hypaque centrifugation (density of 1.077g/cm^3) method was utilized to isolate peripheral blood mononuclear cells (PBMCs) from intravenous blood. PBMCs were further cultured in RPMI 1640 medium containing 10% FBS for 3 hours to aspirate nonadherent cells, and adherent cells were further incubated with complete medium (100 ng/mL granulocyte/macrophage colony-stimulating factor and 50 ng/mL interleukin 4) for 6 days to induce the differentiation of dendritic cells (DCs). Irradiated A549 and H1299 cells (10K Rads) and autologous CD8$^+$ T cells isolated with CD8$^+$ T Cell Isolation Kit (Milteny) mixed in a ratio of 1:20 were pipetted onto the DCs culture system (50 mL culture flask), and supplemented with interleukin 2 (20 ng/mL). The cells cultured for 5–6 days were collected as tumor-specific CTL, which were further incubated with A549 and H1299 cells to indicate the direct cytotoxicity effect.

2.13. TUNEL assay

A FragEL DNA fragmentation detection kit (Merck) was used to detect the labeling of DNA breaks in the apoptotic nuclei. In brief, A549 or H1299 cells cultured with or without CTL were incubated with deoxynucleotidyl transferase (TdT) reaction mix and digoxigenin-11-dUTP at 37°C for 1 hour, which was further incubated with anti-digoxigenin-AP, Fab fragments (1:100) at room temperature for 45 minutes. FAST BCIP/NBT buffered substrate was utilized to detect apoptosis.

2.14. Statistical analysis

PD-L1 and SFPQ protein expression in NSCLC tissues was analyzed with Pearson correlation. Difference in quantitative parameters between groups was detected with a *t*-test. The significance level was set as P value < .05. All statistical analyses were performed using GraphPad Prism.

3. Results

3.1. SFPQ promotes lung cancer cell survival and proliferation

In order to testify the function of SFPQ, SFPQ silencing A549 and H1299 cells were successfully constructed as indicated with diminished SFPQ protein expression (Fig. 1A). SFPQ silencing could significantly inhibit the survival of A549 cells (Fig. 1B) and H1299 cells (Fig. 1C) demonstrated with CCK-8 assay. On the other hand, the proliferation ability of A549 cells (Fig. 1D) and H1299 cells (Fig. 1E) was prohibited by the SFPQ silencing. All of these data indicated that SFPQ could promote the survival and proliferation of lung cancer cells.

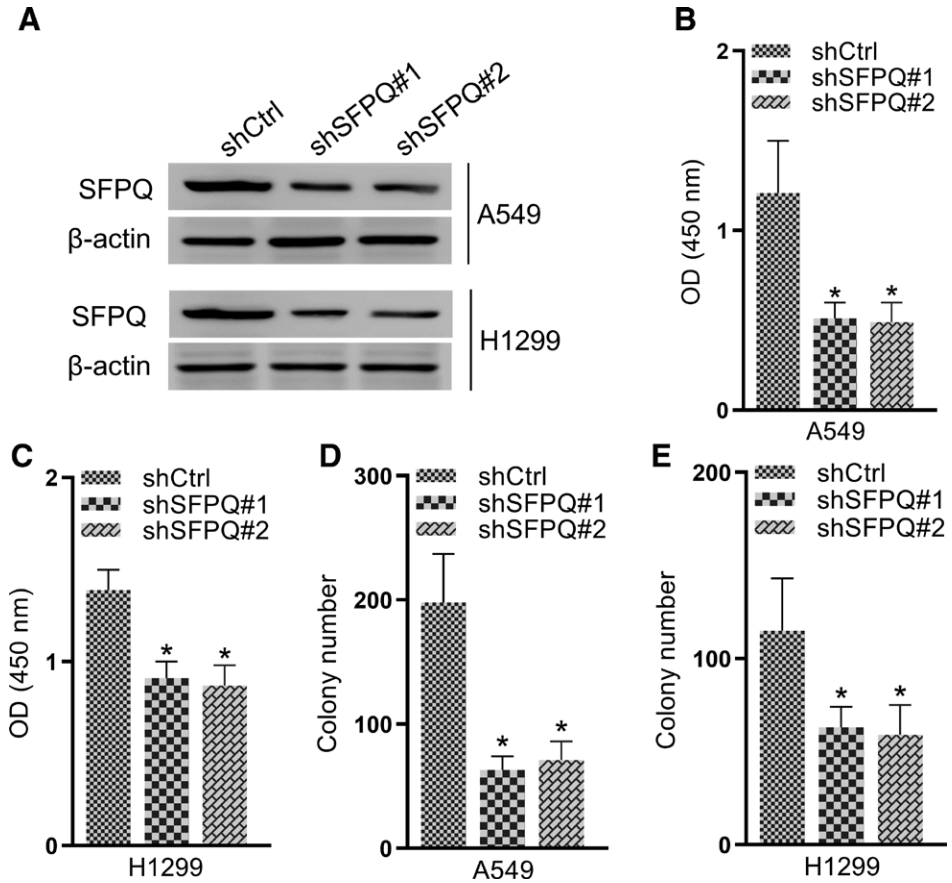

Figure 1. SFPQ-KD suppresses lung cancer proliferation. (A) Western blotting analyses of SFPQ expression in SFPQ-silencing (sh#1 and sh#2) and control (shCtrl) lung cancer cells. (B) A549 and (C) H1299 cells were applied with CCK8 assay after plating in 6-well plates for 72 h. (D) A549 and (E) H1299 cells were subjected to cell colony formation after plating in 6-well plates for 2 wk. Data were presented as mean ± S.D. of 3 independent experiments. *P < .01, Student *t* test. SFPQ = splicing factor proline- and glutamine-rich.

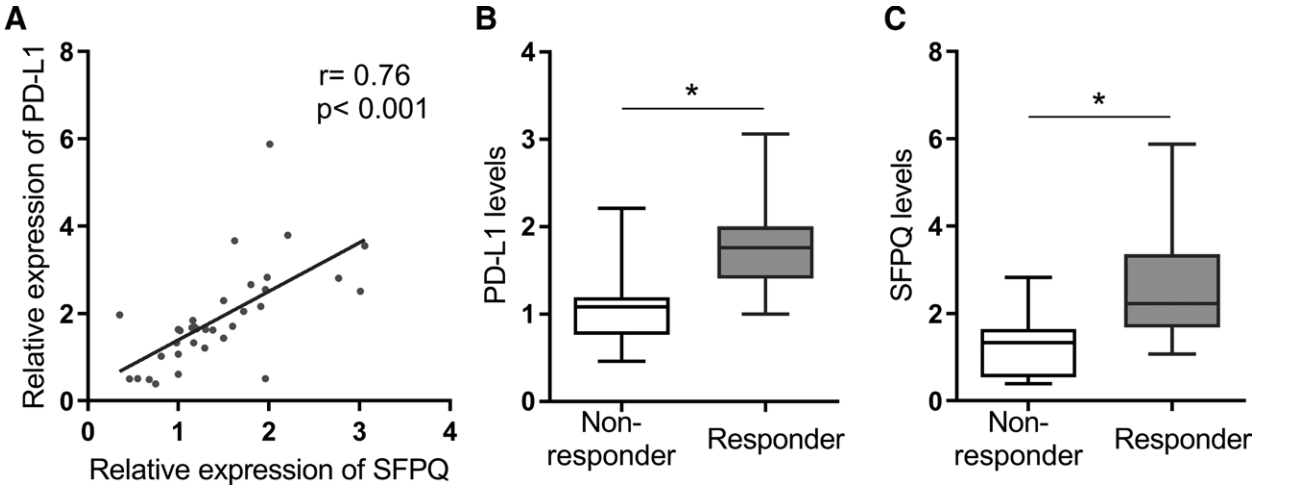

Figure 2. Analysis of PD-L1 and SFPQ expression and the association with checkpoint blockade response. (A) The correlation between PD-L1 and SFPQ expression in lung cancer tissues. Comparison of (B) PD-L1 and (C) SFPQ expression between responders and non-responders. Statistical significance between groups was defined by Student 2-tailed *t* test. *$P < .05$. PD-L1 = programmed death-ligand 1, SFPQ = splicing factor proline- and glutamine-rich.

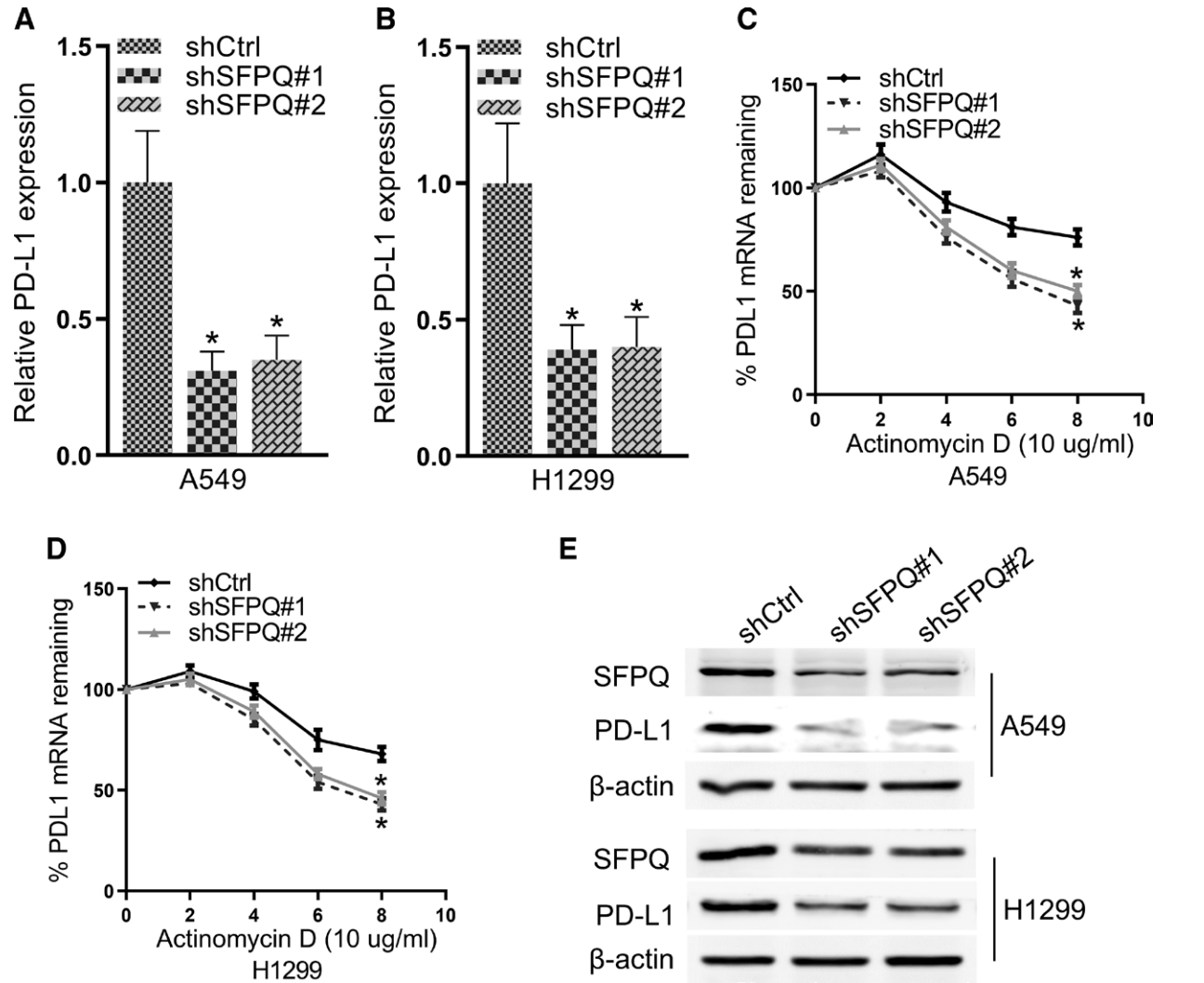

Figure 3. SFPQ-silencing reduces PD-L1 level. (A, B) qRT-PCR analyses of PD-L1 expression in SFPQ-silenced (sh#1 and sh#2) and control (shCtrl) cells. GAPDH was utilized as an endogenous control. (C) A549 and (D) H1299 cells were treated with 10 μg/mL actinomycin D for the indicated times (hour). PD-L1 expression was measured by qRT-PCR in SFPQ-silenced and control cells. (E) PD-L1 expression in SFPQ-silenced and control cells. Data were mean ± S.D. of 3 independent experiments, and each was measured in triplicate. *$P < .01$, Student *t* test. PD-L1 = programmed death-ligand 1, SFPQ = splicing factor proline- and glutamine-rich.

3.2. Analysis of the PD-L1 and SFPQ expression and response to checkpoint blockade

The relative mRNA and protein expression of PD-L1 and SFPQ were positively correlated ($R = 0.76$, $P < .001$, Fig. 2A and Table S1, http://links.lww.com/MD/K490) in NSCLC tumors. It was worth noting that the relative PD-L1 (Fig. 2B, $P < .05$) and SFPQ expression (Fig. 2C, $P < .05$) was significantly high in anti-PD-L1 treatment responders compared to non-responders (Fig. 2B, $P < .01$). In other words, patients with high expression of SFPQ protein had a better response to anti-PD-L1 treatment, which indicated that SFPQ might enhance the effect of immunotherapy through PD-L1.

3.3. SFPQ up-regulates the relative PD-L1 expression

SFPQ-silenced A549 (Fig. 3A) and H1299 cells (Fig. 3B) showed diminished PD-L1 expression. The rate of PD-L1 mRNA decay in shSFPQ-treated A549 (Fig. 3C) and H1299 cells (Fig. 3D) was faster than that of control shRNA-treated cells. On the other hand, SFPQ silence could diminish the relative protein expression of PD-L1 in both A549 cells and H1299 cells (Fig. 3E). All of these indicated that SFPQ could up-regulate the relative PD-L1 expression with a post-transcriptional mechanism.

3.4. SFPQ regulates PD-L1 expression through PD-L1 3'UTR

The dual-luciferase assay demonstrated repressed luciferase activity of PD-L1 3'UTR in SFPQ-silencing A549 (Fig. 4A) and H1299 cells (Fig. 4B). RNA immunoprecipitation assay was utilized to verify the direct binding of SFPQ with PD-L1 3'UTR region. PD-L1 transcript was strongly enriched in SFPQ IPs prepared from A549 (Fig. 4C) and H1299 cells (Fig. 4D). Using mRNA pull-down assay with A549 cell lysates (Fig. 4E), we found that SFPQ could interact with PD-L1 3'UTR (Fig. 4D), which demonstrated that SFPQ up-regulated PD-L1 expression by binding with PD-L1 3'UTR.

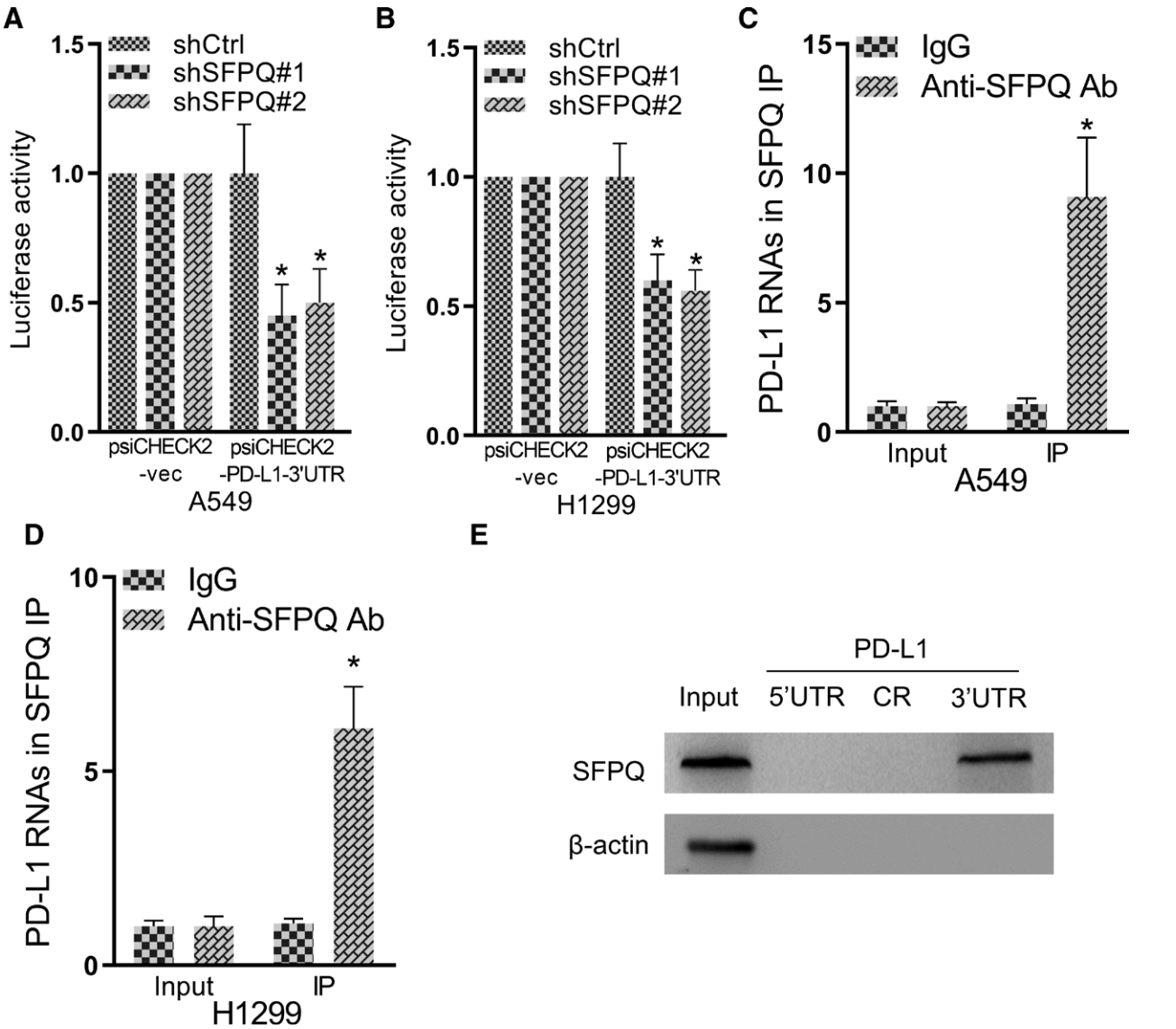

Figure 4. SFPQ regulates the relative PD-L1 expression by binging with PD-L1 3'UTR. (A, B) SFPQ-silencing repressed PD-L1 3'UTR luciferase activity. (C, D) RNA immunoblotting assay was performed in A549 and H1299 cells. (E) RNA pull-down assay was conducted in the A549 cell. Data were presented as mean ± S.D. of 3 independent experiments. *$P < .01$, Student t test. PD-L1 = programmed death-ligand 1, SFPQ = splicing factor proline- and glutamine-rich.

3.5. SFPQ silencing enhances the cytotoxicity of CTL on lung cancer cells

Induced tumor-specific CTL showed cytotoxicity effect on A549 (Fig. 5A) and H1299 cells (Fig. 5B), which could be strengthened by the SFPQ silencing treatment in A549 and H1299 cells with increased TUNEL-positive cells detection. CCK8 assay also indicated that SFPQ silencing enhanced the effector of CTL cells on lung cancer cells with diminished survival in both A549 (Fig. 5C) and H1299 cells (Fig. 5D). All of these indicated that SFPQ silencing could be considered as a treatment option to enhance the cytotoxicity of CTL on lung cancer cells.

4. Discussion

PD-L1 signaling is a vital component of tumor immunosuppression to inhibit CTL activation and result in immune tolerance. Our results indicate that SFPQ could up-regulate PD-L1 expression through a post-transcriptional mechanism to slow the PD-L1 mRNA decay, and SFPQ silencing could enhance the cytotoxicity effector of CTL on lung cancer cells with diminished cell survival. Mechanically, dual-luciferase assays, RNA immunoblotting, and RNA pull-down assay further testify the direct binding of SFPQ with the PD-L1 3'UTR region. These results indicate that the direct binding of SFPQ with the PD-L1 3'UTR region could slow PD-L1 mRNA decay and increase PD-L1 mRNA and PD-L1 protein expression.

SFPQ plays multiple regulatory roles as an RNA-binding protein, such as transcriptional regulation, paraspeckle formation, and DNA damage repair in the nucleus.[18,19] As indicated in our analysis, SFPQ silencing may inhibit lung cancer cell survival. Loss of SFPQ is also testified to promote $BRAF^{V600E}$-driven colorectal cancer cells apoptosis.[20] All of these indicate the survival-promoting effect of SFPQ.

It is worth noting that SFPQ may dissociate from the *Rela* promoter region to promote the transcriptional activation of *Rela* upon endogenous retrovirus viral infection in bone marrow-derived macrophages (BMDMs).[21] Upon influenza virus and herpes simplex virus infection, SFPQ may relocate from promoter region to paraspeckle to mediate interleukin (IL)-8 expression.[22] While in our analysis, the cytoplasmic role of SFPQ in post-transcriptional regulation is identified.

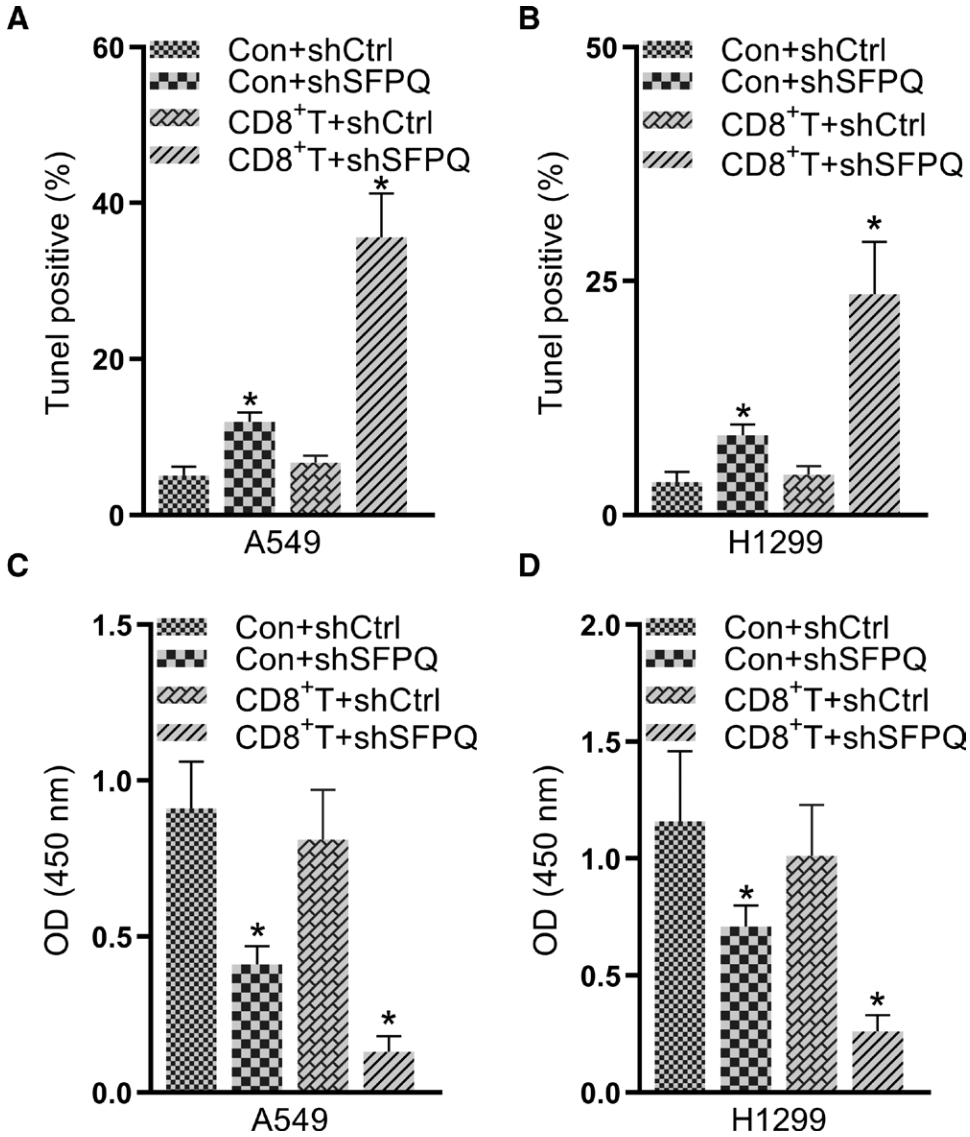

Figure 5. SFPQ silencing enhances the effector of CTL cytotoxicity on lung cancer cells. (A, B) Nuclear TUNEL staining in H1299 and A549 cells with or without CTL incubation. The percentage of TUNEL-positive apoptosis cells was measured and indicated on the histogram. (C) A549 and (D) H1299 cells were subjected to CCK8 assay after plating in 6-well plates for 72 h with or without CTL. Data were mean ± S.D. of 3 independent experiments, and each was measured in triplicate. *$P < .01$, Student t test. CTL = cytotoxic T lymphocytes, SFPQ = splicing factor proline- and glutamine-rich.

We demonstrate that SFPQ might act as a stabilizer of PD-L1 mRNA, which is a vital determinant of the steady-state concentration of PD-L1 mRNA. mRNA decay is not just one aspect of metabolism but may link to translation and mRNA re-localization.[23] All of these demonstrate that SFPQ binding or relocation may seriously affect the expression of the downstream gene.

SFPQ silencing could promote CTL-mediated lung cancer cytotoxicity both in A549 (P53-wt) and H1299 cells (P53-null), which indicates that the function of SFPQ is independent of P53 status. SFPQ is demonstrated to mediate homology-directed DNA damage and repair response resulting from DNA alkylating agents or crosslinking agents.[24] Whether the utilization of SFPQ silencing combined with platinum-based chemotherapy can maintain the genome integrity needs further detailed analysis.

There are some limitations that should be indicated here. The potential synergistic effect with anti-PD-L1 treatment is not deciphered in this investigation. And those patients with high PD-L1 expression have better responses to anti-PD-L1 therapy,[25] whether SFPQ expression could be utilized as an independent prognostic factor to discriminate the responder from nonresponder will need further multivariate analysis. It is worth noting that the tumor immune microenvironment is greatly affected by the surrounding in vivo component, and subcutaneous carcinoma inoculation and orthotopic transplantation model are needed to verify the treatment benefit of SFPQ silencing in vivo. In addition, it would be more convincing to use a list of cell lines to screen an ideal model for studying the interaction between SFQP and PD-L1.

In summary, our investigation indicates that SFPQ silencing can promote the CTL-mediated cytotoxicity to enhance the anti-tumor efficacy, which can be considered as a strategy to improve treatment benefit to NSCLC.

5. Conclusions

SFPQ up-regulates PD-L1 mRNA and protein expression by directly binding to the region of PD-L1 3'UTR, and SFPQ silencing could improve CTL-mediated lung cancer cells cytotoxicity. Our findings indicate that SFPQ silencing could be utilized as an adjuvant therapy to enhance the anti-PD-L1 treatment.

Author contributions

Data curation: Yanming Pan, Yongxia Cheng.
Funding acquisition: Yongxia Cheng.
Investigation: Yongxia Cheng.
Resources: Yongxia Cheng.
Supervision: Yongxia Cheng.
Validation: Yanming Pan, Yongxia Cheng.
Writing – original draft: Yanming Pan, Yongxia Cheng.
Writing – review & editing: Yanming Pan, Yongxia Cheng.

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