Deubiquitination and Stabilization of PD-L1 by USP21

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Abstract

Background: The immunotherapy for different types of cancers that targeting programmed death protein-1 (PD-1) and programmed death ligand-1 (PD-L1) has highlighted the importance of suppressing specific T cell responses. Recently, several studies have shown that the expression level of PD-L1 in tumor cells is positively correlated with tumor metastasis as well as recurrence rate. The potent effects of post-translational modifications (PTMs) for PD-L1, such as ubiquitination, glycosylation, phosphorylation and palmitoylation, have been reported to be related to immunosuppression. However, the regulation of PD-L1 degradation in cancers is still not well understood. In this paper, we mainly investigate the deubiquitination regulation of PD-L1.

Methods: The protein levels of PD-L1 and USP21 were detected by Immunoblotting and immunohistochemistry. The interaction between PD-L1 and USP21 was determined by co-immunoprecipitation. The deubiquitination of PD-L1 was determined by *in vitro* deubiquitination assay. The deubiquitination sites of PD-L1 were identified by mass spectrometry analysis. The expression of mRNA in target tissues was presented by bioinformatics analysis.

Results: Overexpression of USP21 significantly increased PD-L1 abundance and knockdown of USP21 induced degradation of PD-L1. *In vitro* deubiquitination assay showed that USP21-WT reduced polyubiquitin chains from PD-L1 while USP21-C221A did not. Furthermore, five lysines in intracellular segment of PD-L1 are potential deubiquitin sites and cancer-derived mutations of PD-L1 in Asp276 have the ability to enhance the deubiquitination of PD-L1 mediated by USP21. Finally, we found that USP21 is the frequently amplified deubiquitinase in lung cancer, especially in lung squamous cell carcinoma, and its amplification co-occurs with the upregulation of PD-L1 levels. Moreover, IHC analysis showed stronger staining of PD-L1 and USP21 in lung cancer samples than adjacent tissues.

Conclusion: We identified USP21 as a novel deubiquitinase of PD-L1. Hopefully, targeting PD-L1 by inhibiting USP21 might be a potentially novel strategy for the treatment of lung cancer.

Introduction

Programmed cell death ligand-1 (PD-L1), also known as cluster of differentiation 274 (CD274) and B7 homolog 1 (B7-H1), is a transmembrane protein expressed on the surface of B cells, activated T cells, macrophages, dendritic cells and tumor cells (Huang et al. 2019). The binding of immune inhibitory receptor PD-1 (programmed death protein-1) with its ligands triggers the inhibition of effector T cells, results in cancer cells evasion from anti-tumor immunity (Petrelli et al. 2018).

In order to recover the response of cytotoxic T cells on tumors, a great many of monoclonal antibodies have been developed to prevent the interaction between PD-1 and PD-L1 (Huang et al. 2019; Ribas and Wolchok 2018; Salmaninejad et al. 2019). Although PD-1/PD-L1 blocking therapy has shown significant clinical benefits, the response rates have rarely exceeded 40% in multiple cancer types (Yarchoan, Hopkins, and Jaffee 2017; Pitt et al. 2016; Hsu et al. 2018). It is necessary to further explore the

mechanisms of resistance to immune-checkpoint inhibition. Previous studies have shown that post-translational modifications (PTMs), which include ubiquitination, glycosylation, phosphorylation and palmitoylation, are usually therapeutic targets for pharmacological inhibition of cancers (Huang et al. 2019; Yang et al. 2019; Zhang, Dang, et al. 2018; Hsu et al. 2018). In addition, accumulating evidence prove that PD-L1 is widely regulated by ubiquitin/proteasome pathway (Li et al. 2016; Lim et al. 2016; Mezzadra et al. 2017; Burr et al. 2017). These findings indicate that targeting of PD-L1 ubiquitination is a potential strategy to boost anti-tumor immunity. In this paper, we mainly investigate the deubiquitination of PD-L1.

At present, approximately 100 deubiquitinases (DUBs) have been found in humans and there are six structurally distinct DUB families (Mevissen and Komander 2017). These include five cysteine proteases subfamilies: the ubiquitin-specific proteases (USPs), the ubiquitin C-terminal hydrolases (UCHs), the ovarian tumor proteases (OTUs), the Josephin family and the motif interacting with ubiquitin (MIU)-containing novel DUB family (MINDYs). In addition, a family of JAB1/MPN/MOV34 metalloprotease DUBs (JAMMs) exists (Mevissen and Komander 2017; Chen, Zhou, and Chen 2017). USPs are usually involved in the regulation of cell signaling pathways, and the family contains tumor suppressors and oncogenes (Komander, Clague, and Urbe 2009; Reyes-Turcu, Ventii, and Wilkinson 2009). Up to now, some selective DUB inhibitors have been developed and tested in clinical trials, reminding of their potential druggability (Kategaya et al. 2017; Turnbull et al. 2017; Arceci et al. 2019).

USP21 belongs to USPs family and has a C-terminal catalytic DUB domain, is a nuclear/cytoplasmic shuttling protein that exhibits the ability to deubiquitinate RIPK1, FOXM1, and STING, as well as histone H2A (Ye et al. 2011; Chen et al. 2017; Xu et al. 2010; Arceci et al. 2019; Nakagawa et al. 2008). In addition, we noted that USP21 is located in 1q21 of chromosome 1, which is frequently magnified in human cancers (Nguyen et al. 2017; Chen, Chan, and Guan 2010). This region contains a variety of oncogenes such as MDM2 and creb3l4 and the elevation of their copy number has a relation with worse survival in many human cancers (Chen, Chan, and Guan 2010; Nilsson et al. 2004; Shah et al. 2017; Nguyen et al. 2017).

Lung cancer is the main cause of cancer-related mortality around the world, 80–85% of human lung cancer is non-small cell lung cancer (NSCLC), lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC) are the main subtypes (Cheng et al. 2019; Cancer Genome Atlas Research 2012). In China, there were ~ 0.77 million newly diagnosed and 0.69 million death due to lung cancer in 2018 (Feng et al. 2019). Immune checkpoint inhibitors that target PD-1 and PD-L1 have demonstrated significant overall survival benefit in advanced NSCLC (Gandara et al. 2018; Rittmeyer et al. 2017; Fehrenbacher et al. 2016; Reck et al. 2016).

In this study, we investigate the effects of USP21 on the deubiquitination and stabilization of PD-L1 and imply that targeting USP21 is a potential treatment for lung cancer.

Methods

Cell culture and transfection.

Human embryonic kidney cell line (HEK293T), human cervical epithelioid carcinoma cell line (HeLa) and non-small cell lung adenocarcinoma cancer cell line (H1975) were obtained from Dr. JunYing Yuan (Harvard medical school, Boston). HEK293T cells were cultured in DMEM (#SH30022.01, HyClone) supplemented with 10% FBS (#3682, Internegocios-sa) and 1% Penicillin /Streptomycin (#15140-122, GIBCO). H1975 cells were cultured in RPMI-1640 (#SH30809.01, HyClone) supplemented with 10% FBS and 1% Penicillin /Streptomycin. All cell lines were incubated at 37 °C with 5% CO₂. For transfection, HEK293T cells were seeded in 6-well plates and transfected with indicated plasmids by using Lipofectamine 2000 (Invitrogen). MG-132 (SelleckChem) were used at a concentration of 20 µM for 6 h.

Reagents and antibodies.

MG-132 (#S2619) was purchased from SelleckChem, Ltd. The following antibodies were used: Anti-PD-L1 (#66248, HUABIO, WB 1:1000, IHC 1:200), anti-USP21 (#sc-515991, Santa Cruz Biotechnology, WB 1:200), anti-USP21 (#EM-1901-37, HUABIO, IHC 1:200), anti-Flag-HRP (#M185-7, MBL, WB 1:20000), anti-HA (#0906, HUABIO, WB 1:5000), anti-K48 (#05-1307, Milipore, WB 1:1000), anti-Ub (P4D1) (#SC-8017, Santa Cruz Biotechnology, WB 1:200), anti-β-tubulin (#M1305-2, HUABIO, WB 1:5000), goat anti-mouse IgG (H + L) secondary antibody, HRP (#31430, Thermo Fisher Scientific, 1:20000), goat anti-rabbit IgG (H + L) secondary antibody, HRP (#31460, Thermo Fisher Scientific, 1:20000).

Plasmids.

Full-length USP21 cDNA was provided by Life Sciences Institute, Zhejiang University. USP21 was cloned into pCMV3 by PCR amplifying from cDNA templates. USP21 C221A mutation was introduced by PCR and confirmed by DNA sequencing. The mutation primer sequences were: 5'-CCTGGGAAACACGGCCTTCCTGAATGCTG-3' and 5'-CAGCATT CAGGAAGGCCGTGTTTCCCAGG - 3'. Full-length PD-L1 was provided by Life Sciences Institute, Zhejiang University. PD-L1 fragments 1–6 were PCR-amplified from pCMV3-3XFlag-PD-L1 plasmid. The primer sequences were: 1-F 5'-GGCATTGCTGAACGCATTGGTAATTCTGGGAGCCAT-3', 1-R 5'-TGCGTTCAGCAAATGCCAGTAGGTCATGAA-3'; 2-F 5'-GGCATTGCTGAACGCATACAGACAGAGGGCCCGGCT-3', 2-R 5'-TGCGTTCAGCAAATGCCAGTAGGTCATGAA-3'; 3-F 5'-AGGTT CAGCATAGTAGCCCCAAGGCCGAAGTCATCTG-3', 3-R 5'-GCTACTATGCTGAACCTTCAGGTCTTCCTC-3'; 4-F 5'-GTCAGGCTGAGGGCTACTTGGTAATTCTGGGAGCCAT-3', 4-R 5'-GTAGCCCTCAGCCTGACATGTCAGTTCATG-3'; 5-F 5'-CAAATGAAAGGACTCACAGAAAAGGGAGAATGATGGA-3', 5-R 5'-GTGAGTCCTTTCATTTGGAGGATGTGCCAG-3'; 6-F 5'-CATTCATCTTCCGTTTATAATGATCTAGAGCGGCCGC-3', 6-R 5'-TAAACGGAAGATGAATGTCAGTGCTACACC-3'. The PD-L1 mutation (K263R, K270R, K271R, K280R, K281R) was introduced by PCR and confirmed by DNA sequencing. The mutation primer sequences were: K263R-F: 5'-

ATTCATCTTCCGTTTAAGAAGAGGGAGAATGATGGATGTGA-3', K263R-R: 5'-TCACATCCATCATTCTCCCTCTTCTTAAACGGAAGATG AAT-3'; K270R-F: AGGGAGAATGATGGATGTGAGAAAATGTGGCATCCAAGATA-3', K270R-R: TATCTTGGATGCCACATTTTCTCACATCCATCATTCTCCCT-3'; K271R-F: GAGAATGATGGATGTGAAAAGATGTGGCATCCAAGATACAA-3', K271R-R: TTGTATCTTGGATGCCACATCTTTTCACATCCATCATTCTC-3'; K280R-F: CATCCAAGATACAACTCAAGGAAGCAAAGTGATACACATT-3', K280R-R: AATGTGTATCACTTTGCTTCCTTGAGTTTGTATCTTGGATG-3'; K281R-F: CCAAGATACAACTCAAAGAGGCAAAGTGATACACATTTGG-3', K281R-R: CCAAATGTGTATCACTTTGCCTCTTTGAGTTTGTATCTTGG-3'. The PD-L1 5K mutation primer sequences were: 5K-F: 5'-CATTCATCTTCCGTTTAAGAAGAGGGAGAATGATGGATGTGAGAAGATGTGGCATCCAA, 5K-R: 5'-TCCAAATGTGTATCACTTTGCCTCCTTGAGTTTGTATCTTGGATGCCACATCTTCTCACATCCA. The shRNA used to knock down USP21 expression of human was purchased from Vigene Biosciences, Inc. The shRNA target sequences for USP21 knockdown was 5'-GCTCCACCGATACTTGCCAATTTCAAGAGAATTGGCAAGTATCGGTGGAGCTTTTTT-3'.

Immunoblotting.

Samples were lysed in RIPA buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM NaF, 1 mM Na₃VO₄) and heated at 100 °C for 10 min in 2 × SDS loading buffer. Next, samples were separated by gel electrophoresis using 8–12% SDS-PAGE gels and transferred to nitrocellulose membranes for 1–2 h at 0.3A current on the ice. Membranes were blocked in 5–10% nonfat dried milk (Wandashan) diluted in PBS-T (PBS, 1% Tween-20) and probed with the indicated primary antibodies in PBST containing 5% (w/v) BSA (#9048-46-8, Sangon Biotech) overnight at 4 °C. All HRP-conjugated secondary antibodies were incubated at room temperature for 1 h. All washing steps were performed using PBS-T (10 min, 3 times). Detection was performed using HRP-conjugated secondary antibodies and chemiluminescence reagents (#4AW001-500, Beijing 4A Biotech Co, Ltd.).

Immunoprecipitations.

HEK293T cells were transfected with indicated plasmids and collected in RIPA buffer at 4 °C for 30 min. After 15,000 g centrifugation at 4 °C, supernatant and following conjugated beads were combined and rotated at 4 °C for 6 h: anti-Flag (DYKDDDDK) affinity gel (#B23102, Bimake) and anti-HA magnetic (#B26202, Bimake) beads. The beads were collected and washed with RIPA buffer 4 times and heated at 100 °C for 10 min in 2 × SDS loading buffer prior to IB. HeLa cells were collected in RIPA buffer at 4 °C for 40 min. After 15,000 g centrifugation at 4 °C, supernatant from samples were pre-cleared to remove non-specific interactions with protein A/G beads rotating at 4 °C for 2 h. Following pre-clearing and PD-L1 antibody conjugation (4 °C for 2 h), lysate and beads were combined and rotated at 4 °C for 6 h. Following incubation, A/G beads were collected and washed with RIPA buffer 4 times and heated at 100 °C for 10 min in 2 × SDS loading buffer prior to IB.

In vitro deubiquitination assay.

HEK293T cells were transfected with Flag-PD-L1, HA-USP21, HA-USP21-C221A expression plasmids. Cells were collected 48 h post transfection following 6 h MG-132 (20 μ M) treatment. Cells were lysed using a RIPA lysis buffer with protease inhibitor and centrifuged at 15,000 g for 10 min. The supernatant containing protein was incubated with Flag/HA-beads for 6 h. PD-L1 and USP21 were purified from the cell extracts with Flag/HA-beads in lysis buffer. For deubiquitination assay, ubiquitinated PD-L1 was incubated with HA-USP21 or HA-USP21-C221A in DUB Buffer (50 mM Tris pH 7.5, 50 mM NaCl, 5 mM DTT, 5 mM $MgCl_2$). Reactions were incubated at 30 °C for 2 h on rotary shaker. Next, proteins were eluted from beads and heated at 100 °C. Ubiquitination status of PD-L1 was assessed by IB as described above.

Immunohistochemistry.

The IHC of human tumor samples was done by HUABIO, China, with mouse anti-PD-L1 (#66248, HUABIO, IHC 1:200) and mouse anti-USP21 antibody (#EM-1901-37, HUABIO, IHC 1:200) imaging with OLYMPUS BX61 microscopy.

Clinical samples.

The Lung cancer samples were acquired from The First Affiliated Hospital, College of Medicine, Zhejiang University. Tissue samples were rapidly frozen in liquid nitrogen after surgical resection and stored at -80 °C for further analysis.

Bioinformatics analysis.

Cancer genomics-related data were obtained from The Cancer Genome Atlas (TCGA, <http://cancergenome.nih.gov>) and Catalogue of Somatic Mutations in Cancer (<https://cancer.sanger.ac.uk/cosmic>) databases. In the result analysis, no sample was eliminated.

Statistics.

Statistical analyses were performed using the two-tailed Student's t-test. Data were analyzed using the Prism software (GraphPad, San Diego, CA, USA) and expressed as the mean $\pm$ S.D. $p < 0.05$ was considered to be significant.

Results

USP21 binds and regulates the stability of PD-L1.

In our previous research, we have identified USP21 as a potential effector of PD-L1. To determine whether PD-L1 stability is positively controlled by USP21, we examined protein accumulation of PD-L1 in HEK293T and H1975 cells. Immunoblot analysis reveal that PD-L1 abundance significantly increased with overexpression of USP21 (Fig. 1a-b). In addition, knockdown of USP21 induced degradation of

endogenous PD-L1 in HEK293T cells (Fig. 1c). These results indicate that PD-L1 abundance is regulated by USP21. Next, a robust endogenous interaction between PD-L1 and USP21 was detected in HeLa cells (Fig. 1d). Correspondingly, an exogenous interaction between PD-L1 and USP21 was also detected in HEK293T cells by co-immunoprecipitation (Fig. 1e). These results reveal that USP21 directly interacted with PD-L1. Furthermore, to determine the domain on PD-L1 recognized by USP21, we analyzed the interaction between USP21 and different truncations of PD-L1 (Fig. 1f). These results reveal that USP21 binds to PD-L1 through the intracellular (C-terminal 262–290 aa) and extracellular domains (N-terminal 19–241 aa) (Fig. 1g).

USP21 functions as a PD-L1 DUB.

USP21 is a deubiquitinase that remove polyubiquitin chains from its substrates and has been shown to cleave K48 polyubiquitin linkages *in vitro* (Ye et al. 2011). Previous studies have shown that PD-L1 levels were regulated by K48-linked polyubiquitination and proteasomal degradation (Li et al. 2016). To determine whether USP21 affects the binding of polyubiquitin chains on PD-L1, we analyzed PD-L1 ubiquitination in the presence of MG132, a proteasome inhibitor. We found that MG132-induced PD-L1 ubiquitination was abolished by USP21 overexpression (Fig. 2a). However, the level of polyubiquitinated PD-L1 was restored when USP21-C221A (catalytically inactive mutant) was overexpressed, confirming the involvement of the catalytic activity of this deubiquitinase (Fig. 3a). Next, we performed an *in vitro* deubiquitination assay using USP21-WT and USP21-C221A immunopurified from HEK293T cells. Similarly, USP21-WT reduced polyubiquitin chains of PD-L1 (Fig. 2b), while USP21-C221A did not (Fig. 3b). These data suggest USP21 is the deubiquitinase of PD-L1.

PD-L1 is a transmembrane protein, including N-terminal signal peptide, extracellular domain, transmembrane domain and intracellular domain (Zhang, Bu, et al. 2018). To explore the mechanism behind USP21-induced PD-L1 stability, we detected whether PD-L1 undergoes any deubiquitination changes following co-incubating with USP21. As determined by mass spectrometry, USP21 induced several PD-L1 deubiquitination events (Fig. 2c). We found the deubiquitination sites are mainly located in the intracellular domain of PD-L1, such as K280, K281 and K270 (Fig. 2c). Thus, we hypothesized that USP21 deubiquitinates PD-L1 mainly through intracellular domain and we found five potential sites (Fig. 2d). Mutating these residues to ubiquitination-resistant arginine showed that single-site mutant (K263R, K270R, K271R, K280R or K281R) had no significant effect on the ubiquitination level of PD-L1 (Fig. 2e), while 5KR mutation almost abolished ubiquitination levels (Fig. 2f). These results indicate that these five lysines are potential deubiquitin sites of PD-L1.

Cancer-derived mutations of PD-L1 disrupt the regulation of deubiquitination.

Catalogue of Somatic Mutations in Cancer (COSMIC) database reveal that PD-L1 mutations occur in 1.6% of human cancers and are largely clustered within the intracellular domain (Fig. 3c). To further explore the effect of cancer-derived mutations of PD-L1 in deubiquitination mediated by USP21, we constructed mutants in intracellular domain of PD-L1, we found these mutants (R260C, I274V, D276Y, D276H, T277S, T277K, T290M, K280N) had little effect on ubiquitination of PD-L1, while the two mutants

in Asp276 (D276Y and D276H) enhanced deubiquitination levels (Fig. 3d-e). These results suggest that inhibition of USP21 may provide a better therapeutic strategy on cancers with PD-L1 Asp276 mutations.

PD-L1 and USP21 are up-regulated in lung cancer.

Finally, we analyzed the gene expression frequency of PD-L1 and USP21 in 17 types of cancers through The Cancer Genome Atlas (TCGA) database (Fig. 4a). We found the proportions of highly expressed PD-L1 and USP21 were higher in lung cancer and mRNA levels of PD-L1 and USP21 were overexpressed when compared to normal tissues ($p < 0.001$) (Fig. 4a-b). In addition, it should be noted that the mRNA levels of PD-L1 and USP21 were higher in lung squamous cell carcinoma (LUSC) than that in lung adenocarcinoma (LUAD) (Fig. 4c). USP21 amplification was also observed in other cancer types (Fig. 4a), which is consistent with previous studies that USP21 regulates cell proliferation and metastasis in colorectal cancer and pancreas cancer (Yun et al. 2020; Hou et al. 2019). Next, we confirmed the expression levels of PD-L1 and USP21 in LUSC and found that upregulated expression of PD-L1 accounted for 80% (395 of 494) of LUSC samples, while USP21 accounted for 76% (375 of 494) of LUSC samples (Fig. 4d). Furthermore, the expression frequency of USP21 in different clinical stages of lung cancer is consistent with that in PD-L1. (Fig. 4e-f). Together, these results suggest that USP21 is up-regulated in lung cancer, especially in LUSC and its amplification co-occurs with upregulation of PD-L1. Then we measured PD-L1 and USP21 levels in two paired lung cancer tumor samples and their adjacent normal tissues, IHC analysis showed stronger staining of PD-L1 and USP21 in lung cancer samples than adjacent tissues (Fig. 4g).

Discussion

DUBs have been proposed as new anti-cancer targets and several small-molecule DUB inhibitors have been developed as anti-cancer drugs (D'Arcy, Wang, and Linder 2015; Harrigan et al. 2018). Recent studies suggest that USP21 is closely related to the development of cancers, such as hepatocellular carcinoma, pancreas cancer and breast cancer (Xu et al. 2010; Zhang et al. 2013; Pannu et al. 2015; Chen, Zhou, and Chen 2017; Liu et al. 2017; Peng et al. 2016). However, the details of molecular mechanism need further exploration. In this study, we identified PD-L1 as a novel substrate of USP21.

Ubiquitination is one of the most important PTMs of proteins and protein ubiquitination involves a variety of cellular processes, such as protein degradation, immune signal transduction and transcriptional activation or inhibition pathways (Hershko and Ciechanover 1998; Sun 2008; Cao and Yan 2012; Mevissen and Komander 2017). In addition to USP21, the ubiquitin level of PD-L1 is regulated by several other proteins, including deubiquitinase CNS5 (Lim et al. 2016) and E3 ubiquitin ligases, such as Cul3^{SPOP} and β -TrCP (Zhang, Bu, et al. 2018; Li et al. 2016). Recent clinical studies showed that the success of blocking PD-1 and PD-L1 is related to the expression level of PD-L1 in tumor cells (Zhang, Bu, et al. 2018; Iwai et al. 2002; Herbst et al. 2014). Together, we hypothesized that combination treatments that target DUB and E3 ubiquitin ligases might enhance therapeutic efficacy on anti-tumor immunity.

In further research, we determined the deletion of last 28 amino acids of PD-L1 (262–290), impairs the interaction between PD-L1 and USP21, indicating that 262–290 region of PD-L1 is critical for the interaction with USP21. Notably, among the eight cancer-derived PD-L1 mutants located within intracellular domain, mutations at Asp276 enhanced the deubiquitination of PD-L1, which may lead to accumulation of PD-L1, allowing escape from anti-tumor immunity. These results suggest that the intracellular domain of PD-L1 is essential for the regulation of deubiquitination.

USP21 has been shown to exhibit carcinogenesis and execute its carcinogenic function via its deubiquitination activity (Hou et al. 2019). However, the role of USP21 in lung cancer remains unclear. In this paper, our results suggest that USP21 is up-regulated in lung cancer, especially in lung squamous cell carcinoma, the high expression rates of PD-L1 and USP21 both exceed 70%. Previous studies proved that CSN5 is the DUB of PD-L1 and the correlation between PD-L1 and CSN5 was confirmed in breast cancer (Lim et al. 2016). However, we found no correlation between PD-L1 and CSN5 in lung cancer. This interesting cancer-specific difference in PTMs of PD-L1 makes the further research to explore how it regulates stabilization of PD-L1 and escape from anti-tumor immunity more valuable.

Conclusion

In conclusion, we identified USP21 as a novel deubiquitinase of PD-L1. USP21 induces PD-L1 stabilization and cancer escape from immunity via its deubiquitination levels. Importantly, USP21 is up-regulated in lung cancer and co-occurs with the upregulation of PD-L1. Hopefully, targeting PD-L1 by inhibiting USP21 might be a potentially novel strategy for the treatment of lung cancer.

Abbreviations

Co-IP
Co-immunoprecipitation; COSMIC: Catalogue of Somatic Mutations in Cancer; DUBs: deubiquitinases; IB: Immunoblot; NSCLC: non-small cell lung cancer; LUAD: lung adenocarcinoma; LUSC: lung squamous cell carcinoma; MS: mass spectrometry; PD-1: programmed death protein-1; PD-L1: programmed death ligand 1; PTMs: post-translational modifications; TCGA: The Cancer Genome Atlas; USP21: ubiquitin-specific protease 21; USPs: ubiquitin-specific proteases.

Declarations

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Authors' contributions

H.X. conceived and coordinated the project. H.X., S.Y., Q.W. designed the experiments. S.Y. and H.Y. performed most of the experiments, data collection and analysis. S.Y. and Y.W. wrote the manuscript. B.S., D.Z., X.L., X.M., S.Z., Q.Z. assisted with the experiments.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The research was approved by the Ethics Committee of Zhejiang University. All patients provided written informed consent before this study.

Consent for publication

All authors consent for publications.

Competing interests

The authors declare that they have no competing interests.

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References

1. Arceci, A., T. Bonacci, X. Wang, K. Stewart, J. S. Damrauer, K. A. Hoadley, and M. J. Emanuele. 2019. 'FOX M1 Deubiquitination by USP21 Regulates Cell Cycle Progression and Paclitaxel Sensitivity in Basal-like Breast Cancer', *Cell Reports*, 26: 3076-86 e6.
2. Burr, M. L., C. E. Sparbier, Y. C. Chan, J. C. Williamson, K. Woods, P. A. Beavis, E. Y. N. Lam, M. A. Henderson, C. C. Bell, S. Stolzenburg, O. Gilan, S. Bloor, T. Noori, D. W. Morgens, M. C. Bassik, P. J.

- Neeson, A. Behren, P. K. Darcy, S. J. Dawson, I. Voskoboinik, J. A. Trapani, J. Cebon, P. J. Lehner, and M. A. Dawson. 2017. 'CMTM6 maintains the expression of PD-L1 and regulates anti-tumour immunity', *Nature*, 549: 101-05.
3. Cancer Genome Atlas Research, Network. 2012. 'Comprehensive genomic characterization of squamous cell lung cancers', *Nature*, 489: 519-25.
 4. Cao, J., and Q. Yan. 2012. 'Histone ubiquitination and deubiquitination in transcription, DNA damage response, and cancer', *Front Oncol*, 2: 26.
 5. Chen, L., T. H. Chan, and X. Y. Guan. 2010. 'Chromosome 1q21 amplification and oncogenes in hepatocellular carcinoma', *Acta Pharmacologica Sinica*, 31: 1165-71.
 6. Chen, Y., L. Wang, J. Jin, Y. Luan, C. Chen, Y. Li, H. Chu, X. Wang, G. Liao, Y. Yu, H. Teng, Y. Wang, W. Pan, L. Fang, L. Liao, Z. Jiang, X. Ge, B. Li, and P. Wang. 2017. 'p38 inhibition provides anti-DNA virus immunity by regulation of USP21 phosphorylation and STING activation', *Journal of Experimental Medicine*, 214: 991-1010.
 7. Chen, Y., B. Zhou, and D. H. Chen. 2017. 'USP21 promotes cell proliferation and metastasis through suppressing EZH2 ubiquitination in bladder carcinoma', *Oncotargets and Therapy*, 10: 681-89.
 8. Cheng, Z., C. Yu, S. Cui, H. Wang, H. Jin, C. Wang, B. Li, M. Qin, C. Yang, J. He, Q. Zuo, S. Wang, J. Liu, W. Ye, Y. Lv, F. Zhao, M. Yao, L. Jiang, and W. Qin. 2019. 'circTP63 functions as a ceRNA to promote lung squamous cell carcinoma progression by upregulating FOXM1', *Nature Communications*, 10: 3200.
 9. D'Arcy, P., X. Wang, and S. Linder. 2015. 'Deubiquitinase inhibition as a cancer therapeutic strategy', *Pharmacology & Therapeutics*, 147: 32-54.
 10. Fehrenbacher, L., A. Spira, M. Ballinger, M. Kowanetz, J. Vansteenkiste, J. Mazieres, K. Park, D. Smith, A. Artal-Cortes, C. Lewanski, F. Braiteh, D. Waterkamp, P. He, W. Zou, D. S. Chen, J. Yi, A. Sandler, A. Rittmeyer, and Poplar Study Group. 2016. 'Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicentre, open-label, phase 2 randomised controlled trial', *Lancet*, 387: 1837-46.
 11. Feng, R. M., Y. N. Zong, S. M. Cao, and R. H. Xu. 2019. 'Current cancer situation in China: good or bad news from the 2018 Global Cancer Statistics?', *Cancer Commun (Lond)*, 39: 22.
 12. Gandara, D. R., S. M. Paul, M. Kowanetz, E. Schleifman, W. Zou, Y. Li, A. Rittmeyer, L. Fehrenbacher, G. Otto, C. Malboeuf, D. S. Lieber, D. Lipson, J. Silterra, L. Amler, T. Riehl, C. A. Cummings, P. S. Hegde, A. Sandler, M. Ballinger, D. Fabrizio, T. Mok, and D. S. Shames. 2018. 'Blood-based tumor mutational burden as a predictor of clinical benefit in non-small-cell lung cancer patients treated with atezolizumab', *Nat Med*, 24: 1441-48.
 13. Harrigan, J. A., X. Jacq, N. M. Martin, and S. P. Jackson. 2018. 'Deubiquitylating enzymes and drug discovery: emerging opportunities', *Nature Reviews Drug Discovery*, 17: 57-77.
 14. Herbst, R. S., J. C. Soria, M. Kowanetz, G. D. Fine, O. Hamid, M. S. Gordon, J. A. Sosman, D. F. McDermott, J. D. Powderly, S. N. Gettinger, H. E. Kohrt, L. Horn, D. P. Lawrence, S. Rost, M. Leabman,

- Y. Xiao, A. Mokatrini, H. Koeppen, P. S. Hegde, I. Mellman, D. S. Chen, and F. S. Hodi. 2014. 'Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients', *Nature*, 515: 563-7.
15. Herskho, A., and A. Ciechanover. 1998. 'The ubiquitin system', *Annu Rev Biochem*, 67: 425-79.
16. Hou, P., X. Ma, Q. Zhang, C. J. Wu, W. Liao, J. Li, H. Wang, J. Zhao, X. Zhou, C. Guan, J. Ackroyd, S. Jiang, J. Zhang, D. J. Spring, Y. A. Wang, and R. A. DePinho. 2019. 'USP21 deubiquitinase promotes pancreas cancer cell stemness via Wnt pathway activation', *Genes Dev*, 33: 1361-66.
17. Hsu, J. M., C. W. Li, Y. J. Lai, and M. C. Hung. 2018. 'Posttranslational Modifications of PD-L1 and Their Applications in Cancer Therapy', *Cancer Research*, 78: 6349-53.
18. Huang, X., Q. Zhang, Y. Lou, J. Wang, X. Zhao, L. Wang, X. Zhang, S. Li, Y. Zhao, Q. Chen, T. Liang, and X. Bai. 2019. 'USP22 Deubiquitinates CD274 to Suppress Anticancer Immunity', *Cancer Immunol Res*, 7: 1580-90.
19. Iwai, Y., M. Ishida, Y. Tanaka, T. Okazaki, T. Honjo, and N. Minato. 2002. 'Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade', *Proc Natl Acad Sci U S A*, 99: 12293-7.
20. Kategaya, L., P. Di Lello, L. Rouge, R. Pastor, K. R. Clark, J. Drummond, T. Kleinheinz, E. Lin, J. P. Upton, S. Prakash, J. Heideker, M. McClelland, M. S. Ritorto, D. R. Alessi, M. Trost, T. W. Bainbridge, M. C. M. Kwok, T. P. Ma, Z. Stiffler, B. Brasher, Y. Tang, P. Jaishankar, B. R. Hearn, A. R. Renslo, M. R. Arkin, F. Cohen, K. Yu, F. Peale, F. Gnad, M. T. Chang, C. Klijn, E. Blackwood, S. E. Martin, W. F. Forrest, J. A. Ernst, C. Ndubaku, X. Wang, M. H. Beresini, V. Tsui, C. Schwerdtfeger, R. A. Blake, J. Murray, T. Maurer, and I. E. Wertz. 2017. 'USP7 small-molecule inhibitors interfere with ubiquitin binding', *Nature*, 550: 534-38.
21. Komander, D., M. J. Clague, and S. Urbe. 2009. 'Breaking the chains: structure and function of the deubiquitinases', *Nat Rev Mol Cell Biol*, 10: 550-63.
22. Li, C. W., S. O. Lim, W. Xia, H. H. Lee, L. C. Chan, C. W. Kuo, K. H. Khoo, S. S. Chang, J. H. Cha, T. Kim, J. L. Hsu, Y. Wu, J. M. Hsu, H. Yamaguchi, Q. Ding, Y. Wang, J. Yao, C. C. Lee, H. J. Wu, A. A. Sahin, J. P. Allison, D. Yu, G. N. Hortobagyi, and M. C. Hung. 2016. 'Glycosylation and stabilization of programmed death ligand-1 suppresses T-cell activity', *Nature Communications*, 7: 12632.
23. Lim, S. O., C. W. Li, W. Y. Xia, J. H. Cha, L. C. Chan, Y. Wu, S. S. Chang, W. C. Lin, J. M. Hsu, Y. H. Hsu, T. Kim, W. C. Chang, J. L. Hsu, H. Yamaguchi, Q. Q. Ding, Y. Wang, Y. Yang, C. H. Chen, A. A. Sahin, D. H. Yu, G. N. Hortobagyi, and M. C. Hung. 2016. 'Deubiquitination and Stabilization of PD-L1 by CSN5', *Cancer Cell*, 30: 925-39.
24. Liu, J., A. Kruswick, H. Dang, A. D. Tran, S. M. Kwon, X. W. Wang, and P. Oberdoerffer. 2017. 'Ubiquitin-specific protease 21 stabilizes BRCA2 to control DNA repair and tumor growth', *Nature Communications*, 8: 137.
25. Mevissen, T. E. T., and D. Komander. 2017. 'Mechanisms of Deubiquitinase Specificity and Regulation', *Annu Rev Biochem*, 86: 159-92.
26. Mezzadra, R., C. Sun, L. T. Jae, R. Gomez-Eerland, E. de Vries, W. Wu, M. E. W. Logtenberg, M. Slagter, E. A. Rozeman, I. Hofland, A. Broeks, H. M. Horlings, L. F. A. Wessels, C. U. Blank, Y. L. Xiao, A. J. R.

- Heck, J. Borst, T. R. Brummelkamp, and T. N. M. Schumacher. 2017. 'Identification of CMTM6 and CMTM4 as PD-L1 protein regulators', *Nature*, 549: 106-+.
27. Nakagawa, T., T. Kajitani, S. Togo, N. Masuko, H. Ohdan, Y. Hishikawa, T. Koji, T. Matsuyama, T. Ikura, M. Muramatsu, and T. Ito. 2008. 'Deubiquitylation of histone H2A activates transcriptional initiation via trans-histone cross-talk with H3K4 di- and trimethylation', *Genes Dev*, 22: 37-49.
 28. Nguyen, H. T., J. M. Kugler, A. C. Loya, and S. M. Cohen. 2017. 'USP21 regulates Hippo pathway activity by mediating MARK protein turnover', *Oncotarget*, 8: 64095-105.
 29. Nilsson, M., L. A. Meza-Zepeda, F. Mertens, A. Forus, O. Myklebost, and N. Mandahl. 2004. 'Amplification of chromosome 1 sequences in lipomatous tumors and other sarcomas', *Int J Cancer*, 109: 363-9.
 30. Pannu, J., J. I. Belle, M. Forster, C. U. Duerr, S. Y. Shen, L. Kane, K. Harcourt, J. H. Fritz, S. Clare, and A. Nijnik. 2015. 'Ubiquitin Specific Protease 21 Is Dispensable for Normal Development, Hematopoiesis and Lymphocyte Differentiation', *Plos One*, 10.
 31. Peng, L., Y. Hu, D. M. Chen, S. C. Jiao, and S. K. Sun. 2016. 'Ubiquitin specific peptidase 21 regulates interleukin-8 expression, stem-cell like property of human renal cell carcinoma', *Oncotarget*, 7: 42007-16.
 32. Petrelli, F., M. Maltese, G. Tomasello, B. Conti, K. Borgonovo, M. Cabiddu, M. Ghilardi, M. Ghidini, R. Passalacqua, S. Barni, and M. Brighenti. 2018. 'Clinical and Molecular Predictors of PD-L1 Expression in Non-Small-Cell Lung Cancer: Systematic Review and Meta-analysis', *Clin Lung Cancer*, 19: 315-22.
 33. Pitt, J. M., M. Vetizou, R. Daillere, M. P. Roberti, T. Yamazaki, B. Routy, P. Lepage, I. G. Boneca, M. Chamaillard, G. Kroemer, and L. Zitvogel. 2016. 'Resistance Mechanisms to Immune-Checkpoint Blockade in Cancer: Tumor-Intrinsic and -Extrinsic Factors', *Immunity*, 44: 1255-69.
 34. Reck, M., D. Rodriguez-Abreu, A. G. Robinson, R. Hui, T. Csoszi, A. Fulop, M. Gottfried, N. Peled, A. Tafreshi, S. Cuffe, M. O'Brien, S. Rao, K. Hotta, M. A. Leiby, G. M. Lubiniecki, Y. Shentu, R. Rangwala, J. R. Brahmer, and Keynote- Investigators. 2016. 'Pembrolizumab versus Chemotherapy for PD-L1-Positive Non-Small-Cell Lung Cancer', *N Engl J Med*, 375: 1823-33.
 35. Reyes-Turcu, F. E., K. H. Ventii, and K. D. Wilkinson. 2009. 'Regulation and cellular roles of ubiquitin-specific deubiquitinating enzymes', *Annu Rev Biochem*, 78: 363-97.
 36. Ribas, A., and J. D. Wolchok. 2018. 'Cancer immunotherapy using checkpoint blockade', *Science*, 359: 1350-+.
 37. Rittmeyer, A., F. Barlesi, D. Waterkamp, K. Park, F. Ciardiello, J. von Pawel, S. M. Gadgeel, T. Hida, D. M. Kowalski, M. C. Dols, D. L. Cortinovis, J. Leach, J. Polikoff, C. Barrios, F. Kabbinavar, O. A. Frontera, F. De Marinis, H. Turna, J. S. Lee, M. Ballinger, M. Kowanetz, P. He, D. S. Chen, A. Sandler, D. R. Gandara, and O. A. K. Study Group. 2017. 'Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial', *Lancet*, 389: 255-65.

38. Salmaninejad, A., S. F. Valilou, A. G. Shabgah, S. Aslani, M. Alimardani, A. Pasdar, and A. Sahebkar. 2019. 'PD-1/PD-L1 pathway: Basic biology and role in cancer immunotherapy', *Journal of Cellular Physiology*, 234: 16824-37.
39. Shah, G. L., H. Landau, D. Londono, S. M. Devlin, S. Kosuri, A. M. Lesokhin, N. Lendvai, H. Hassoun, D. J. Chung, G. Koehne, S. C. Jhanwar, O. Landgren, R. Levine, and S. A. Giralt. 2017. 'Gain of chromosome 1q portends worse prognosis in multiple myeloma despite novel agent-based induction regimens and autologous transplantation', *Leuk Lymphoma*, 58: 1823-31.
40. Sun, S. C. 2008. 'Deubiquitylation and regulation of the immune response', *Nat Rev Immunol*, 8: 501-11.
41. Turnbull, A. P., S. Ioannidis, W. W. Krajewski, A. Pinto-Fernandez, C. Heride, A. C. L. Martin, L. M. Tonkin, E. C. Townsend, S. M. Buker, D. R. Lancia, J. A. Caravella, A. V. Toms, T. M. Charlton, J. Lahdenranta, E. Wilker, B. C. Follows, N. J. Evans, L. Stead, C. Alli, V. V. Zarayskiy, A. C. Talbot, A. J. Buckmelter, M. Wang, C. L. McKinnon, F. Saab, J. F. McGouran, H. Century, M. Gersch, M. S. Pittman, C. G. Marshall, T. M. Raynham, M. Simcox, L. M. D. Stewart, S. B. McLoughlin, J. A. Escobedo, K. W. Bair, C. J. Dinsmore, T. R. Hammonds, S. Kim, S. Urbe, M. J. Clague, B. M. Kessler, and D. Komander. 2017. 'Molecular basis of USP7 inhibition by selective small-molecule inhibitors', *Nature*, 550: 481-86.
42. Xu, G. F., X. J. Tan, H. M. Wang, W. J. Sun, Y. Shi, S. Burlingame, X. Gu, G. W. Cao, T. Zhang, J. Qin, and J. H. Yang. 2010. 'Ubiquitin-specific Peptidase 21 Inhibits Tumor Necrosis Factor alpha-induced Nuclear Factor kappa B Activation via Binding to and Deubiquitinating Receptor-interacting Protein 1', *Journal of Biological Chemistry*, 285: 969-78.
43. Yang, Y., J. M. Hsu, L. Sun, L. C. Chan, C. W. Li, J. L. Hsu, Y. Wei, W. Xia, J. Hou, Y. Qiu, and M. C. Hung. 2019. 'Palmitoylation stabilizes PD-L1 to promote breast tumor growth', *Cell Res*, 29: 83-86.
44. Yarchoan, M., A. Hopkins, and E. M. Jaffee. 2017. 'Tumor Mutational Burden and Response Rate to PD-1 Inhibition', *New England Journal of Medicine*, 377: 2500-01.
45. Ye, Y., M. Akutsu, F. Reyes-Turcu, R. I. Enchev, K. D. Wilkinson, and D. Komander. 2011. 'Polyubiquitin binding and cross-reactivity in the USP domain deubiquitinase USP21', *EMBO Rep*, 12: 350-7.
46. Yun, S. I., H. K. Hong, S. Y. Yeo, S. H. Kim, Y. B. Cho, and K. K. Kim. 2020. 'Ubiquitin-Specific Protease 21 Promotes Colorectal Cancer Metastasis by Acting as a Fra-1 Deubiquitinase', *Cancers*, 12.
47. Zhang, J., X. Bu, H. Wang, Y. Zhu, Y. Geng, N. T. Nihira, Y. Tan, Y. Ci, F. Wu, X. Dai, J. Guo, Y. H. Huang, C. Fan, S. Ren, Y. Sun, G. J. Freeman, P. Sicinski, and W. Wei. 2018. 'Cyclin D-CDK4 kinase destabilizes PD-L1 via cullin 3-SPOP to control cancer immune surveillance', *Nature*, 553: 91-95.
48. Zhang, J., C. Chen, X. X. Hou, Y. Y. Gao, F. Lin, J. Yang, Z. M. Gao, L. N. Pan, L. Q. Tao, C. J. Wen, Z. J. Yao, A. Tsun, G. C. Shi, and B. Li. 2013. 'Identification of the E3 Deubiquitinase Ubiquitin-specific Peptidase 21 (USP21) as a Positive Regulator of the Transcription Factor GATA3', *Journal of Biological Chemistry*, 288: 9373-82.
49. Zhang, J., F. Dang, J. Ren, and W. Wei. 2018. 'Biochemical Aspects of PD-L1 Regulation in Cancer Immunotherapy', *Trends Biochem Sci*, 43: 1014-32.

Figures

Figure 1

USP21 binds and regulates the stability of PD-L1. (a) Western blot analysis of PD-L1 levels in HEK293T cells co-transfected with Flag-PD-L1 and HA-USP21 for 48 h. Cell lysates were immunoblotted with indicated antibodies. (b) Western blot analysis of PD-L1 levels in H1975 cells transfected with HA-USP21 for 48 h. Cell lysates were immunoblotted with indicated antibodies. (c) Western blot analysis of PD-L1 levels in HEK293T cells transfected with USP21-shRNA or control-shRNA (shNC) for 72 h. Cell lysates were immunoblotted with indicated antibodies. shNC: scrambled shRNA. (d) Co-immunoprecipitation (Co-IP) analysis for the interaction of PD-L1 and USP21 in HeLa cells. Endogenous PD-L1 and USP21 were immunoprecipitated and immunoblotted with indicated antibodies. IgG was used as a negative control. (e) Co-IP analysis for the interaction of PD-L1 and USP21 in HEK293T cells co-transfected with Flag-PD-L1 and HA-USP21 for 48 h. (f) A schematic of PD-L1 fragments is shown. (g) Co-IP analysis for the

interaction of USP21 and indicated PD-L1 fragments in HEK293T cells co-transfected with HA-USP21 and Flag-PD-L1 (indicated fragments) for 48 h.

Figure 2

USP21 functions as a PD-L1 DUB. (a) Regulation of PD-L1 ubiquitination by USP21. HEK293T cells were co-transfected with Flag-PD-L1 and HA-USP21 for 48 h and treated with or without MG132 (20 μ M) for 6 h. Cell lysates were immunoprecipitated and immunoblotted with indicated antibodies. (b)

Deubiquitination of PD-L1 in vitro by USP21. Ubiquitinated PD-L1 was incubated with purified USP21 in vitro. The ubiquitination of PD-L1 was detected using indicated antibodies. (c) Mapping PD-L1 ubiquitination sites following USP21 treatment. HEK293T cells were transfected with Flag-PD-L1, HA-USP21-WT or USP21-C221A for 48 h. PD-L1 and USP21 (WT/C221) were purified with Flag- or HA-beads in total cell lysates. Then ubiquitinated PD-L1 was incubated with HA-USP21-WT or HA-USP21-C221A in DUB Buffer and the binding proteins were analyzed by mass spectrometry. Peptide ionization data corresponding to (K280, K281, K270, K189, K75, K271) is shown. (d) Candidate ubiquitin sites in the intracellular domain of PD-L1. (e-f) Co-IP analysis for the interaction of PD-L1 and USP21 in HEK293T cells co-transfected with Flag-PD-L1 (WT or indicated mutants) and HA-USP21 for 48 h, treated with or without MG132 (20 μ M) for 6 h. Cell lysates were immunoprecipitated and immunoblotted with indicated antibodies. 5KR: K263R, K270R, K271R, K280R, K281R.

Figure 3

Mutations of USP21 and PD-L1 disrupt the regulation of deubiquitination. (a) Co-IP analysis for the interaction of PD-L1 and USP21 in HEK293T cells co-transfected with Flag-PD-L1 and HA-USP21-WT or HA-USP21-C221A for 48 h, treated with or without MG132 (20 μ M) for 6 h. Cell lysates were immunoprecipitated and immunoblotted with indicated antibodies. (b) Deubiquitination of PD-L1 in vitro by USP21-WT or USP21-C221A. Ubiquitinated PD-L1 was incubated with purified USP21-WT or USP21-

C221A in vitro. The ubiquitination of PD-L1 were immunoblotted with indicated antibodies. (c) Schematic diagram of cancer-derived mutation sites in the intracellular domain of PD-L1 analyzed with Catalogue of Somatic Mutations in Cancer (COSMIC) database. Mutations without changing amino acid sequence have been discarded. (d-e) Co-IP analysis for the interaction of USP21 and PD-L1 (WT or mutants) in HEK293T cells co-transfected with Flag-PD-L1 (WT or mutants) and HA-USP21 for 48 h, treated with or without MG132 (20 μ M) for 6 h. Cell lysates were immunoprecipitated and immunoblotted with indicated antibodies.

Figure 4

PD-L1 and USP21 are up-regulated in lung cancer. (a) The gene expression frequency (Low/High expression) of PD-L1 and USP21 across 17 cancer types analyzed with The Cancer Genome Atlas (TCGA) database. L: Low expression; H: High expression. (b) The mRNA expression of PD-L1 and USP21 analyzed with TCGA database between lung cancer patients and normal individuals. N represents the number of patients. Data represent mean $\pm$ S.D, two-tailed t-test. (c) The TPM (Transcripts Per Million) of PD-L1 and USP21 analyzed with TCGA database across the patients with different types of lung cancer. N represents the number of patients. Data are mean $\pm$ S.D., two-tailed t-test, **p< 0.01, ***p< 0.001. (d) The gene expression frequency (Low/High expression) of PD-L1 and USP21 across lung cancer analyzed with TCGA database. N represents the number of patients. (e-f) The gene expression frequency (Low/High expression) of PD-L1 and USP21 across lung cancer patients in different clinical stages analyzed with TCGA database. (g) Representative images of PD-L1 and USP21 immunohistochemistry staining in tumor tissues and adjacent normal tissues. P1: patient 1; P2: patient 2; Scales bar, 100 μ m.