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RESEARCH PAPER

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FUBP1 promotes the proliferation of lung squamous carcinoma cells and regulates tumor immunity through PD-L1

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Abstract
Background and aim: Lung cancer is a common malignancy. Non-small cell lung cancer (NSCLC) is divided into lung squamous cancer (LUSC), large cell carcinoma, and adenocarcinoma. More than 85% of lung cancer cases are NSCLC patients. Further exploration of the pathogenesis of lung cancer is of great significance. In this study, functions of far upstream element-binding protein 1 (FUBP1) on the proliferation and tumor immunity of LUSC cells were evaluated.
Materials and methods: The Cancer Genome Atlas (TCGA) database, Western blot analysis, and immunohistochemistry (IHC) analysis were used to examine the overexpression levels of FUBP1 in LUSC and paracancerous tissues, LUSC cell line, and human normal lung cell line. Then, Western blot assay was employed to validate the transfection efficiency of FUBP1 knockdown in SK-MES-1 cells. Cell counting kit-8 and colony formation assays were used to detect the viability and proliferation of SK-MES-1 cells. Transwell assay was used to examine the migrative and invasive abilities of SK-MES-1 cells. Finally, the xenograft tumor mice model was applied to explore the role of FUBP1 *in vivo*. IHC assay was used to determine the expression levels FUBP1, PD-L1, and Ki-67. Flow cytometry technology was employed to detect the proportion of $CD4^+$ and $CD8^+$ cells in short sequence negative control (sh-NC) and sh-FUBP1 groups.
Results: Collectively, the results first indicated that FUBP1 was up-regulated in LUSC tissues and cells. It was also demonstrated that knockdown of FUBP1 suppressed cell migration, invasion, and proliferation in lung squamous carcinoma cells. Finally, knockdown of FUBP1 regulated tumor immunity *in vivo*.
Conclusion: This research suggested that FUBP1 promotes the proliferation of LUSC cells and regulates tumor immunity through programmed death-ligand 1 (PD-L1).

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Introduction

Lung cancer is one of the most common malignant tumors with the highest fatality rate worldwide. Non-small cell lung cancer (NSCLC) can be divided into lung squamous cancer (LUSC), adenocarcinoma, and large cell carcinoma. More than 85% of lung cancer patients are NSCLC cases. At present, the treatment of NSCLC includes surgery, chemotherapy, radiotherapy, etc. These treatment options have limitations as well as adverse reactions, and less than 5% of patients survive after 5 years. Therefore, there is an urgent requirement to develop therapies to improve antitumor outcome in NSCLC patients. In recent years, with the development of antitumor immunology research, promising treatment options for NSCLC using immune checkpoint inhibitors (ICI) to reverse cancer immunosuppression have been discovered; particularly successful examples are drugs that interfere with the programmed cell death protein-1 (PD-1)/PD-L1 pathway.[1]

Programmed cell death protein-1 is an immune checkpoint receptor expressed on the cell surface of immune cells and has a fundamental role in immune regulation. PD-L1 is one of the ligands of PD-1. T cell tolerance and avoidance of host immunity through down-regulating $CD8^+$ T cell viability and accelerating the binding of PD-1/PD-L1. In cancer cells, PD-L1 can escape antitumor immune response through engaging the PD-1/PD-L1 axis, preventing the immune system from killing cancer cells. Thus, immune checkpoint inhibitors that binding the PD-1/PD-L1 axis have been developed as anticancer therapies.[2]

Far upstream element-binding protein 1 (FUBP1) is upregulated in a variety of tumors, including liver, squamous cell, renal cell, breast, prostate, and bladder cancers. FUBP1 can form complexes with FUSE sites that regulate gene expression, including that of *c-Myc*, *p21*, *usp29*, et al., and exhibit a wide range of activities, such as promoting tumor cell proliferation and cell cycle.[3] FUBP1 was validated to be involved in tumor cell proliferation and migration, and increased *c-Myc*-mediated PD-L1 expression in pancreatic cancer cells through modulating cancer cell immunity.[4] Compared with nontumor lung tissues, the expression of FUBP1 is significantly increased in 70% of NSCLC patients.[5] However, its role in lung cancer is unclear.

In this study, the effects of FUBP1 on the proliferation and tumor immunity of lung squamous carcinoma cells were studied.

Materials and methods

Tissue sample

Lung squamous cancer and adjacent tissues were randomly obtained from 50 LUSC patient samples at the Guizhou Provincial People's Hospital. The clinicopathological characteristics of 50 patients with lung squamous carcinoma cells are shown in Table 1. Study protocols were approved by the Ethics Committee of Guizhou Provincial People's Hospital according to the principles expressed in the Declaration of Helsinki.[6]

Table 1 Clinicopathological characteristics of 50 patients with lung squamous cell carcinoma.

Clinicopathological characteristics	Cases (%)
Age	
≤60	26 (52%)
>60	24 (48%)
Gender	
Male	30 (60%)
Female	20 (40%)
Smoking	
Yes	20 (40%)
No	30 (60%)
TNM stage	
IA	8 (16%)
IB	6 (12%)
IIA	17 (34%)
IIB	14 (28%)
IIIA	5 (10%)
Lymph node metastasis	
Yes	28 (56%)
No	22 (44%)
Differentiation degree	
High	19 (38%)
Moderate	15 (30%)
Low	16 (32%)

Cell culture

Normal human lung epithelial cell line BEAS-2B, and LUSC cell lines, NCI-H520, SK-MES-1, and NCI-H2170, were obtained from the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified eagle medium (DMEM; Gibco, Carlsbad, CA, USA), which contained 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco), was applied to culture cells. Cells were cultured in a humidified 5% CO_2 incubator at 37°C.

Western blot analysis

Total protein was extracted and separated through radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). The protein was identified by incubating with specific primary antibodies FUBP1 (ab213525, 1:2000; Abcam, Cambridge, MA, USA), PD-L1 (ab237726, 1:1000; Abcam), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ab9485, 1:2500; Abcam). Next, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G secondary antibody (ab205718, 1:3000; Abcam). GAPDH was used to normalize analyzed samples.

Cell transfection

Single-stranded FUBP1 (si-FUBP1) and short sequence-FUBP1 (sh-FUBP1; Wuhan GeneCreate Biological Engineering Co.

Ltd., Hubei Province, China) were used for the knockdown of FUBP1, And 3×10^4 SK-MES-1 cells were seeded into 6-well plates. After 1 day, lipofectamine 3000 (Thermo Fisher Scientific, Grand Island, USA) was applied to transfected aimed plasmids into seeding cells. Finally, fluorescence imaging was preceded under fluorescence microscope (Olympus Dmi-8, Japan).

Colony formation assay

Briefly, we seeded 1×10^6 SK-MES-1 cells into 6-well plates. Supernatant was removed after 2 weeks; 4% formaldehyde was added drop-wise, and waited for 10 min. Finally, 0.25% crystal violet solution was used for 30 min to stain cells. The total number of cells was obtained after drying of culture plate.

Transwell assay

In all, 2×10^4 cells were seeded into transwell inserts (Costar, Manassas, VA, USA) and treated under different conditions. For invasion assay, the chambers were coated with 10-μL Martrigel (Invitrogen, USA) and incubated at 37°C for 1 h. Then 200-μL serum-free medium was applied into transwell inserts, and 600-μL medium with 10% fetal bovine serum (FBS) was applied into the well. After culturing for 1 day, in the lower chamber, migrated cells were fixed in 4% paraformaldehyde for 10 min and dyed through 0.2% crystal violet solution for 20 min.

Immunohistochemistry

Tumor tissues were parceled in 4% paraformaldehyde, embedded in paraffin, and cut into slices and antibodies added. The rabbit anti-mouse FUBP1 antigen rabbit polyclonal antibodies (ab213525, 1:16,000; Abcam), rabbit anti-mouse PD-L1 antigen polyclonal antibodies (ab233482, 1:100; Abcam), and rabbit anti-mouse Ki-67 antigen polyclonal antibodies (ab15580, 1:400; Abcam) were applied to detect FUBP1, PD-L1, and Ki-67 protein expressions.

Flow cytometry

The cells were collected and incubated with recombinant anti-CD4 antibody (ab207755, 1:60; Abcam), recombinant anti-CD3 antibody (ab135372, 1:200; Abcam), or recombinant anti-CD8 alpha antibody (ab217344, 1:500; Abcam). The cells were fixed and permeabilized with BD Cytofix/Cytoperm (BD Biosciences, San Jose, CA, USA) for intracellular staining. Flow cytometry was performed on BD AccuriC6 (BD Biosciences), and data were analyzed by the FlowJo software (Tree Star, OR, USA).

Xenograft experiments

Male BALB/c nude mice, 5-week old (n = 3), were obtained from Shanghai Experimental Animal Center (Shanghai, China). The animals were subcutaneously injected with short sequence negative control (sh-NC)- or sh-FUBP1-transfected SK-MES-1 cells (2×10^6). Tumor volume was measured by ruler every week until mice were sacrificed. The animals were sacrificed after being injected subsequently for 28 days; finally, tumor tissues were obtained, measured, and photographed. All animal experiments were approved by the Ethics Committee of Guizhou Provincial People's Hospital for the use of animals, and conducted in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines.

Statistical analysis

Data were represented as mean ± standard deviation (SD) obtained from three independent assays. Student's *t*-test was applied to calculate comparisons between two groups. $P < 0.05$ was considered as statistically significant. Data were analyzed by SPSS.

Results

FUBP1 was highly expressed in lung squamous carcinoma tissues and cells

Results from the Cancer Genome Atlas (TCGA) database demonstrated that the expression level of FUBP1 was significantly increased in LUSC samples (n=503) compared with normal samples (n=52) (Figure 1A). The FUBP1 protein expression in 50 LUSC and paracancerous tissues was examined through immunohistochemistry (IHC) technique, and the images revealed that the FUBP1 protein expression was up-regulated in 50 LUSC tumor tissues compared to that in normal tissues (Figure 1B). Western blot assay results exhibited that the FUBP1 protein expression was elevated in LUSC cell lines, SK-MES-1, NCI-H2170, and NCI-H520, compared with normal human lung cell line BEAS-2B (Figure 1C). These outcomes suggested that FUBP1 was up-regulated in lung squamous carcinoma tissues and cells.

FUBP1 knockdown inhibited the proliferation of lung squamous carcinoma cells

Western blot assay was employed to validate the transfection efficiency of FUBP1 knockdown in SK-MES-1 cells. Compared to si-NC group, the FUBP1 protein expression was notably down-regulated in SK-MES-1 cells after transfection of si-FUBP1-1# or si-FUBP1-2# (Figure 2A). Cell counting kit-8 (CCK-8) assay revealed that the viability of SK-MES-1 cells in control or si-NC group was significantly induced as compared to that in si-FUBP1-1# or si-FUBP1-2# group (Figure 2B). Colony formation assay results demonstrated that the proliferative abilities of SK-MES-1 cells were weakened in si-FUBP1-1# or si-FUBP1-2# group as compared to that in control or si-NC group (Figure 2C). These results demonstrated that FUBP1 knockdown suppressed the proliferation of lung squamous carcinoma cells.

FUBP1 knockdown suppressed the migration and invasion of lung squamous carcinoma cells

Transwell experimental outcomes revealed that the migration and invasion of SK-MES-1 cells demonstrated no

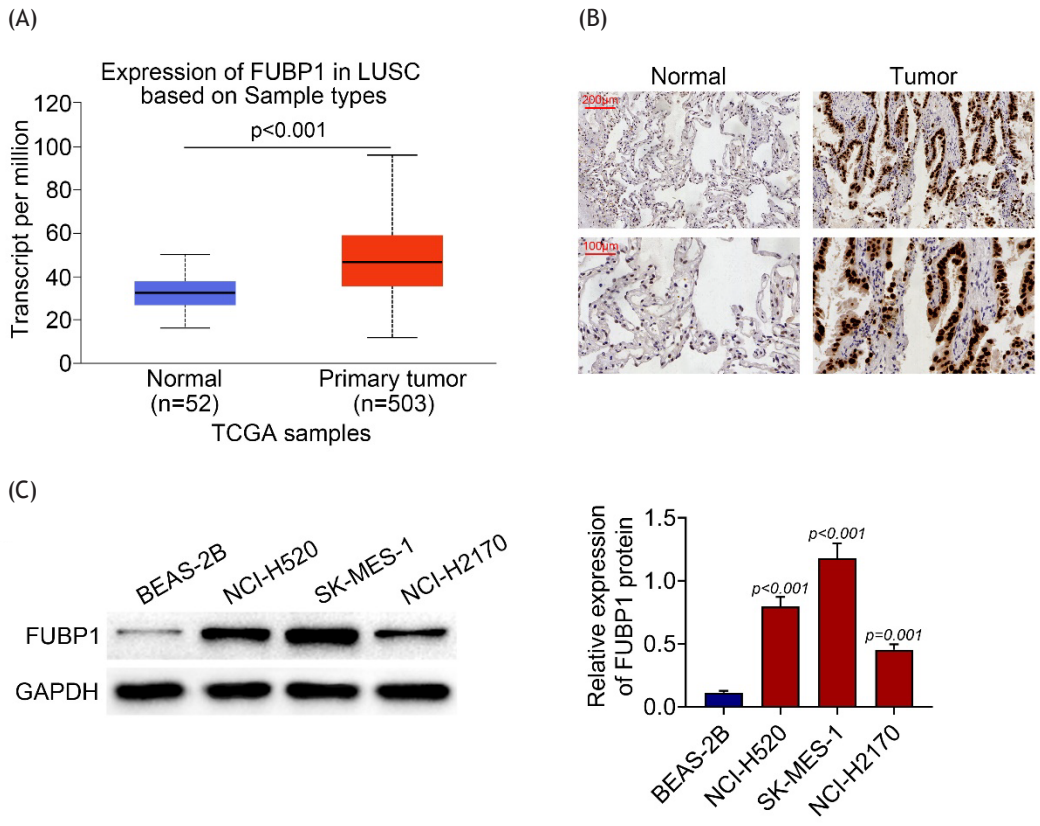

Figure 1 FUBP1 was highly expressed in lung squamous carcinoma tissues and cells. (A) TCGA database expressed FUBP1 expression in LUSC (n = 503) and normal samples (n = 52), P < 0.001. (B) IHC images revealed the protein expression level of FUBP1 in 50 LUSC tumor and paracancerous tissues, scale bar = 100 μm and 200 μm. (C) Western blot analysis exhibited the protein expression level of FUBP1 in three LUSC cell lines, NCI-H520, SK-MES-1, and NCI-H2170, and normal human lung cell line BEAS-2B, P < 0.001, GAPDH was normalized as an internal control.

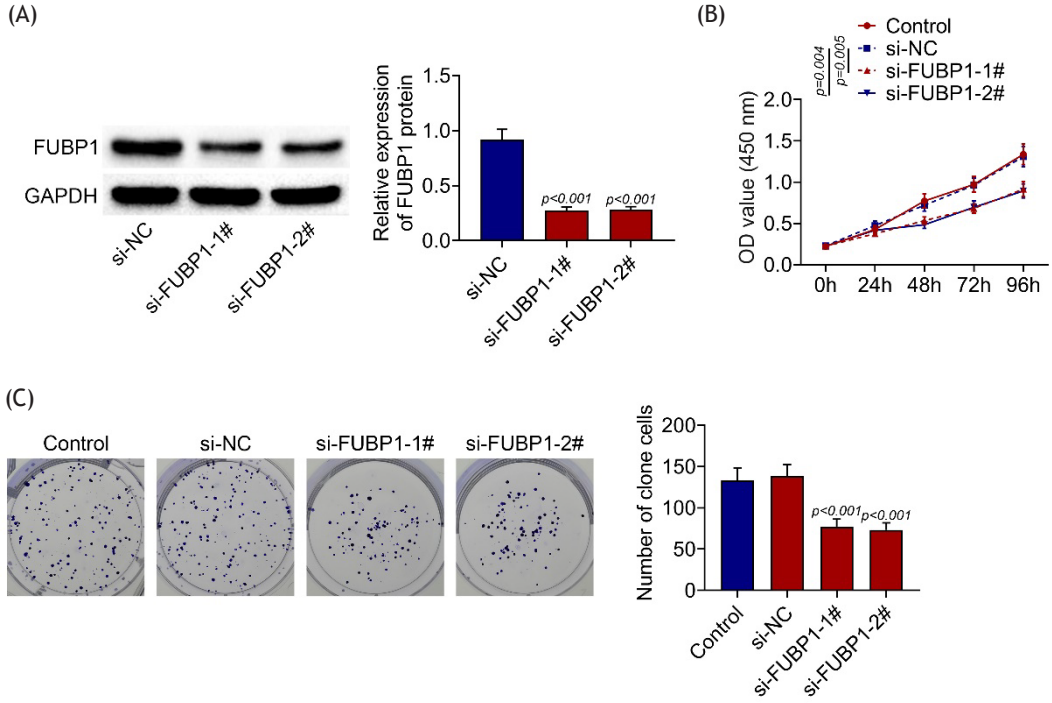

Figure 2 FUBP1 knockdown inhibited the proliferation of lung squamous carcinoma cells. (A) Western blot analysis indicated the transfection efficiency of FUBP1 knockdown in SK-MES-1 cells, P < 0.001, GAPDH was normalized as an internal control. (B) CCK-8 assay exhibited the viability of SK-MES-1 cells in control, si-NC, si-FUBP1-1#, and si-FUBP1-2# groups, P < 0.001. (C) Colony formation assay demonstrated the proliferation of SK-MES-1 cells in control, si-NC, si-FUBP1-1#, and si-FUBP1-2# groups, P < 0.001.

significant difference between control and si-NC groups. However, both migrative and invasive abilities of SK-MES-1 cells were decreased in si-FUBP1-1# and si-FUBP1-2# groups as compared with control or si-NC group (Figure 3). These results collectively suggested that FUBP1 knockdown suppressed the migrative and invasive abilities of lung squamous carcinoma cells. FUBP1 may have a primary function in the development and progression of lung squamous carcinoma cells.

FUBP1 knockdown inhibited tumor growth in lung squamous carcinoma cells through regulating tumor immunity

Western blot assay revealed that the expression level of PD-L1 was declined in si-FUBP1-1# and si-FUBP1-2# groups as compared to that in control and si-NC groups (Figure 4A). To explore the function of FUBP1 *in vivo*, 5-week-old male BALB/c nude mice (n = 3) were transplanted with SK-MES-1 cells, which was established as a mice model of lung squamous carcinoma cells. After subcutaneous injection of SK-MES-1 cells for 28 days, animals were euthanized and tumors were taken out and recorded. It was observed in the recorded photos that the size (Figure 4B), volume (Figure 4C), and weight (Figure 4D) of tumors in sh-FUBP1 group were obviously smaller than that in sh-NC group. Then, the IHC assay was applied to detect the expression of FUBP1, PD-L1, and Ki-67, and the results established that, after FUBP1 knockdown, both PD-L1 and Ki-67 protein expressions were down-regulated as compared to sh-NC group (Figure 4E). Meanwhile, the outcomes of flow cytometry suggested that $CD4^+$ and $CD8^+$ cells were increased in sh-FUBP1 group compared with that in sh-NC group (Figures 4F and G). These outcomes revealed that knockdown of FUBP1 regulated tumor immunity.

Discussion

Despite the advancements in technology and science, the molecular mechanism of LUSC is not clear. Nowadays, several studies have demonstrated that FUBP1 regulates the progression of several cancers. For example, FUBP1 is involved in cell survival and cell cycle progression.[7] *RARS-MAD1L1* fusion gene might serve as an attractive target for therapeutic intervention for nasopharyngeal carcinoma (NPC) through the FUBP1-*c-Myc* axis.[8] FUBP1 serves as a cancer operator and widespread regulator of tumor suppressor, and FUBP1 participates in regulating mechanistically N6-methyladenosine (m6A) RNA methylation.[9] However, no research has been conducted on the role of FUBP1 in LUSC. In this study, it was discovered that FUBP1 was up-regulated in LUSC tissues and cells. FUBP1 knockdown suppressed the proliferation of lung squamous carcinoma cells SK-MES-1. Previous research has identified FUBP1 as an oncoprotein or a tumor suppressor, and its overexpression was observed in a growing number of cancers and resulted in a deregulation of targets such as fine-tuned *c-Myc* oncogene.[10] In the

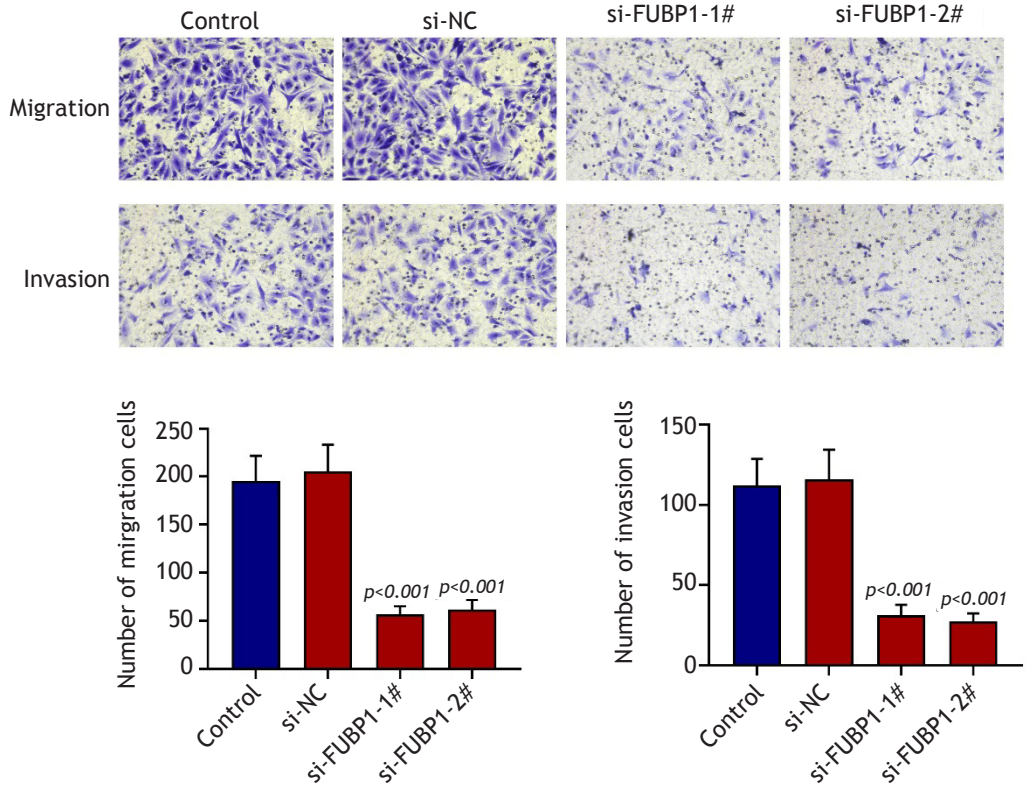

Figure 3 FUBP1 knockdown suppressed the migration and invasion of lung squamous carcinoma cells. Transwell assay demonstrated the migration and invasion of SK-MES-1 cells in control, si-NC, si-FUBP1-1#, and si-FUBP1-2# groups, P < 0.001.

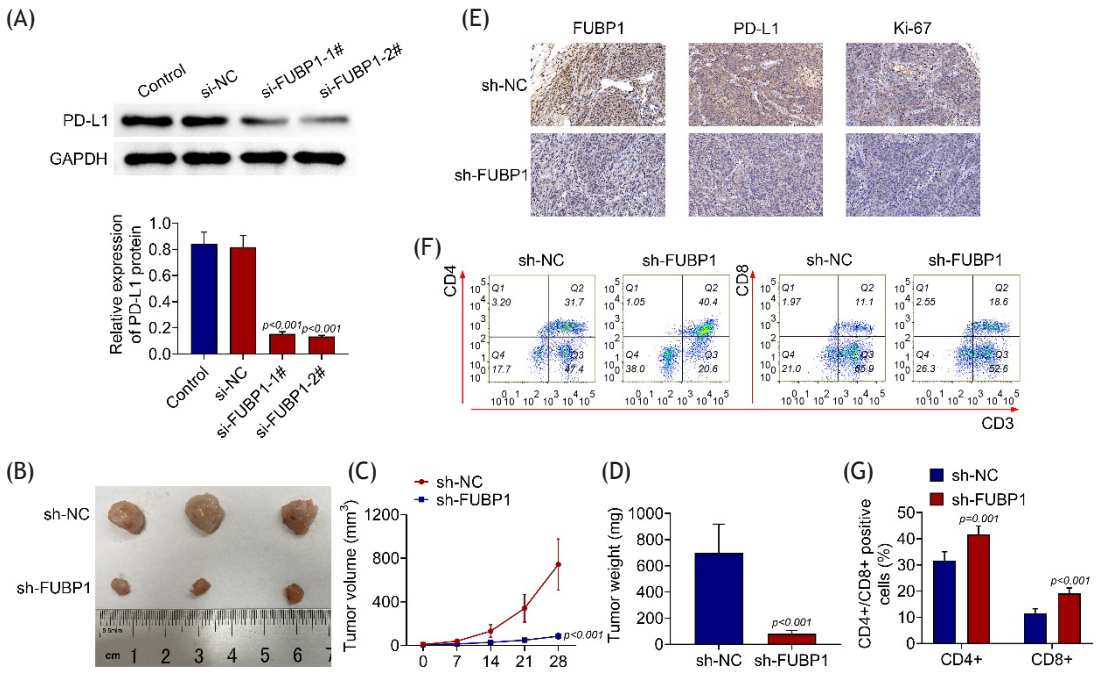

Figure 4 FUBP1 knockdown inhibited tumor growth in lung squamous carcinoma cells through regulating tumor immunity. (A) Western blot analysis indicated that FUBP1 negatively regulated PD-L1 protein expression; P < 0.001, and GAPDH was normalized as an internal control. (B) Tumor size, (C) volume, and (D) weight of xenograft mice in sh-NC and sh-FUBP1 groups were obtained, P < 0.001. (E) IHC results indicated the protein expressions of FUBP1, PD-L1, and Ki-67 in sh-NC and sh-FUBP1 groups. (F, G) Flow cytometry analysis exhibited the percentage of CD4+ and CD8+ cells in sh-FUBP1 and sh-NC groups, P < 0.001.

present study, knockdown of FUBP1 suppressed migration and invasion in lung squamous carcinoma cells, suggesting that FUBP1 may have a regulatory function in the development and progression of LUSC.

The expression levels of PD-L1 and PD-L2 were correlated to the genes in NSCLC.[11] PD-L2 and PD-L1 play important roles in evading antitumor immunity, hinting that PD-1/PD-L2 blockade can be considered as optimal immunotherapy in LUSC.[12] Gene expression serves as potential biomarkers of response to PD-1 blockade in NSCLC.[13] PD-L1 expression was related to increased antitumor immune signatures ACE2 upregulation, and favorable anti-PD-1/PD-L1/CTLA-4 immunotherapy response.[14] The present research revealed that knockdown of FUBP1 could down-regulate the expression levels of PD-L1, thus regulating tumor immunity in LUSC. The possible mechanism could be that FUBP1 regulates the ubiquitin enzyme OTUB1, thus participates in the ubiquitination of immune checkpoint protein PD-L1.

Conclusion

In summary, the present study discovered that FUBP1 was expressed highly in LUSC tissues and cells. Moreover, FUBP1 knockdown suppressed the proliferation, migration, and invasion of lung squamous carcinoma cells. Finally, FUBP1 knockdown regulated tumor immunity *in vivo*. Besides, results also indicated that knockdown of FUBP1 declined expressions of PD-L1 and Ki-67 compared to sh-NC group. Meanwhile, the flow cytometry demonstrated that CD4+ and CD8+ cells were increased in sh-FUBP1 group as compared to that in sh-NC group. All these results illustrated that FUBP1 promotes the proliferation of LUSC cells and regulates tumor immunity through PD-L1, which could provide novel ideas for clinical therapeutic targets for LUSC.

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Competing interests

The authors stated that there were no conflicts of interest to disclose.

Statement of Informed Consent

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this study.

Author Contribution

Jie Yu and Wen Peng designed and carried out the study. Yingbo Xue supervised data collection, and analyzed and

interpreted the data. Yun Li, Lei Yang, and Yang Geng prepared the manuscript for publication and reviewed its draft. All authors read and approved the final manuscript.

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