

# PD-L1 promotes head and neck squamous cell carcinoma cell growth through mTOR signaling

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**Abstract.** Programmed death-ligand 1 (PD-L1), an immune co-stimulatory molecule, is expressed on various cancer cells and the surface of immune cells. Its overexpression on tumor cells suppresses the immune response to promote tumor cell immune escape. The present study demonstrated that PD-L1 was critical in head and neck squamous cell carcinoma (HNSCC) carcinogenesis. Immunohistochemical analysis of HNSCC tissue microarrays revealed that PD-L1 was overexpressed in tumor tissue, and its expression increased as tumor malignancy progressed (from grade I to IV). Subsequently, the expression of PD-L1 was knocked down or overexpressed in the HNSCC cell lines Cal-27 and Fadu. It was demonstrated that PD-L1 significantly induced HNSCC cell proliferation and colony forming ability. Cell proliferation was also promoted in Cal-27 cell xenograft BALB/c nude mice. In addition, it was determined by western blotting that the PD-L1-mediated increase in HNSCC cell proliferation may have been associated with the activation of mammalian target of rapamycin (mTOR) signaling pathway. Furthermore, mTOR inhibitor (rapamycin) prevented the increase in proliferation. Based on these results, it was concluded that PD-L1 promoted cell proliferation of HNSCC cells through mTOR signaling, and blocking PD-L1 may be conducive in HNSCC therapy.

## Introduction

Head and neck squamous cell carcinoma (HNSCC) is the seventh most common cancer type worldwide, with >500,000 new cases of HNSCC diagnosed worldwide every year.

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Approximately 60% of HNSCC are diagnosed at an advanced stage, and the prognosis is poor, despite the numerous forms of treatment available (1,2). In recent decades, the 5-year survival rate has improved (3). Although the development of new therapies and advanced examination techniques has prolonged the life of the patients, HNSCC is considered difficult to treat, with the exception of early stage tumors (3). In order to increase patient quality of life, a strategy for the early detection of HNSCC is urgently required. Several biomarkers have been associated with the diagnosis and prognosis of HNSCC, but few have demonstrated adequate clinical efficacy (4).

Programmed death-ligand 1 (PD-L1) is an immune co-stimulatory molecule that belongs to the B7-H gene family (5). It is expressed on many tumor cell types and the surface of immune cells, including B cells, T cells, myeloid dendritic cells and macrophages (6). PD-L1 serves an important role in regulating cellular immunity. PD-L1 combined with programmed cell death protein 1 (PD-1) inhibits the migration and proliferation of T cells, as well as the secretion of cytotoxic mediators, thus limiting its antitumor effects on tumor cells (7,8). Therefore, anti-PD-L1 monoclonal antibodies ( $\alpha$ PD-L1) are effective in oncotherapy, and antitumor immunity may be enhanced by inhibiting the expression of PD-L1 (9). Anti-PD-L1 monoclonal antibodies have shown considerable promise in the treatment of melanoma, renal cell carcinoma and non-small cell lung cancer (10). The PD-L1/PD-1 axis has gained increasing attention in cancer immunotherapy and immunopathogenesis (11). The majority of research has focused on antitumor immunity, particularly in T cells. However, tumor-intrinsic PD-L1 signals have not been extensively investigated (12). Recently, the regulation of tumor cell proliferation by PD-L1 family members has gained increasing attention; it has been revealed that PD-L1 participates in epithelial-mesenchymal transition (EMT) regulation (13,14), and is closely associated with cell cycle progression (15,16) and the expression of proliferation marker Ki-67 in human breast cancer (16). Based on these previous findings, it was speculated that tumor-intrinsic PD-L1 signaling may have broad biological effects, which require further investigation.

In the present study, the function of PD-L1 in HNSCC was investigated. Immunohistochemical analysis was used to detect PD-L1 expression in HNSCC tissues. Next, it was demonstrated that PD-L1 promoted HNSCC cell proliferation

*in vitro* and *in vivo*. In addition, the potential mechanisms underlying the PD-L1-mediated increase in HNSCC cell proliferation were explored. The current research aimed to provide experimental evidence for the use of PD-L1 as a therapeutic target in HNSCC.

## Materials and methods

**Patient selection and tissue microarray (TMA).** The independent tissue microarrays were purchased (LP803 and LP804; US Biomax, Inc., Rockville, MD, USA). The tissue microarrays consisted of 110 laryngocarcinoma tissues, five normal laryngeal tissues and five samples of normal adjacent laryngeal tissue (NAT), which included 106 men and 14 women. Detailed tissue microarray information is presented in Tables I and II.

**Immunohistochemical staining and evaluation.** PD-L1 antibody (cat. no. 13684; Cell Signaling Technology, Inc., Danvers, MA, USA) was used for immunohistochemical staining, and the method of immunohistochemical staining was performed as previously described (17). To further analyze the immunohistochemical staining results, all TMAs were scored for frequency (0-4) and intensity (0-3) under an Olympus BX51 microscope (Olympus Corp., Tokyo, Japan). The scores of frequency were assigned when 0-25, 26-50, 51-75 and 76-100% of the tumor cells stained positive. The scores of intensity (0-3) respectively represented: 0, negative; 1, weak; 2, moderate; and 4, strong. The composite expression scores (CES) utilized the following formula: CES = intensity x frequency.

**Cell culture and transfection.** Two HNSCC cell lines (Cal-27 and FaDu) were purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 20 mg/ml ampicillin and 20 mg/ml kanamycin at 37°C in an incubator with 5% CO<sub>2</sub>.

To generate the stable PD-L1-overexpressing (PD-L1<sup>over</sup>) and PD-L1 knockdown (PD-L1<sup>RNAi</sup>; lentivirus transduction particles containing PD-L1 shRNA) cell lines, as well as negative control (NC) groups (PD-L1<sup>over NC</sup> and PD-L1<sup>RNAi NC</sup>), lentivirus transduction particles containing GFP label (cat. nos. GOCL3581014115 and GICL2481014115; Shanghai GeneChem Co., Ltd., Shanghai, China) were transfected (multiplicity of infection=20) into Cal-27 and FaDu cells (2x10<sup>5</sup> cells/well), and the stable transfected Cal-27 and FaDu cell lines were selected by culturing for 1 week in complete medium (DMEM with 10% FBS, ampicillin and kanamycin), which also contained puromycin (2 µg/ml).

**Cell proliferation and colony formation assay.** Cell proliferation was determined using Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) and EdU incorporation (cat. no. C10310-1; Guangzhou RiboBio Co., Ltd., Shanghai, China) assays, according to the manufacturer's instructions. For the CCK-8 assay, cells were seeded in 96-well plates at 1x10<sup>3</sup> cells/well in DMEM with 10% FBS, and treated with rapamycin (10 nM) for the total culture period of 72 h. The absorbance was measured at 450 nm by a microplate

Table I. LP803 tissue microarray patient characteristics.

Characteristic	Number of patients (%)
Number of patients	80 (100)
Mean age, years	57.35±8.47
Sex	
Female	9 (11.25)
Male	71 (88.75)
Localization	
Larynx	80 (100)
Differentiation	
1	24 (33.8)
2	42 (59.2)
3	5 (7.0)
Nine cases did not include differentiation information.	

Table II. LP804 tissue microarray patient characteristics.

Characteristic	Number of patients (%)
A, Patients with laryngocarcinoma	
Number of patients	40 (100)
Mean age, years	55.25±13.39
Sex	
Female	5 (12.5)
Male	35 (87.5)
Localization	
Larynx	40 (100)
T classification	
T1	3 (10.3)
T2	11 (38)
T3	3 (10.3)
T4	12 (41.4)
N classification	
N0	22 (73.3)
N+	8 (26.7)
Differentiation	
1	12 (41.4)
2	15 (51.7)
3	2 (6.9)
B, Healthy controls	
Number of patients	10 (100)
NAT	5 (50)
Normal laryngeal tissue	5 (50)
Of the laryngocarcinoma cases, one did not include T classification; another did not include differentiation information.	

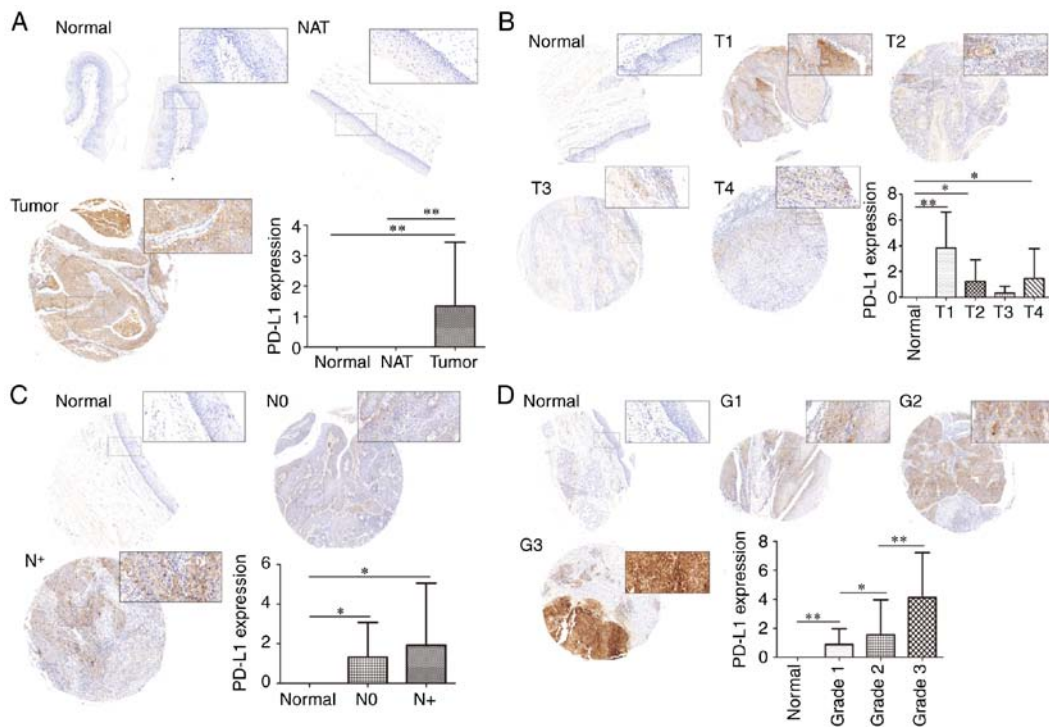


Figure 1. PD-L1 expression in HNSCC. Representative images of immunohistochemical staining for PD-L1 protein expression in (A) normal, NAT and tumor tissue, as well as in (B) different T-stages, (C) N-stages and (D) tumor grades. Magnifications, x40 and x200. \*P<0.05, \*\*P<0.01. PD-L1, programmed death-ligand 1; HNSCC, head and neck squamous cell carcinoma; NAT, normal adjacent laryngeal tissue.

reader (Perkin Elmer) at 0, 24, 48 and 72 h. For the EdU proliferation assay, tumor cells ( $5 \times 10^6$  cells/well) were plated into 6-well plates in DMEM with 10% FBS, and directly labeled using the Cell-Light™ EdU Apollo® 567 *in vitro* Imaging kit, according to the manufacturer's instructions.

For the colony formation assay, cells were seeded into 6-well plates (200 cells/well) and incubated in complete medium for 12 days at 37°C. The 6-well plates were washed with PBS and stained with 0.1% crystal violet at room temperature for 15 min. Colonies which consisted of >50 cells were counted under an Olympus IX51 microscope (Olympus Corp.).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated with TRIzol™ reagent (Thermo Fisher Scientific, Inc.). RNA (1 μg) was reverse transcribed using the Super RT Reverse Transcriptase reagent kit (Beijing CoWin Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. qPCR was conducted in a 25 μl reaction system, using the 7500 Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and amplified with transcript-specific primers and SYBR®-Green Master Mix (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Relative gene expression was calculated using the  $2^{-\Delta\Delta C_q}$  method (18), with GAPDH as the internal control. PD-L1 (cat. no. HQP008443) and GAPDH (cat. no. HQP006940) primers were purchased from GeneCopoeia, Inc. (Rockville, MD, USA). The primer sequences were as follows: PD-L1 forward, 5'-TAGAATTCA TGAGGATATTTGCTGTCTT-3' and reverse, 5'-TAGGAT CCTTACGTCTCCTCCAAATGTG-3'; GAPDH forward, 5'-TGACTTCAACAGCGACACCCA-3' and reverse, 5'-CAC CCTGTTGCTGTAGCCAAA-3.

**Xenograft study.** Female BALB/c nude mice (n=20; 4 weeks old; 16-18 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and underwent adaptive feeding 1 week before the experiment. Mice were housed at constant temperature (20-25°C) and humidity (40-70%) in a 12 h light/dark cycle, with free access to sterile water and standard chow. The nude mice were randomly divided into four groups (PD-L1<sup>over</sup> NC, PD-L1<sup>over</sup>, PD-L1<sup>RNAi</sup> NC and PD-L1<sup>RNAi</sup>; n=5 each). Cal-27 cells were selected to establish subcutaneous xenotransplanted tumor model since Cal-27 cells are more superior than FaDu cells in establishing a subcutaneous xenotransplanted tumor model. Cells ( $2 \times 10^6$ ) were suspended in PBS (200 μl cell suspension) and injected into the right side of the mice's backs. Xenograft tumor diameters were measured every week, and tumor volumes were calculated using the following equation: Volume = 1/2 x length x width<sup>2</sup>. The maximum tumor size was 20 mm. Nude mice were sacrificed and tumors surgically removed 12 weeks after inoculation.

**Western blotting.** Cal-27 and FaDu cells were harvested and lysed in radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.) supplemented with protease and phosphatase inhibitors (Roche Applied Science, Penzberg, Germany). Protein concentration was determined by the bicinchoninic acid protein assay. Lysates (20 μg of protein loaded per lane) were resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes and immunoblotted with specific primary antibodies (all 1:800) overnight at 4°C against PD-L1 (cat. no. 9234T; Cell Signaling Technology, Inc.), protein kinase B (Akt; cat. no. 4691T; Cell Signaling Technology, Inc.), phosphorylated (p)-Akt<sup>S473</sup>

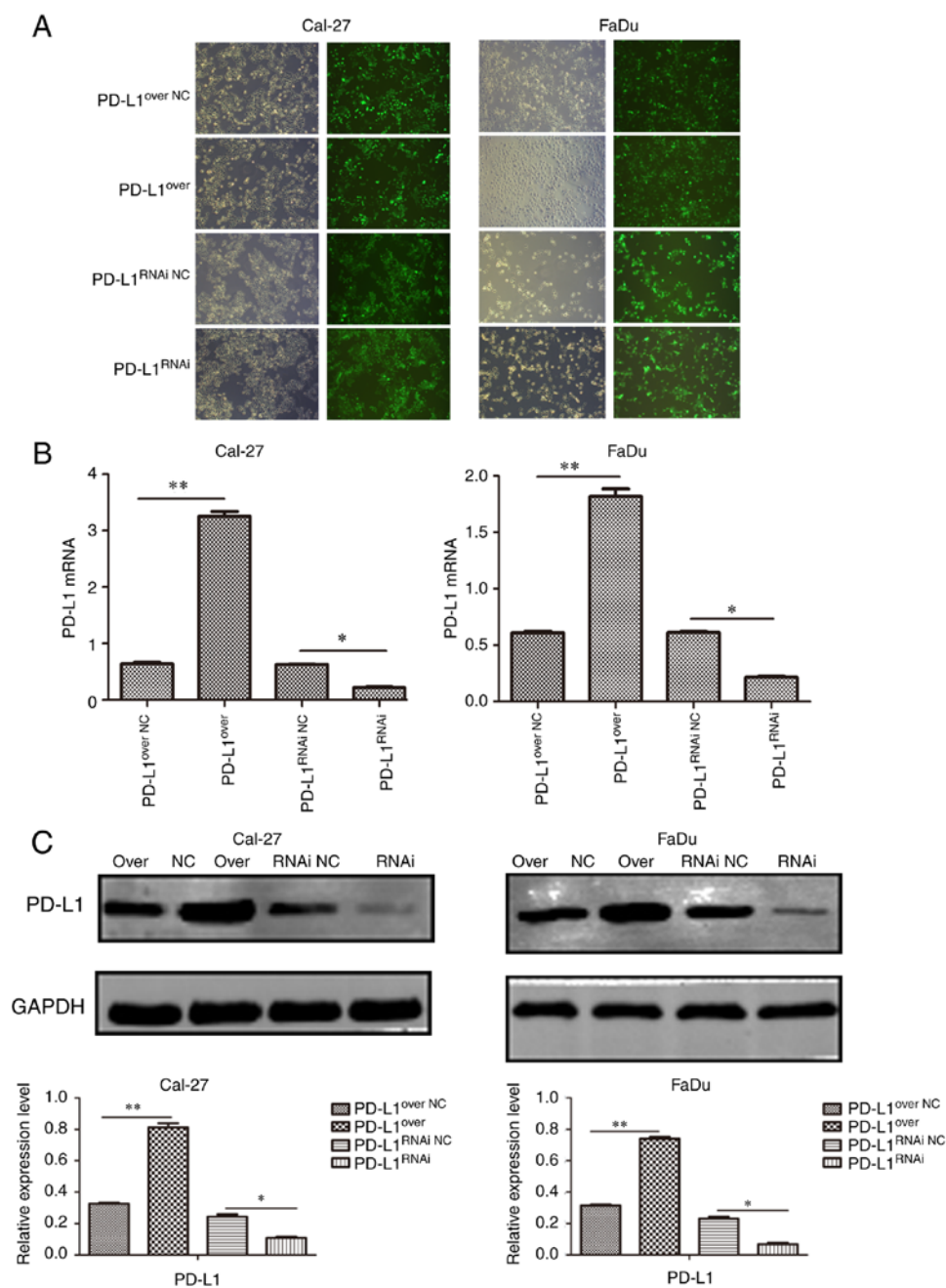


Figure 2. Lentivirus transduction particles were transfected into Cal-27 and FaDu cells. (A) Representative green fluorescent images of transfected cells. (B) Relative expression of PD-L1 mRNA was detected by quantitative polymerase chain reaction. Data were expressed as the mean  $\pm$  standard deviation. (C) Western blot analysis of PD-L1 protein expression in PD-L1<sup>over</sup>, PD-L1<sup>RNAi</sup>, PD-L1<sup>over</sup>NC and PD-L1<sup>RNAi</sup>NC expression. \* $P < 0.05$  and \*\* $P < 0.01$ . PD-L1, programmed death-ligand 1; PD-L1<sup>over</sup>, PD-L1-overexpressing; PD-L1<sup>RNAi</sup>, PD-L1 knockdown; NC, negative control.

(cat. no. 4060T; Cell Signaling Technology, Inc.), 70 kDa ribosomal protein S6 kinase 1 (P70S6K; cat. no. 2708S; Cell Signaling Technology, Inc.), p-P70S6K<sup>T389</sup> (cat. no. 9234T; Cell Signaling Technology, Inc.) and GAPDH (cat. no. 5174S; Cell Signaling Technology, Inc.). Following immunoblotting with IRDye<sup>®</sup> goat-anti rabbit IgG fluorescence secondary antibodies (dilution 1:20,000; cat. no. 926-32211; LI-COR Biosciences, Lincoln, NE, USA) at room temperature for 1 h, the membranes were scanned by an Odyssey infrared imaging system (LI-COR Biosciences).

**Statistical analysis.** All values are expressed as the mean  $\pm$  standard deviation of three independent experimental repeats.

Statistical analyses were performed in SPSS 19.0 (SPSS, Inc., Chicago, IL, USA), using one-way analysis of variance with Tukey's post hoc test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**PD-L1 expression in HNSCC.** In order to examine the expression of PD-L1 in HNSCC, NAT and normal tissues, TMAs of HNSCCs stained for PD-L1 were analyzed, and the CES of every clinical sample was measured. PD-L1 expression was compared and associated with clinical characteristics, including tumor grade and TNM staging.

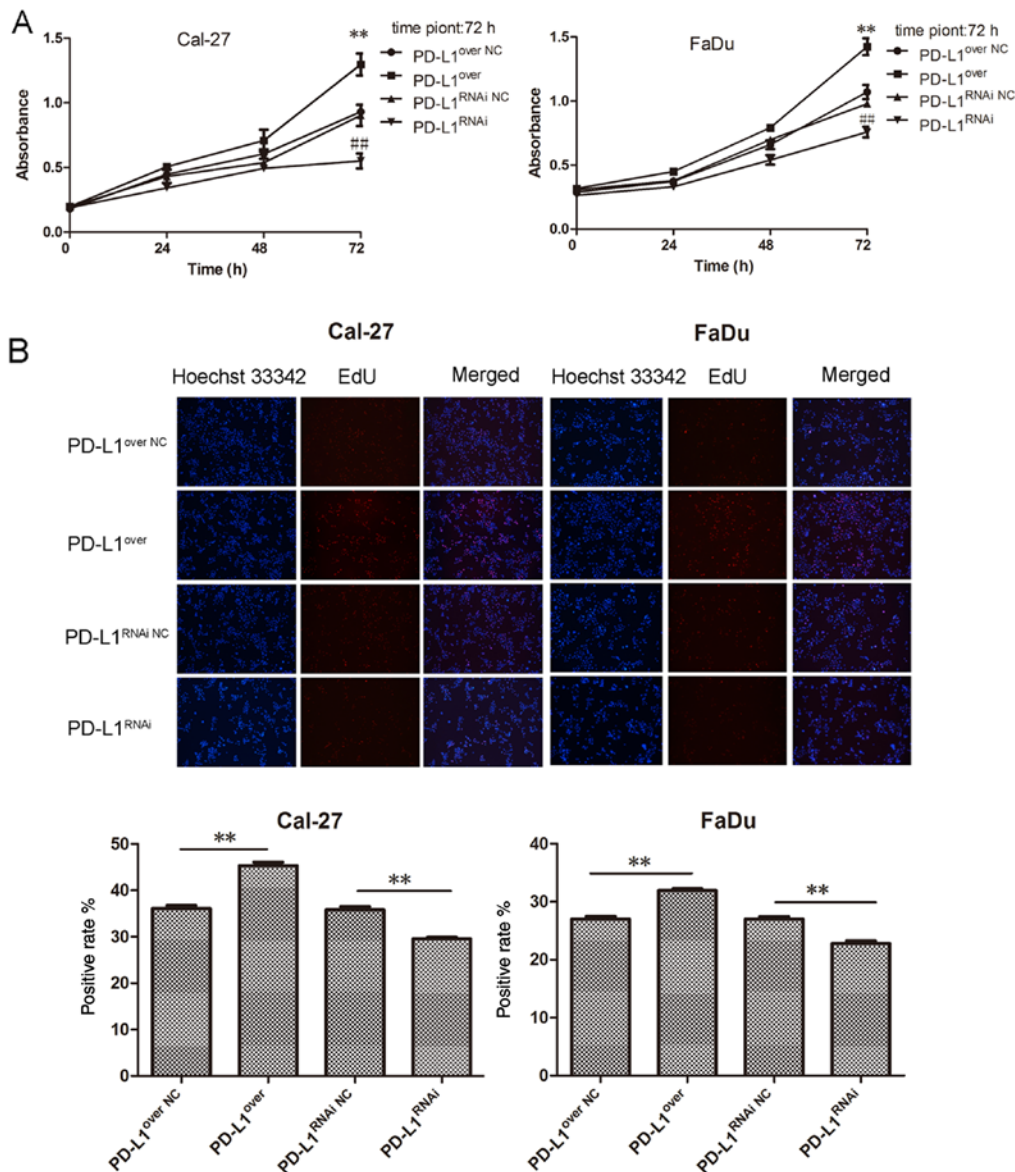


Figure 3. PD-L1 overexpression promotes the proliferation of HNSCC cell lines. (A) HNSCC cell proliferation was detected with Cell Counting Kit-8 assays, \*\*P<0.01 vs. PD-L1<sup>over</sup> NC, ##P<0.01 vs. PD-L1<sup>RNAi</sup> NC. (B) HNSCC cells were labeled with Cell-Light™ EdU (red), and nuclei were stained by Hoechst 33342 (blue). Histograms represent the positive rate of EdU staining.

PD-L1 expression in tumor tissue was significantly higher than in normal tissue and NAT (P<0.01), with no significant difference in PD-L1 expression between normal tissue and NAT (P>0.05; Fig. 1A). T-stage analysis revealed that although the expression of PD-L1 in normal tissue was significantly higher in T1 (P<0.01), T2 (P<0.05) and T4 (P<0.05), there was no statistical significance in PD-L1 expression in T3 (P>0.05; Fig. 1B). It is likely that a larger number of samples are required to fully investigate the relationship between T-stage and PD-L1, as the sample sizes of T1 and T3 were insufficient, and the expected increase in PD-L1 expression with T stage progression was not observed. For the N-stage, representative images were presented in Fig. 1C. The CES of the PD-L1 protein expression revealed that there was no significant differences between the N-stages (P>0.05). As N-stage indicates regional lymph node metastasis, these results suggest that PD-L1 may have no relationship with the regional metastasis of HNSCC. Finally, the expression of PD-L1 among different tumor grades

was compared respectively, demonstrating that an increased tumor grade was associated with increased PD-L1 expression (Fig. 1D).

*PD-L1 regulates the proliferation and colony formation in HNSCC cell lines in vitro.* To explore the function of PD-L1, stable PD-L1-overexpressing (PD-L1<sup>over</sup>), PD-L1 knock-down (PD-L1<sup>RNAi</sup>) and negative control cells (PD-L1<sup>over</sup> NC, PD-L1<sup>RNAi</sup> NC) were generated, and the expression of PD-L1 was detected by RT-qPCR and western blotting. The stably transfected Cal-27 and FaDu cells were established and representative micrographs showed the immunofluorescence of GFP in cells (Fig. 2A). It was demonstrated that the lentiviral transduction particles successfully altered PD-L1 protein (Fig. 2B) and gene (Fig. 2C) expression. PD-L1<sup>RNAi</sup> HNSCC cell proliferation was significantly reduced, compared with its respective control group, with the PD-L1<sup>over</sup> cells exhibiting the highest rate of proliferation (Fig. 3A).

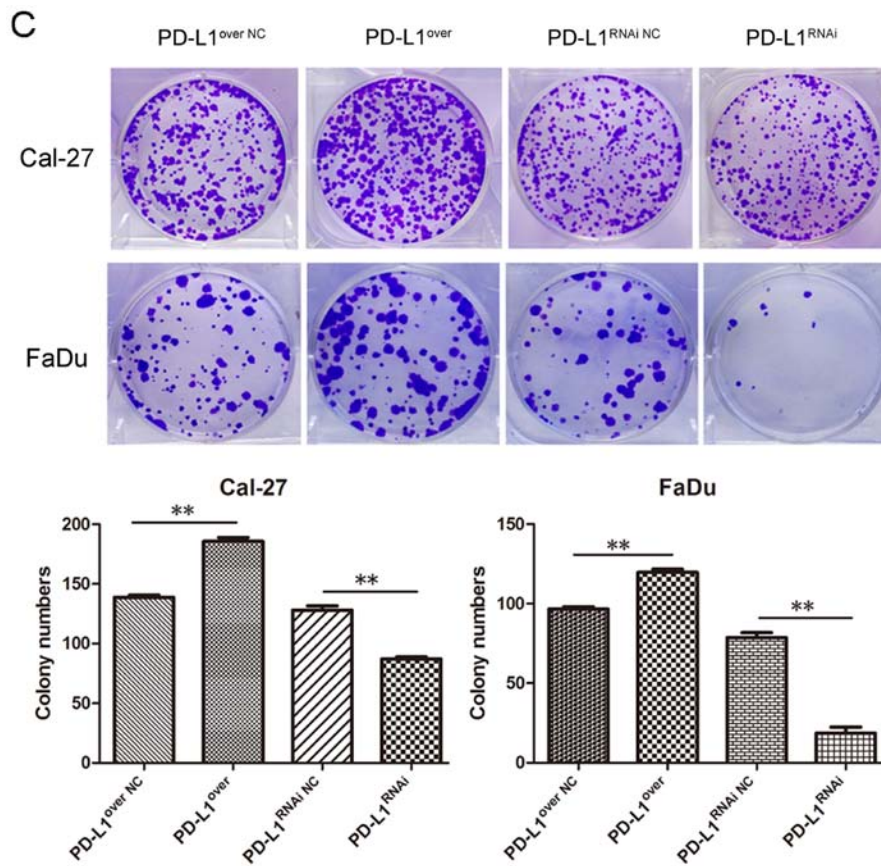


Figure 3. Continued. PD-L1 overexpression promotes the proliferation of HNSCC cell lines. (C) Colony formation assay. Cell colony numbers (>50 cells/unit) were counted. Data were expressed as the mean  $\pm$  standard deviation. \*\* $P < 0.01$ . PD-L1, programmed death-ligand 1; HNSCC, head and neck squamous cell carcinoma; PD-L1<sup>over</sup>, PD-L1-overexpressing; PD-L1<sup>RNAi</sup>, PD-L1 knockdown; NC, negative control.

Furthermore, the EdU proliferation assay revealed a similar trend, in which the PD-L1-overexpressing cells exhibited the highest red fluorescence (Fig. 3B). Furthermore, the colony forming ability of PD-L1-overexpressing cells was remarkably increased compared with the PD-L1<sup>over</sup> NC group, and colony numbers in the PD-L1 knockdown group were significantly reduced, compared with the PD-L1<sup>RNAi</sup> NC group (Fig. 3C).

**PD-L1 upregulates mTOR signaling in HNSCC cell lines.** To investigate the potential mechanism by which PD-L1 promoted cell growth in HNSCC cells, the expression levels of proteins associated with mTOR signaling and cell proliferation were detected by western blotting (Fig. 4A). Compared with their corresponding control groups, p-P70S6K<sup>T389</sup> and p-Akt<sup>S473</sup> expression was significantly increased on the PD-L1<sup>over</sup> group, but significantly decreased in the PD-L1<sup>RNAi</sup> group (Fig. 4B).

**Effect of PD-L1 expression on tumor growth in vivo.** To validate whether the expression of PD-L1 affected tumor growth *in vivo*, Cal-27 cells were used to establish a xenograft mouse model. At the 12 week end point, the tumor growth curve (Fig. 5A) showed that the tumor volume in the Cal-27-PD-L1<sup>over</sup> group was significantly larger than the control group ( $P < 0.01$ ), and the tumor volume in the PD-L1<sup>RNAi</sup> group was the smallest ( $P < 0.05$ ). The tumors were removed and measured on week 12 (Fig. 5B), and the average volume was calculated (Fig. 5C). These results demonstrated that PD-L1

accelerated tumor growth, suggesting an important role for PD-L1 in regulating the tumor cell growth *in vivo*. No significant differences in animal weight were detected (Fig. 5D).

**PD-L1 enhances the sensitivity of HNSCC cells to mTOR inhibitor in vitro.** As PD-L1 upregulated the expression of proteins involved in mTOR signaling, the effects of mTOR inhibitor rapamycin were investigated. Cellular proliferation was significantly inhibited in the PD-L1<sup>over</sup> group following treatment with mTOR inhibitor. PD-L1<sup>over</sup> Cal-27 cells were the most sensitive to rapamycin in the four groups, and the PD-L1<sup>RNAi</sup> tumor cells were the most tolerant to rapamycin-mediated proliferation inhibition (Fig. 6A-C). The result obtained in Cal-27 and FaDu cells were consistent. Although there was no significant statistical difference between PD-L1<sup>RNAi</sup> and PD-L1<sup>RNAi</sup> NC groups in FaDu cells, it was still indicated that PD-L1 exerted some regulatory action on tumor cell proliferation, and mTOR inhibitor was effective in preventing the PD-L1-driven proliferative effects. As presented in Fig. 4, PD-L1 knockdown reduced the activation of mTORC1 and mTORC2. In response to rapamycin treatment, p-P70S6K<sup>T389</sup> expression was markedly suppressed, and p-P70S6K<sup>T389</sup> could mediate a negative feedback loop on phosphoinositide 3-kinase (PI3K)/Akt which was de-repressed by rapamycin, and elevated the level of p-Akt<sup>S473</sup> (Fig. 7A and B). The proportion of p-P70S6K<sup>T389</sup> appeared the most markedly decreased in PD-L1<sup>over+RP</sup> cells, compared with PD-L1<sup>over</sup> cells;

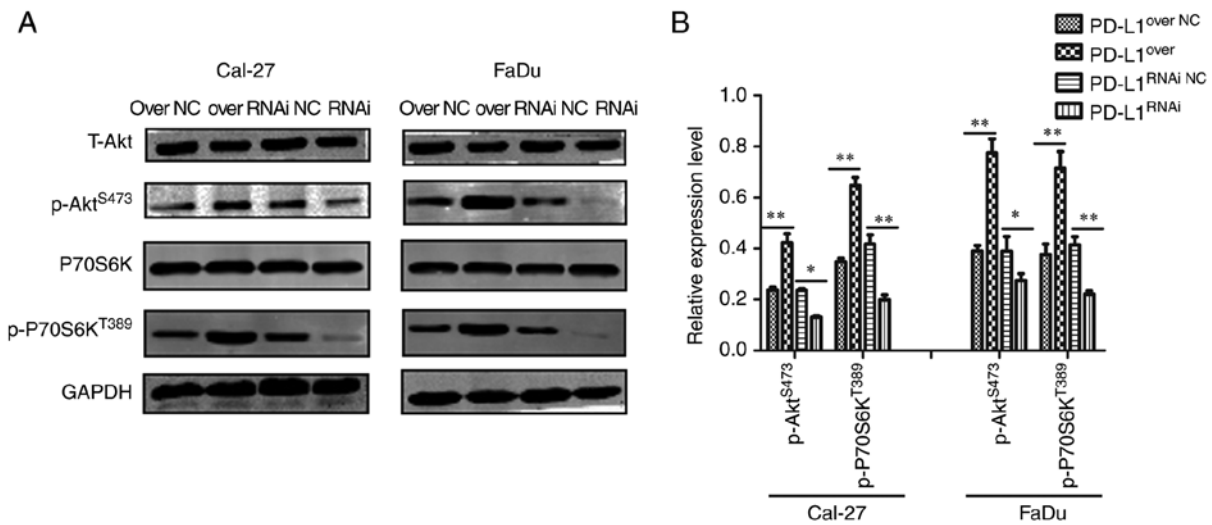


Figure 4. PD-L1 upregulates mTOR signaling in HNSCC cell lines. (A) Western blot analysis of T-Akt, P70S6K, p-P70S6K<sup>T389</sup>, p-Akt<sup>S473</sup> and GAPDH expression in PD-L1<sup>over</sup>, PD-L1<sup>RNAi</sup> and respective NC cells. (B) p-P70S6K<sup>T389</sup> and p-Akt<sup>S473</sup> expression in each group was quantified. \*P<0.05 and \*\*P<0.01. PD-L1, programmed death-ligand 1; T-, total; P70S6K, 70 kDa ribosomal protein S6 kinase 1; p-, phosphorylated; Akt, protein kinase B; PD-L1<sup>over</sup>, PD-L1-overexpressing; PD-L1<sup>RNAi</sup>, PD-L1 knockdown; NC, negative control.

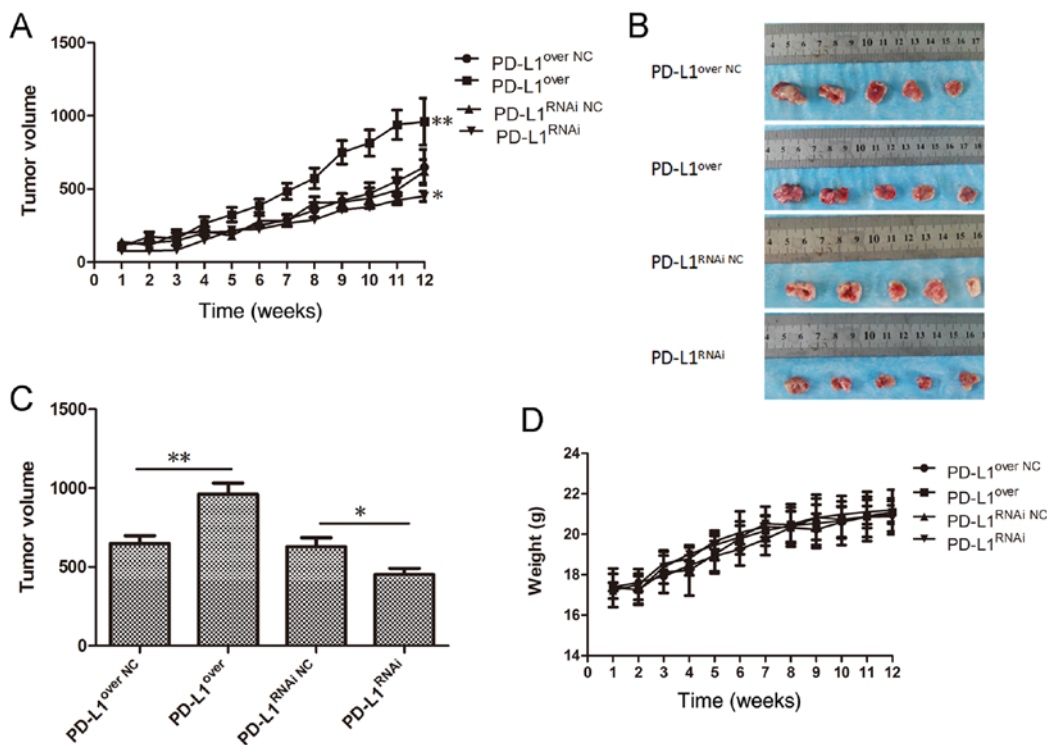


Figure 5. Effects of PD-L1 expression on tumor growth *in vivo*. (A) PD-L1 expression and tumor growth in the subcutaneous xenotransplanted tumor models. (B) Tumors were removed from nude mice and (C) the average tumor volume was presented in the histogram. (D) The average weight of nude mice was presented. \*\*P<0.01 and \*P<0.05. PD-L1, programmed death-ligand 1; PD-L1<sup>over</sup>, PD-L1-overexpressing; PD-L1<sup>RNAi</sup>, PD-L1 knockdown; NC, negative control.

and p-Akt<sup>S473</sup> of PD-L1<sup>RNAi</sup>, which increased, was more than PD-L1<sup>RNAi+RP</sup> (Fig. 7B), further indicating that PD-L1 may have promoted cell growth through mTOR signaling upregulation.

**Discussion**

PD-1 is predominantly expressed in activated T/B cells, where its function is to suppress immune cell activation via a physiological self-stabilization mechanism (19). Overactivation of

T/B cells lead to autoimmune diseases, and thus PD-1 has a protective role (19,20). In the tumor microenvironment, infiltrating T cells overexpress PD-1, and PD-L1 and PD-L2 are highly expressed by tumor cells; the ligation of PD-1/PD-L1 inhibits T cell activation (21). T cell migration and proliferation is inhibited, as well as the secretion of perforin and granzymes by cytotoxic T cells, which greatly limits their killing effects on tumor cells (22). Blocking the binding of PD-1 to PD-L1 would likely prevent immune escape,

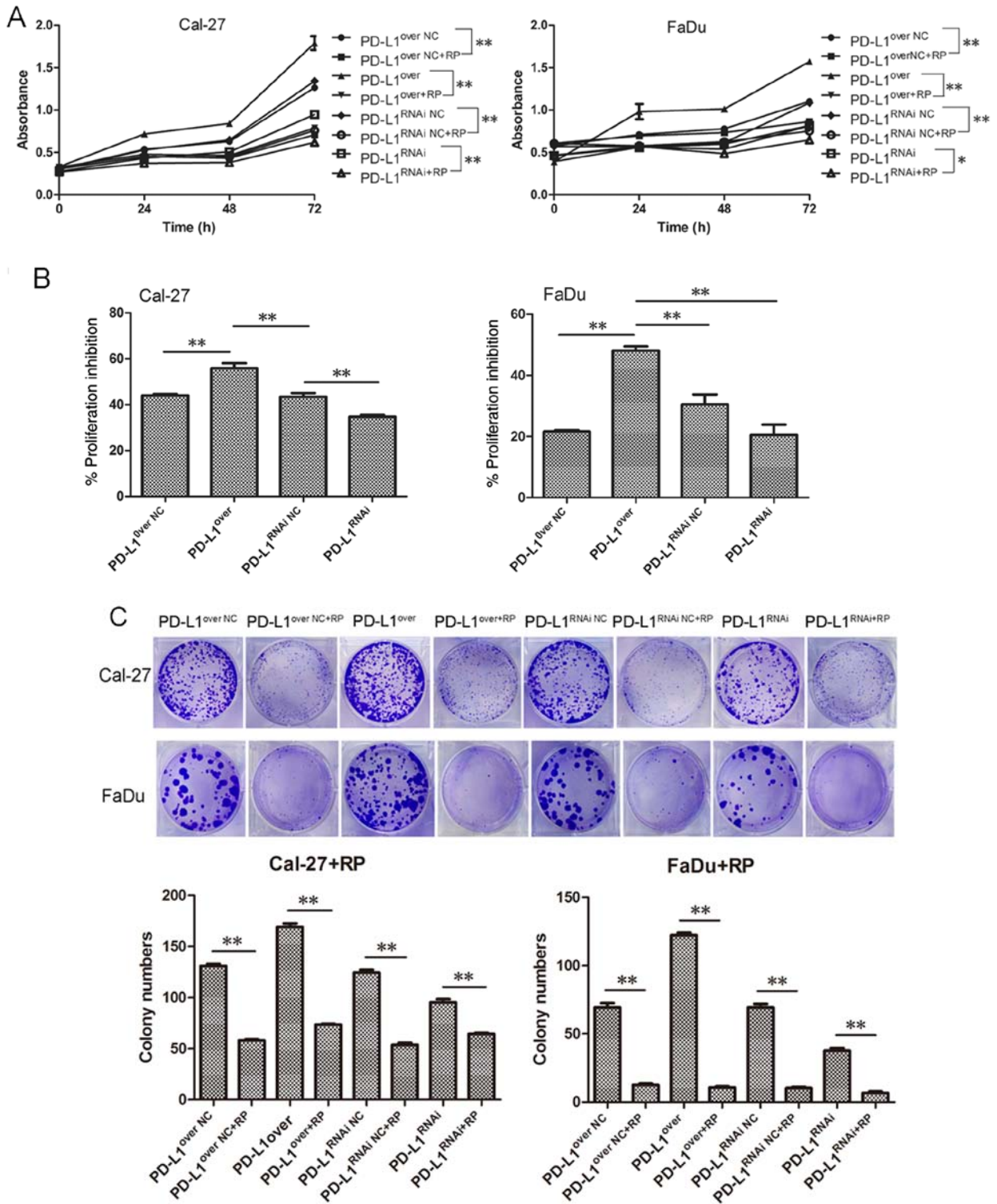


Figure 6. PD-L1 enhances the sensitivity of HNSCC cells to mTOR inhibitor *in vitro*. (A) HNSCC cells were cultured with rapamycin and cell proliferation was detected by Cell Counting Kit-8 assay (B) and the proliferation inhibition rate was calculated. (C) Colony formation assay results. Cell colony numbers (>50 cells/unit) were counted \*P<0.05 and \*\*P<0.01. PD-L1, programmed death-ligand 1; HNSCC, head and neck squamous cell carcinoma; mTOR, mammalian target of rapamycin; RP, rapamycin; PD-L1<sup>over</sup>, PD-L1-overexpressing; PD-L1<sup>RNAi</sup>, PD-L1 knockdown; NC, negative control.

enhance antitumor immunity and inhibit tumor progression. At present, the efficacy of PD-1-targeted therapy is very low as a monotherapy, with an overall response rate of 20-50% in cases with multiple tumors (23,24). However, it has been suggested that combining PD-1-targeted therapy with other therapeutic methods may significantly augment the curative

effect and the overall response rate (25). Therefore, the combination of PD-1/PD-L1 inhibition with other therapies requires further research.

As a novel target of immunotherapy, tumor-expressed PD-L1 mediates cancer immunopathogenesis by negatively regulating T cells (26). However, increasing evidence has

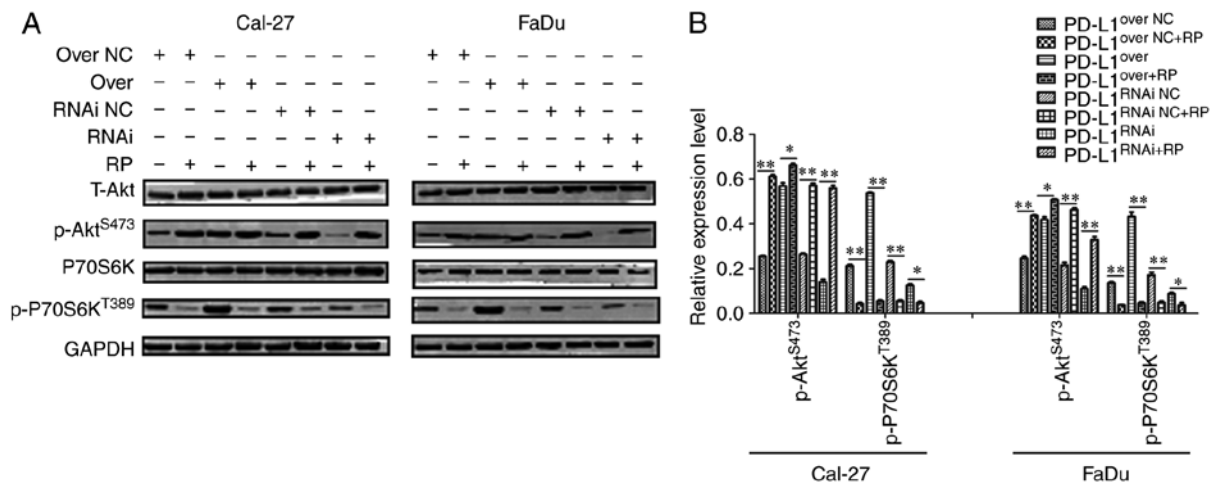


Figure 7. Rapamycin prevents the PD-L1-mediated effects on mTOR expression. (A) Western blot analysis of T-Akt, P70S6K, p-P70S6K<sup>T389</sup>, p-Akt<sup>S473</sup> and GAPDH expression in PD-L1<sup>over</sup>, PD-L1<sup>RNAi</sup> and respective NC cells with or without rapamycin. (B) p-P70S6K<sup>T389</sup> and p-Akt<sup>S473</sup> expression in each group was quantified. \*P<0.05 and \*\*P<0.01. PD-L1, programmed death-ligand 1; T-, total; P70S6K, 70 kDa ribosomal protein S6 kinase 1; p-, phosphorylated; Akt, protein kinase B; PD-L1<sup>over</sup>, PD-L1-overexpressing; PD-L1<sup>RNAi</sup>, PD-L1 knockdown; NC, negative control; mTOR, mammalian target of rapamycin; RP, rapamycin.

suggested that PD-L1 has critical functions in promoting tumor formation and development, without relying on the immune checkpoint (15,27). Previous studies have confirmed PD-L1 mRNA is upregulated in a wide variety of tumors, such as pulmonary adenocarcinoma, breast cancer and squamous lung cancer (13,28,29). A previous study reported that PD-L1 protein expression was increased in squamous cell carcinoma (SCC) of the head and neck, esophagus and lung (30). In addition, PD-1 expression is upregulated in response to proinflammatory cytokines IFN- $\gamma$ , TNF- $\alpha$  and IL-1 $\beta$ , and PD-L1 blockade by a monoclonal antibody efficiently augments the effects of adaptive T cell immunotherapy in a murine model of PD-L1-transfected SCC, and inhibits the growth of *de novo* induced PD-L1<sup>+</sup> SCC (21,30). In the present study, TMAs were obtained to detect PD-L1 expression characteristics in HNSCC. PD-L1 expression was significantly higher in tumor tissue, compared with NAT and normal tissue. Of note, the expression of PD-L1 progressively increased along with the increase of the tumor grades. These results suggested that PD-L1 was associated with HNSCC tumorigenesis. Consistent with the results of the present study, Strome *et al* (31) also reported that PD-L1 protein was upregulated in SCC and highly expressed in 66% (16 of 24) of freshly isolated SCC samples of the head and neck (31). However, the relationship between PD-L1 expression and tumor TNM staging remains unclear in certain reports, as well as its relationship with regional lymph node metastasis (32,33). In the present study, the results revealed that PD-L1 expression was not significantly different between T/N stages. PD-L1/PD-1 plays an important role in directly regulating the tumor microenvironment, and considering the complicated effects of PD-L1/PD-1 in the tumor and tumor microenvironment (9), it is difficult to illuminate the relationship between PD-L1 and T/N stages. Additionally, the small sample size may have had an impact on the results. Hence, further investigation with a larger number of samples is required.

In addition, the role of PD-L1 as a non-immune checkpoint was demonstrated, which may be a potential therapeutic

target in HNSCC. The majority of studies focusing on PD-L1 have predominantly focused on immunity, particularly in T cells (11,24,31). Although it has been reported that blocking PD-L1 affects tumor cell proliferation (12,27,34), it was uncertain whether PD-L1 inhibition suppresses tumor growth in HNSCC. Through the generation of stable PD-L1-overexpressing (PD-L1<sup>over</sup>), and PD-L1 knockdown (PD-L1<sup>RNAi</sup>) cell lines, the present study demonstrated that PD-L1 promoted tumor growth *in vitro* and *in vivo*. In addition, compared with the control group, PD-L1<sup>RNAi</sup> HNSCC cell proliferation decreased, even in the rapamycin-treated RNAi group. Francisco *et al* (35) reported that PD-L1 mediates the development of regulatory T cells via downregulation of the mTOR pathway, coupled with the upregulation of phosphatase and tensin homolog (PTEN). In addition, it has been reported that blocking PD-L1 suppresses mTOR activity (27,34). Our preliminary experiments confirmed that PD-L1 had no significant effects on the expression of key proteins involved in multiple signaling pathways, such as signal transducer and activator of transcription, mitogen-activated protein kinase and nuclear factor- $\kappa$ B. Hence, it was concluded that PD-L1 may have exerted bi-directional effects in regulating the mTOR pathway, although the exact mechanism remains unclear in HNSCC.

The PI3K/Akt/mTOR signaling pathway has critical functions in both solid tumors and hematological malignancies (36). mTOR regulates cell growth, motility, and metabolism; this signaling cascade is frequently upregulated in cancer due to loss of the tumor suppressor PTEN (37). Glycogen synthase kinase-3 (GSK-3) also interacts with and affects the function of downstream components of the PI3K/Akt/mTOR signaling network (38). Bertacchini *et al* (39) reported that dual inhibition of PI3K/mTOR signaling resensitizes resistant cancer cells to chemotherapy (39). mTOR contains two complexes, mTORC1 and mTORC2. p-P70S6K<sup>T389</sup> is the downstream effector of mTORC1, and p-Akt<sup>S473</sup> is the downstream effector of mTORC2. mTORC1 and mTORC2 exert important influences on the growth and survival of tumor cells (40,41). In the present study, it was demonstrated that PD-L1 increased

p-P70S6K<sup>T389</sup> and p-Akt<sup>S473</sup> expression in HNSCC cell lines, and cells overexpressing PD-L1 proliferated faster than control cells. Thus, PD-L1 may have been capable of regulating both mTORC1 and mTORC2 expression. In addition, mTORC1 inhibitor suppressed p-P70S6K<sup>T389</sup> expression and prevented the PD-L1-mediated increase in proliferation. Cell proliferation inhibition rate of the PD-L1<sup>over</sup> cells was the highest in the four groups following treatment with mTOR inhibitor, and PD-L1<sup>RNAi</sup> cells were the most resistance to rapamycin-mediated proliferation inhibition. In addition, p-Akt<sup>S473</sup> expression was increased in the of PD-L1<sup>over+RP</sup> cells, compared with the PD-L1<sup>RNAi+RP</sup> cells, this may be the combined effect of p-P70S6K<sup>T389</sup>-mediated negative feedback loop on phosphoinositide 3-kinase (PI3K)/Akt which was de-repressed by rapamycin and low PD-L1-mediated restraint of p-Akt<sup>S473</sup>. Therefore, further study is required to understand the mutual influence of both these factors.

Accumulating evidence has revealed that the curative effect of anti-PD-L1 treatment is superior to anti-cytotoxic T-lymphocyte protein 4 therapy (42,43). It has been speculated that anti-PD-L1 may not only prevent PD-L1-mediated immune escape, but may also restrain PD-L1-mediated carcinogenesis. This may also be the reason why anti-PD-L1 therapy has been reported to be more effective than immunotherapy in tumors (42). In addition, it suggests that tumor intrinsic PD-L1 signals have an antitumor effect.

In conclusion, the present study revealed that the expression of PD-L1 was significantly higher in HNSCC compared with normal tissues or cell lines, and the expression of PD-L1 increased as the tumor grade progressed. Further, PD-L1 promoted HNSCC cell proliferation both *in vitro* and *in vivo*. PD-L1 depletion led to a downregulation of mTOR signaling, and mTOR inhibitor prevented the PD-L1-mediated proliferative effect. These findings increased the current understanding of PD-L1-mediated carcinogenesis in HNSCC, and may be conducive in discovering novel therapeutic targets in HNSCC.

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#### Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

#### Authors' contributions

ZT and AZ conceived and designed the experiments. AZ, FL, FC, JZ, LW, YW, SC and BX performed the experiments. AZ and ZT analyzed the data and wrote the paper. ZT and FL revised the paper. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

All experimental protocols were approved by the Wuhan University (Wuhan, China).

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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