

## Original Article

# Upregulation of ATF-3 is correlated with prognosis and proliferation of laryngeal cancer by regulating Cyclin D1 expression

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**Abstract:** Objective: This study aimed to investigate the expression and significance of ATF-3 in laryngeal squamous cell carcinoma (LSCC). Methods: Expression of ATF-3 was examined using immunohistochemistry methods in samples from 83 cases of LSCC carcinoma. MTT assay was used to detect proliferation of Hep-2 cells after ATF-3 knocked down by siRNA lentivirus. A mouse model was used to investigate the inhibitive role of ATF-3 siRNA in LSCC xenografts. Realtime PCR was used to detect Cyclin D1 expression after ATF-3 downregulation in Hep-2 cells. Results: The expression of ATF-3 was positively detected in all the 83 cases of LSCC cancer tissues while Only 4 cases of adjacent non-neoplastic tissues were detected with positive ATF-3 expression. The ATF-3 expression was statistically related with T stage, neck nodal metastasis, clinical stage and prognosis of LSCC. Both cell proliferation *in vitro* and tumor growth *in vivo* were suppressed after ATF-3 knockdown. Furthermore, the expression of Cyclin D1 was decreased after ATF-3 downregulation in Hep-2 cells. Conclusion: ATF-3 is involved in the progress of LSCC, and may provide clinical information for evaluation of prognosis of LSCC. The oncologic role of ATF-3 may be correlated with Cyclin D1 regulation.

**Keywords:** Laryngeal squamous cell carcinoma, activating transcription factor 3, Cyclin D1

## Introduction

Laryngeal cancer is one of the most common malignancies of the head and neck, and its incidence is increasing over time. Current treatments, including surgical intervention, radiation therapy, and chemotherapy, have a moderate effect on early stage cases, but less effective in more advanced cases. In addition, surgery might lead to complete or partial loss of vocal function and many patients have to maintain a tracheal cannula for lifetime due to total laryngectomy. Therefore, a better understanding of the molecular mechanisms of LSCC progression and a new strategy for the treatment of LSCC are in urgent demand. ATF-3 (Activating transcription factor 3), a member of the ATF/cyclic AMP response element-binding (ATF/CREB) family of transcription factors, has been demonstrated to play differing roles in cancer development depending on the cell type. For

example, ATF-3 expression was found to be elevated in a significant number of human breast cancers [1], prostate cancer [2], Hodgkin lymphomas [3] and cutaneous squamous cell carcinoma [4]. In contrast, ATF-3 was down-regulated and may play tumor suppressed roles in human colorectal cancer and bladder cancer [5, 6]. However, the biological roles of ATF-3 in LSCC are still poorly understood. Therefore, in this study, we first detected the expression of ATF-3 in LSCC and found a significant up-regulation of ATF-3 in LSCC cancer tissues. Furthermore, ATF-3 knockdown can suppress proliferation and decrease expression of cell cycle gene, Cyclin D1 in LSCC cells. Our findings suggest that ATF-3 plays an oncogenic role in LSCC.

## Samples

Included in the study were 83 patients with laryngeal cancer who underwent partial or total laryngectomy at the Department of Otorhinol-

aryngology of the Second Affiliated Hospital of Harbin Medical University between October 2005 and January 2007. The patients had not received any therapy before admission. After surgery, the matched specimens of LSCC and the corresponding adjacent nonneoplastic tissues obtained from patients were preserved in liquid nitrogen within 5 minutes of excision and then were transported frozen to the laboratory and stored at  $-80^{\circ}\text{C}$ . The study was approved by the Ethics Committee of Harbin Medical University and informed consent was obtained.

#### *Immunohistochemistry*

About 2 mm size specimens cut from the 83 frozen LSCC samples were formalin-fixed, paraffin-embedded with serial sections of  $4\ \mu\text{m}$  in thickness for routine pathologic and immunohistochemical examination. The immunohistochemical procedures were performed using the avidin-biotin-peroxidase complex method. Briefly, endogenous peroxidase activity was abolished by treatment with 0.3%  $\text{H}_2\text{O}_2$  in methanol, and nonspecific binding of antibodies was blocked by preincubation with 5% normal goat serum in phosphate-buffered saline (PBS). Then, the tissue sections were subjected to antigen retrieval by incubating with 0.1% pepsin in PBS for 6 min and boiling in citric acid buffer for 30 min. Anti-ATF-3 antibody was applied as the first antibody, and biotinylated antimouse immunoglobulin as the second antibody. SABC kit and corresponding antibodies were purchased from Bioss (Beijing, China). SABC kit was used according to the manufacturer's recommendation. Finally, the sections were incubated with diaminobenzidine and 0.006%  $\text{H}_2\text{O}_2$  and counterstained with Mayer's hematoxylin. The slides were evaluated independently by two investigators without any information of the patient's clinicopathological features. According to the tumor area that was stained for ATF-3, in ATF-3-positive groups, the cutoff value was set at 30%, the mean value of staining, and those cases with more than 30% of the tumor area stained for ATF-3 were grouped as high ATF-3 (++) and those with less than 30% as low ATF-3 (+).

#### *Lentivirus vectors for ATF-3 siRNA*

SiRNA of human ATF-3 lentivirus vector carrying GFP sequence was provided by Genechem (Shanghai, China). The sequences of the siRNA

for ATF-3 were 5'-AAGAGGCGACGAGAAAGAAAT-3'. The recombinant lentivirus of ATF-3 siRNA and the control lentivirus (GFP-lentivirus) were prepared and titered to  $10^8$  TU/mL (transfection unit).

#### *Cell culture and virus transfection*

Hep-2 cells of human LSCC were kindly provided by the Laboratory of Cell Pathology, Harbin Medical University. Cells were cultured in DMEM medium containing 10% fetal bovine serum (Gibco) in a humidified ( $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ ) incubator. Hep-2 cells were plated in 24-well plates ( $2 \times 10^4$  cells/well) overnight. The lentiviruses were diluted in 0.2 mL ( $10^7$  TU/mL) complete medium containing polybrene (8 mg/mL) and incubated with the cells for 1 h at  $37^{\circ}\text{C}$ , next the cells were incubated with 0.3 mL fresh prepared polybrene-DMEM for another 24 h, the medium was replaced with fresh DMEM medium and the cells were cultured for 48 h.

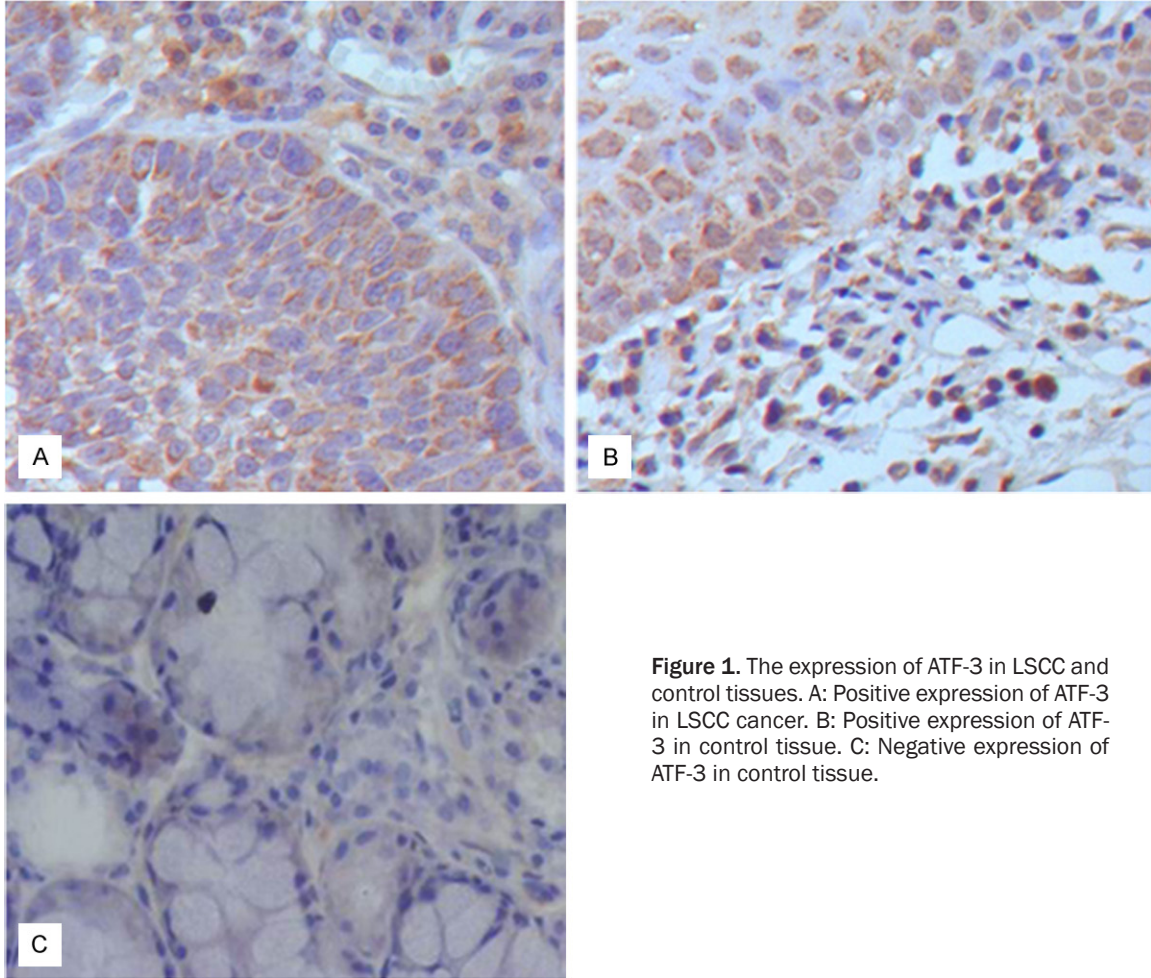
#### *MTT*

After ATF-3 siRNA transfection of Hep-2 cells for varying time periods: 20, 44, 68 and 92 h, 20  $\mu\text{L}$  of sterile MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) dye (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added and incubation continued for another 4 h at  $37^{\circ}\text{C}$ . Then, 150  $\mu\text{L}$  of dimethyl sulfoxide was added to each well and the plates were thoroughly mixed for 10 min. Spectrometric absorbance at a wavelength of 492 nm was measured on an enzyme immunoassay analyzer (model 680; Bio-Rad Laboratories, Hercules, CA, USA). The cell growth rate was calculated using the following formula:

$$\text{Cell growth rate (\%)} = (\text{mean absorbance in six wells of the treatment group} / \text{mean absorbance in six wells of the cells control group}) \times 100$$

#### *Xenografts in mice*

Twelve 5-6 weeks old BALB/c mice were provided by Vital River Laboratories (Beijing, China). They were bred in aseptic conditions and kept at a constant humidity and temperature according to standard guidelines under a protocol approved by Harbin Medical University. All mice were injected subcutaneously in the dorsal scapula region with 100  $\mu\text{L}$  suspension



**Figure 1.** The expression of ATF-3 in LSCC and control tissues. A: Positive expression of ATF-3 in LSCC cancer. B: Positive expression of ATF-3 in control tissue. C: Negative expression of ATF-3 in control tissue.

( $1 \times 10^6$ ) of Hep-2 cells. The size of the tumor was measured twice a week with calipers, and the volume of tumor was determined using the simplified formula of a rotational ellipsoid (length  $\times$  width<sup>2</sup>  $\times$  0.5). Once tumors reached approximately 0.5-0.6 cm<sup>3</sup>, the mice received an injection into the tumor once a week for 3 weeks. The 6 mice in the experimental group were treated with 100  $\mu$ l ATF-3 siRNA lentivirus, while six mice in the control group received an injection of 100  $\mu$ l GFP-lentivirus. Tumors were harvested 1 week after the end of treatment.

#### qPCR

Total RNA was extracted from cancerous or noncancerous specimens and Hep-2 cells. The expression level of ATF-3 and Cyclin D1 were determined by qPCR, as described previously [7]. The primers for ATF-3 were 5'-CTCCTGGTCACTGGTGT-3' and 5'-GTCGCCTCTTTTCCTTCA-3' [8]. The primers for Cyclin D1 were primer: 5'-AGGAGAACAACAGATCA-3' and

5'-TAGGACAGGAAGTTGTTG-3' [9]. The relative expression level was calculated using the  $2^{-\Delta\Delta Ct}$  method, with the CT values normalized using 18S rRNA as internal control.

#### Statistical analysis

Statistical analysis was done with SPSS version 17.0 for Windows. Expression of ATF-3 in LSCC was tested with chi square test and log-rank test was used to evaluate prognosis. Other data were expressed as means  $\pm$  SD, differences between groups were assessed by unpaired, two-tailed Student's t test. *P* values less than 0.05 were considered significant.

#### Results

##### *Expression of ATF-3 is increased in LSCC tissues*

Immunohistochemistry showed that ATF-3 protein was detected in all the 83 LSCC tumor tis-

**Table 1.** Relationship between ATF-3 expression level and clinicopathologic parameters of LSCC

Characteristics (n)	Expression of ATF-3		P
	(+) 46	(++) 37	
Sex			0.516
Male (57)	32	25	
Female (26)	14	12	
Age			0.295
≥ 56 (42)	25	17	
< 56 (41)	21	20	
T classification			0.016
T1-2 (52)	34	18	
T3-4 (31)	12	19	
Differentiation			0.086
G1 (59)	36	23	
G2 (24)	10	14	
Lymph node metastasis			0.017
Negative (54)	35	19	
Positive (29)	11	18	
Primary location			0.174
Supraglottic (35)	22	13	
Glottic (48)	24	24	
Clinical stage			0.013
I-II (46)	31	15	
III-IV (37)	15	22	

sues. Only 4 cases of adjacent non-neoplastic tissues were detected with positive ATF-3 expression (**Figure 1**). The ATF-3 expression was statistically related with T stage, neck nodal metastasis and clinical stage of LSCC (**Table 1**). Tumors with T3-4 grade, lymph node metastasis or advanced clinical stages expressed higher level of ATF-3. To determine the prognostic value of ATF-3 expression, a log-rank test was carried out. Cumulative survival rates were calculated by the Kaplan-Meier method. As shown in **Figure 2**, patients with high ATF-3 expression level had significantly shorter overall survival than those with low ATF-3 expression level ( $\chi^2$ : 6.632, P = 0.010).

#### *ATF-3 siRNA inhibits proliferation of Hep-2 cells*

Lentiviral vector system that incorporates GFP was used as a reporter gene to express siRNA to downregulate ATF-3. The MTT assay results showed after ATF-3 siRNA transfection, the viability of the Hep-2 cells was evidently decreased at each different time-point (24, 48, 72, and 96

h). However, survival rates of the Hep-2 cells in the control groups did not show any obvious alteration during the time course (**Figure 3**) (P < 0.01).

#### *Knockdown of ATF-3 suppresses tumor growth in vivo*

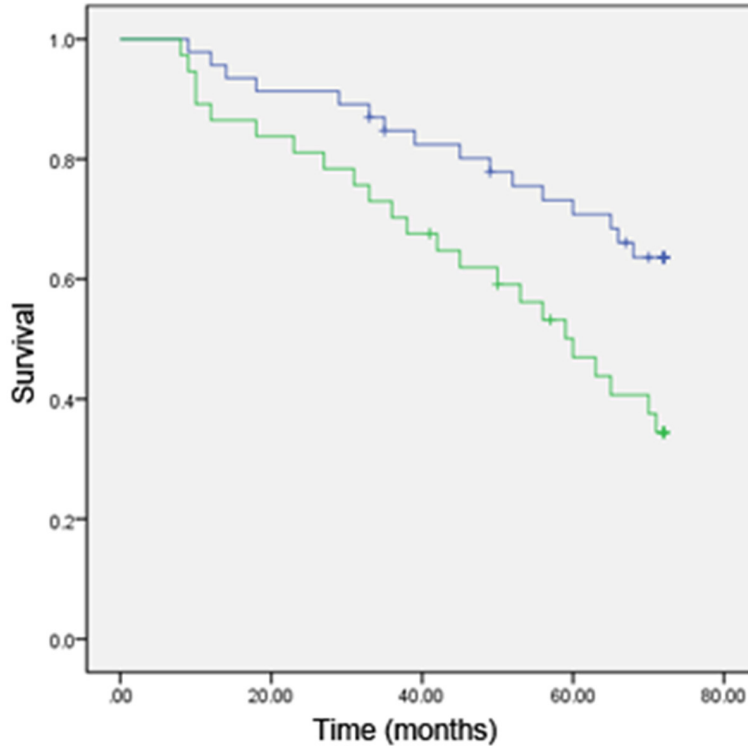
All of the 12 mice developed detectable tumors after they were subcutaneously injected with Hep-2 cells. The growth of the LSCC xenografts was significantly inhibited in mice treated with ATF-3 siRNA lentivirus compared with those treated with GFP-lentivirus (**Figure 4**). The average tumor weight ( $1.408 \pm 0.118$  g) in the ATF-3 siRNA treated LSCC xenografts was statistically lower (P < 0.01) than the tumors in control group ( $2.073 \pm 0.120$  g).

#### *ATF-3 siRNA suppresses Cyclin D1 expression in Hep-2 cells*

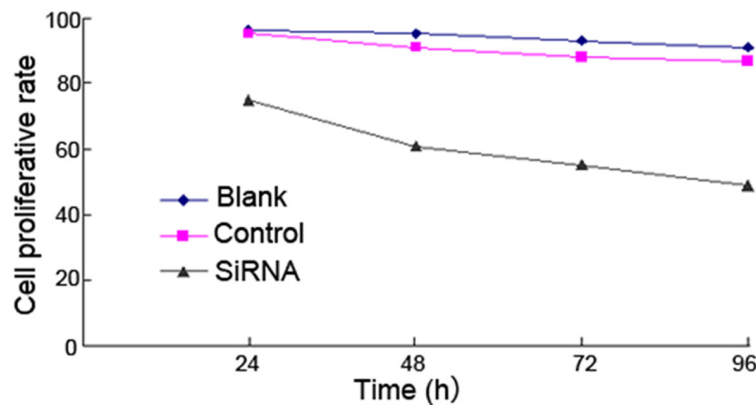
To determine the effect of ATF-3 on Cyclin D1 expression in Hep-2 cells, the mRNA level of Cyclin D1 was detected by realtime PCR. The results showed that the mRNA level of Cyclin D1 was reduced in Hep-2 cells transfected with ATF-3 siRNA lentivirus compared with control whilst similar levels of Cyclin D1 expression were found between the control Hep-2 cells and cells without any treatment (**Figure 5**).

## Discussion

The occurrence and development of cancers are a complex process involving multiple genes and multiple steps, which is as a result of cooperation and interaction of multiple factors. The recurrence and metastasis of tumor are major factors threatening the human health. Thus, to explore the relationship among genes exerting inhibitory effect on cancer is clinically important for the evaluation of prognosis and treatment of cancers [9]. The activating transcription factor (ATF) family represents a large group of basic-region leucine zipper (bZIP) transcription factors and they are critical transcriptional regulators in human cancer [10]. Unlike other ATF family members, recent studies have implicated ATF-3 in host defence against invading pathogens and cancer [11, 12]. ATF-3 has been demonstrated overexpression in many malignant tumors. But in bladder cancer, decreased



**Figure 2.** The Kaplan-Meier overall survival curve for LSCC patients (n = 83). The higher curve represented the patients with tumors that weakly expressed ATF-3, and the lower curve represented the patients with tumors that highly expressed ATF-3 (P = 0.013).

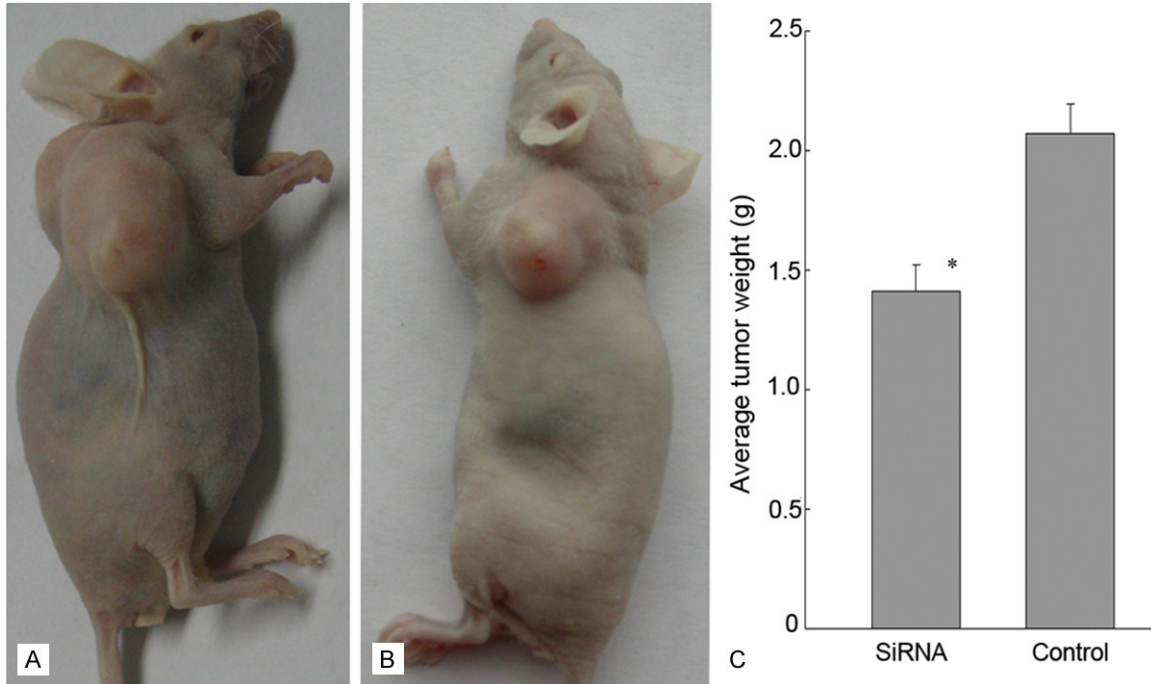


**Figure 3.** Curve of cell proliferative rate. After ATF-3 siRNA lentivirus transfection, the proliferative rate of Hep-2 cells was evidently decreased according to each different time point (24, 48, 72, and 96 h, respectively) compared with the controls.

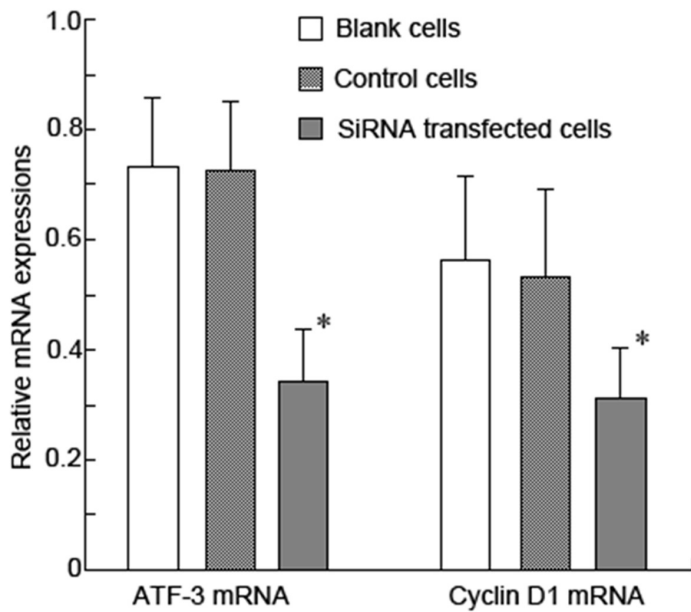
ATF-3 was associated with progression and poor prognosis [6]. Furthermore, ATF-3 upregulation in highly metastatic bladder cancer cells decreased migration in vitro and experimental metastasis in vivo [6]. Similarly, ATF-3 downregulation in colon cancer promotes tumor

growth and metastasis [13]. These results suggest ATF-3 acting either as a tumor suppressor or an oncogene in different cancers. In this study, we found ATF-3 was overexpressed in all the 83 cases of primary LSCC compared with adjacent non-cancerous tissues. Moreover, high level of ATF-3 expression was correlated with T stage, lymph node metastasis or advanced clinical stages of LSCC. Furthermore, we demonstrated that the overexpression of ATF-3 had statistically significant association with decreased overall survival in LSCC. These data suggest that ATF-3 may promote the malignant progression of LSCC. To further understand the biological function of ATF-3 in LSCC progression, both in vitro and in vivo assays were performed. Our in vitro data demonstrated that siRNA mediated downregulation of ATF-3 led to significant decrease of proliferation in Hep-2 cells. Moreover, we injected Hep-2 cells into mice to make xenografts which were treated by ATF-3 siRNA lentivirus. The average tumor weight was significantly lower in these mice compared with control, suggesting that ATF-3 downregulation could effectively suppress the progress of LSCC in vivo. As a key regulator of the cellular integrated stress response, ATF-3 gene is involved in a variety of signal pathways in cancers. It is a target of p53

gene and can also feedback regulate p53 level in non-small cell lung carcinoma cells [14]. In breast cancer cells, ATF-3 is induced by TGF- $\beta$  and it also up-regulates the expression of the TGF- $\beta$  gene itself, forming a positive-feedback loop for TGF- $\beta$  signaling [15]. Cyclin D1 is a key



**Figure 4.** Downregulation of ATF-3 suppresses tumor growth in vivo. A: Representative mouse in the control group. B: Representative mouse in ATF-3 siRNA lentivirus treated group. C: Difference of tumor weight between ATF-3 siRNA lentivirus treated-group and the control. \*:  $p < 0.01$ .



**Figure 5.** ATF-3 decreases Cyclin D1 expression in Hep-2 cells. A: Real-time PCR showed ATF-3 mRNA level decreased after ATF-3 siRNA transfection. B: Cyclin D1 mRNA level also decreased after ATF-3 siRNA transfection. \*:  $P < 0.01$ .

LSCC [9]. In the present study, our data showed Cyclin D1 level decreased in LSCC cells after ATF-3 gene knockdown. This suggests ATF-3 can regulate LSCC cell proliferation through regulating Cyclin D1 related pathway.

In summary, our data suggest that ATF-3 is overexpressed in LSCC tumor tissues and associated with progression and prognosis of LSCC. Moreover, we found that downregulation of ATF-3 can inhibit proliferation and growth of LSCC cells. These oncogenic effects of ATF-3 are related to the regulation of Cyclin D1 signal network. Taken together, these results suggest that ATF-3 can serve as a marker for LSCC prognosis and ATF-3 may be a useful therapeutic target for LSCC.

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cell cycle regulatory protein that governs cell cycle progression from the G1 to S phase and it is overexpressed in many cancers including

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#### Disclosure of conflict of interest

None.

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