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STYK1 promotes the malignant progression of laryngeal squamous cell carcinoma through targeting TGF-β1

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Abstract. – OBJECTIVE: The aim of this study was to uncover the expression characteristic and biological function of STYK1 in the progression of laryngeal squamous cell carcinoma (LSCC), and to explore the underlying mechanism.

PATIENTS AND METHODS: Expression level of STYK1 in 44 paired LSCC and adjacent normal tissues was detected by quantitative real-time polymerase chain reaction (qRT-PCR). The relationship between STYK1 level and clinical parameters of LSCC patients was analyzed. Subsequently, the regulatory effect of STYK1 on the proliferative ability of AMC-HN-8 and Hep-2 cells was evaluated by cell counting kit-8 (CCK-8) assay and colony formation assay. Dual-Luciferase reporter gene assay and rescue experiments were conducted to uncover the role of STYK1/TGF-β1 axis in regulating the progression of LSCC.

RESULTS: STYK1 was significantly up-regulated in LSCC tissues than that of adjacent normal tissues ($p<0.05$). LSCC patients with high expression level of STYK1 exhibited significantly higher clinical stage and lower survival rate ($p<0.05$). Knockdown of STYK1 remarkably attenuated viability and clonality in Hep-2 cells, while overexpression of STYK1 achieved the opposite trends in AMC-HN-8 cells ($p<0.05$). TGF-β1 was confirmed to be the direct target binding STYK1, whose expression level was negatively regulated by STYK1. TGF-β1 was significantly down-regulated in LSCC tissues ($p<0.05$). Meanwhile, its low expression predicted significantly poor prognosis of LSCC patients. In addition, TGF-β1 was responsible for STYK1-regulated malignant progression of LSCC.

CONCLUSIONS: STYK1 is upregulated in LSCC and is closely associated with T stage and poor prognosis. Furthermore, STYK1 promotes the proliferative ability of LSCC cells through targeting TGF-β1, thus aggravating the malignant progression of LSCC.

Key Words:
STYK1, TGF-β1, Laryngeal squamous cell carcinoma (LSCC), Proliferation.

Introduction

Laryngeal carcinoma is the most common malignancy in the clinical practice of otolaryngology department. About 96-98% cases of laryngeal carcinoma belong to laryngeal squamous cell carcinoma (LSCC). LSCC mainly affects the population aged 40-60 years old, with the male-female ratio of 7-9:1[1-3]. Currently, surgery (including microscopic laser operation and open surgery) combined local radiotherapy is the first choice for LSCC patients. For advanced LSCC, comprehensive therapy including systemic chemotherapy, is the optimal strategy[4,5]. With the development of medical technologies, the rate of laryngeal preservation, life quality and 5-year survival of LSCC patients have been greatly improved[6,7]. Nevertheless, the prognosis of advanced LSCC is still far from satisfactory. Nowadays, target drugs are popular in tumor treatment[8,9]. Therefore, it is of clinical significance to develop effective therapeutic targets with low toxicity for LSCC[10,11].

Tumor is a pathological condition of dysphasia. Under normal circumstances, the balance between cell proliferation and apoptosis is maintained by uncontrolled cell cycle progression[12,13]. Once cell cycle progression is damaged, uncontrolled proliferation eventually leads to tumorigenesis[14]. Tyrosine protein kinases (PTKs) transfer r-phosphoric acid from ATP to tyrosine residues of substrate protein molecules by catalysis. Enzyme activities are regulated by phosphorylation of tyrosine

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residues, resulting in recognition sites of downstream genes[15]. PTKs are associated with multiple important intercellular pathways, which also participate in cell-cell communication[16]. Members of the PTKs family have been well concerned due to their critical biological functions[17,18]. STYK1 is a novel subfamily of PTKs, presenting proto-oncogene features. STYK1 (55 kb) is located on human chromosome 12, which consists of 11 exons and encodes 422 amino acids[19,20]. Upregulation of STYK1 leads to tumorigenesis in nude mice, demonstrating its carcinogenic role[21]. Previous studies[22,23] have identified that STYK1 is upregulated in ovarian cancer and lung cancer. However, its potential influence on the progression of LSCC remains unclear.

Transforming Growth factor-β (TGF-β) is a well-known regulator involved in inflammatory response, tissue repair and embryonic development. In recent years, critical functions of TGF-β in cell growth, cell differentiation and immune function have been identified[24,25]. Generally speaking, TGF-β stimulates mesenchymal-derived cells and inhibits activities of epithelial-derived or neuroectodermal-derived cells[26]. Meanwhile, changes in the expression and activity of TGF-β can significantly affect tumor progression[27,28]. In this experiment, 44 paired LSCC tissues and adjacent normal tissues were collected. The potential influence of STYK1 on the prognosis of LSCC patients, and *in vitro* phenotypes of LSCC cells were mainly explored. Our findings might provide references for the development of therapeutic strategies of LSCC.

Patients and Methods

LSCC Patients and Samples

The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). A total of 44 paired LSCC tissues and adjacent normal tissues were collected and stored at -80°C. None of enrolled patients received preoperative anti-tumor therapy. Clinical and follow-up data of enrolled patients were recorded. Informed consent was obtained from patients and their families before the study. This investigation was approved by the Ethics Committee of Weifang People's Hospital.

Cell Culture

LSCC cell lines (AMC-HN-8 and Hep-2) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C. Cell passage was conducted in 1×trypsin+EDTA (ethylenediaminetetraacetic acid) at 80-90% of confluence.

Cell Transfection

Transfection plasmids were provided by GenePharma (Shanghai, China). Briefly, cells were inoculated into 6-well plates and cultured to 40% of confluence. Cell transfection was conducted according to the instructions of Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Transfected cells for 48 h were harvested for the following functional experiments.

Cell Proliferation Assay

Cells were first inoculated into 96-well plates at a density of 2×10^3 cells per well. At appointed time points, absorbance value at 450 nm was recorded using the cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan). Finally, the viability curve was plotted.

Colony Formation Assay

Cells were inoculated into 6-well plates at a density of 2×10^2 cells per well and cultured for 2 weeks. Culture medium was replaced once in the first week and twice in the second week. Afterwards, formed colonies were washed with phosphate-buffered saline (PBS), fixed with methanol for 20 min and dyed with 0.1% crystal violet for 20 min. Visible colonies were captured under a microscope, and the number of formed colonies was calculated.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNAs were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Each sample was performed in triplicate. Relative expression level was calculated by the $2^{-\Delta\Delta Ct}$ method. Primers used in this study

were as follows: STYK1: forward: 5'-CAGTGGGAAGGAGGGACTGA-3', reverse: 5'-TGCAGCCCAGTGAAATTGGA-3'; TGF-β1: forward: 5'-CGCTCTCTGCTCCTCCTGTTC-3', reverse: 5'-ATCCGTTGACTCCGACCTTCAC-3'; GAPDH: forward: 5'-ACAGTCAGCCGCATCTTCTT-3', reverse: 5'-GACAAGCTTCCCGTTCTCAG-3'.

Western Blot

Transfected cells were first centrifuged at 14000×g and 4°C for 15 min for total protein extraction. Subsequently, obtained cellular protein was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking in 5% skim milk for 1 hour, the membranes were incubated with primary antibodies overnight at 4°C. On the next day, the membranes were incubated with corresponding secondary antibody for 2 h at room temperature. Next, the membranes were washed with Tris Buffered Saline- and Tween-20 (TBST) for 1 min. Immunoreactive bands were finally exposed by the chemiluminescent substrate kit.

Dual-Luciferase Reporter Gene Assay

Cells were first inoculated into 24-well plates. On the next day, cells were co-transfected with WT-STYK1/MUT-STYK1 and pcDNA-TGF-β1/NC, respectively. 48 hours later, transfected cells were lysed for determining relative Luciferase activity.

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for data analyses. Experimental data were expressed as mean ± standard deviation. Differences between two groups were analyzed by the *t*-test. Kaplan-Meier method was introduced for survival analysis. Spearman correlation test was performed to assess the relationship between STYK1 and TGF-β1 expressions with clinical parameters of LSCC patients. $p<0.05$ was considered statistically significant.

Results

STYK1 Was Highly Expressed In LSCC Tissues

Compared with adjacent normal tissues, STYK1 was significantly upregulated in LSCC tissues ($p<0.05$, Figure 1A, 1B). This suggested that STYK1 might serve as an oncogene in the progression of LSCC.

STYK1 Expression Was Correlated With Pathological Staging and Overall Survival of LSCC Patients

According to the median level of STYK1, enrolled 44 LSCC patients were assigned into two groups, including high-level group and low-level group. Clinical data were collected from all patients for further analyses. The results showed that STYK1 level was positively correlated with T stage, rather than age, sex,

Table I. Association of STYK1 and TGF-β1 expression with clinicopathologic characteristics of laryngeal squamous cell carcinoma.

Parameters	No. of cases	STYK1 expression		*p*-value	TGF-β1 expression		*p*-value
		Low (%)	High (%)		Low (%)	High (%)	
Age (years)				0.122			0.319
<60	17	11	6		5	12	
≥60	27	11	16		12	15	
Gender				0.228			0.353
Male	22	13	9		7	15	
Female	22	9	13		10	12	
T stage				0.005			0.005
T1-T2	27	18	9		6	21	
T3-T4	17	4	13		11	6	
Lymph node metastasis				0.052			0.085
No	30	18	12		9	21	
Yes	14	4	10		8	6	
Distance metastasis				0.353			0.122
No	27	15	12		8	19	
Yes	17	7	10		9	8	

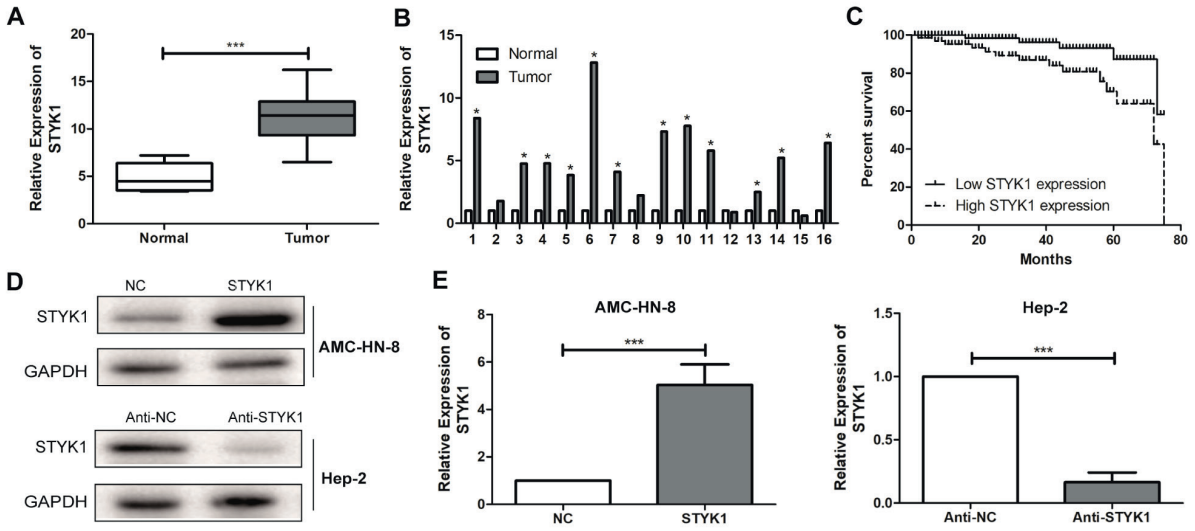

Figure 1. STYK1 was highly expressed in LSCC. **A-B,** STYK1 levels in LSCC tissues and adjacent normal tissues. **C,** Overall survival in LSCC patients expressing high or low level of STYK1. **D-E,** Transfection efficacy of pcDNA-STYK1 and anti-STYK1 in AMC-HN-8 and Hep-2 cells, respectively. Data were expressed as mean ± SD. *p<0.05, **p<0.01, ***p<0.001.

distant metastasis and lymphatic metastasis of LSCC patients (Table I). Besides, Kaplan-Meier curves revealed significantly worse prognosis in LSCC patients of high-level group compared with that of low-level group (p<0.05, Figure 1C).

Promotive Effect of STYK1 on Proliferative Ability of LSCC

In vitro overexpression and knockdown models of STYK1 were established in AMC-HN-8 and Hep-2 cells, respectively. Transfection efficacy was verified by Western blot (Figure 1D) and qRT-PCR (Figure 1E). In AMC-HN-8 cells overexpressing STYK1, cell viability and colony formation ability were markedly elevated than those of controls (p<0.05). Conversely, knockdown of STYK1 significantly decreased the viability and colony formation ability of Hep-2 cells (Figure 2A, 2B).

TGF-Ð1 Directly Bound to STYK1

Through bioinformatics prediction, the binding sequences between TGF-β1 and STYK1 were depicted (Figure 3A). Overexpression of TGF-β1 significantly quenched Luciferase activity in wild-type STYK1 vector (p<0.05). However, the mutant-type one was not affected, verifying the binding relationship between STYK1 and TGF-β1 (Figure 3A). Overexpression of STYK1 markedly downregulated protein and mRNA levels of TGF-β1 in AMC-HN-8 cells (p<0.05). Conversely, knockdown of STYK1 up-regulated TGF-β1 level in Hep-2 cells (p<0.05, Figure 3B, 3C). In LSCC tissues, TGF-β1 was highly expressed compared with adjacent normal tissues (p<0.05, Figure 3D). Subsequent analysis indicated with TGF-β1 level was negatively correlated with T stage, whereas it was not associated with other pathological parameters (Table I). Moreover, low level of TGF-β1 predicted poor prognosis of LSCC patients (Figure 3E).

STYK1/TGF-β1 Axis in LSCC Progression

Up-regulated STYK1 in AMC-HN-8 cells transfected with pcDNA-STYK1 was partially reversed by co-transfection of si-TGF-β1. On the contrary, overexpression of TGF-β1 abolished the down-regulation of STYK1 in Hep-2 cells transfected with anti-STYK1 (Figure 4A). Interestingly, the elevated viability and clonality in AMC-HN-8 cells overexpressing STYK1 were partially reversed by knockdown of TGF-β1. In Hep-2 cells, overexpressed TGF-β1 abolished the attenuated proliferative ability owing to silence of STYK1 (Figure 4B, 4C). As a result, our findings proved that STYK1

promoted the proliferative ability of LSCC *via* negatively regulating TGF-β1.

Discussion

Currently, LSCC is the major subtype of laryngeal carcinoma worldwide. The therapeutic efficacy of advanced LSCC is relatively poor. Meanwhile, impaired laryngeal function may result in severely affected life quality[1-5]. Therefore, it is urgent to clarify the molecular mechanism of LSCC, and to develop precise treatment for alleviating the malignant progression of LSCC[9-11].

Tumor progression is a complicated process involving multiple factors and genes, among which, cell proliferation is an important event[12-14]. TGF-β1 is able to regulate activities of cyclin-dependent kinases, thereby affecting tumorigenesis[19-21]. Previous studies[22,23] have reported that dysregulated STYK1 is linked to the imbalanced proto-oncogenes and tumor-suppressor genes. In addition, STYK1 exerts a certain prognostic potential in multiple malignant tumors[23]. In non-

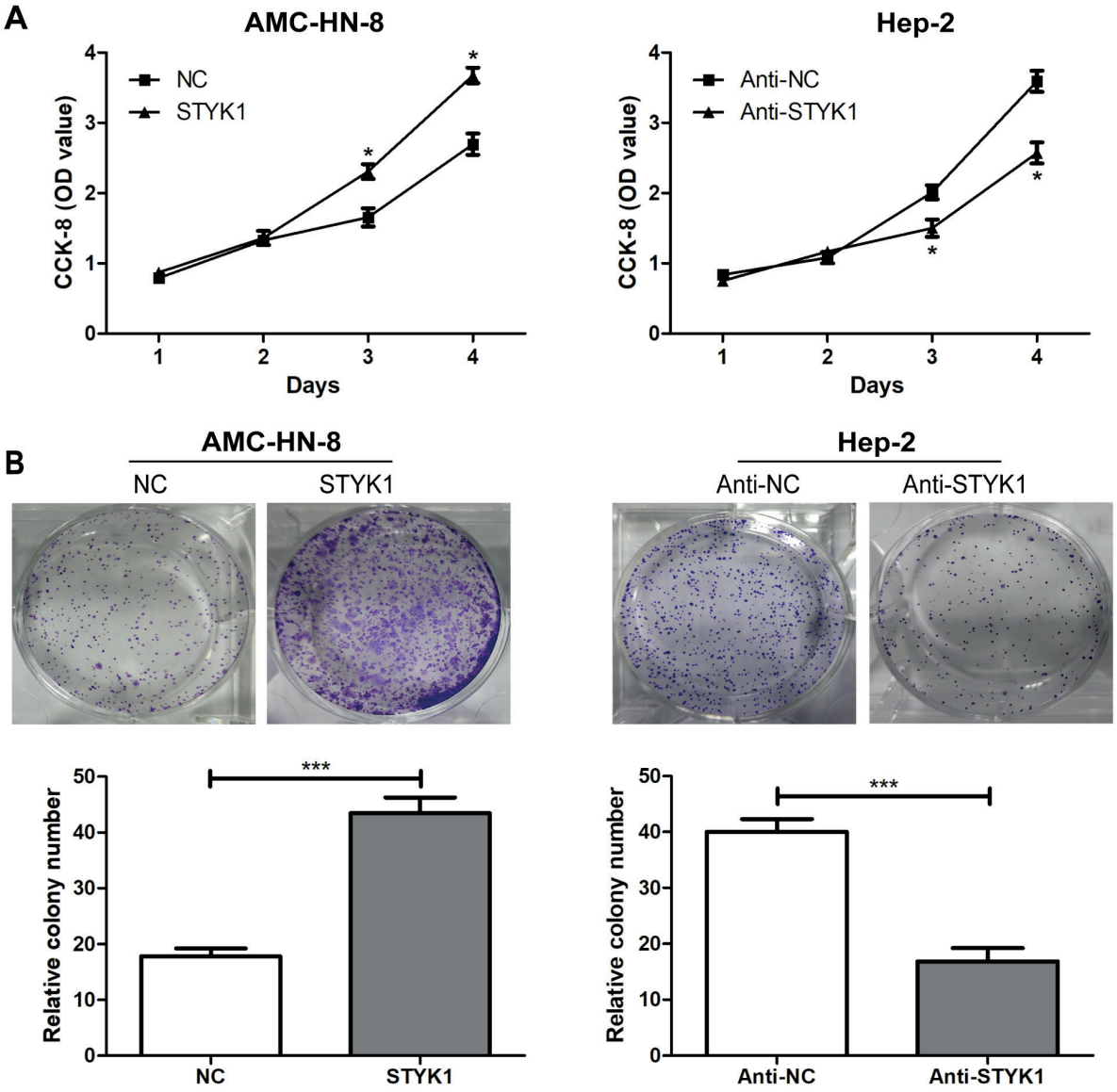

Figure 2. Promotive effect of STYK1 on the proliferative ability of LSCC cells. AMC-HN-8 cells were transfected with NC or pcDNA-STYK1. Hep-2 cells were transfected with anti-NC or anti-STYK1. **A,** Viability in AMC-HN-8 and Hep-2 cells. **B,** Colony number in AMC-HN-8 and Hep-2 cells. Data were expressed as mean ± SD (magnification: 10×). *$p<0.05$, ***$p<0.001$.

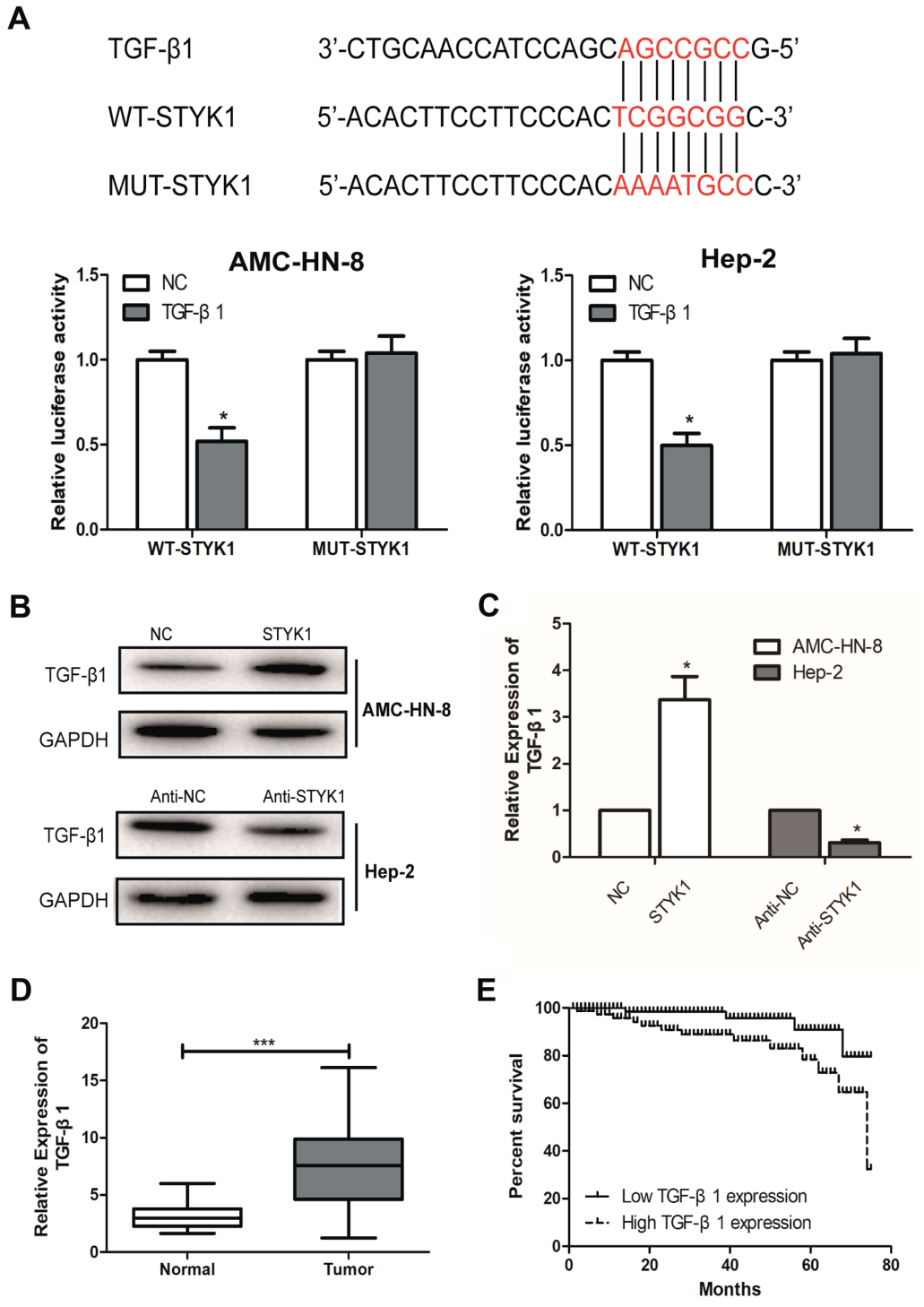

Figure 3. TGF-β1 directly bound to STYK1. **A,** Luciferase activity in AMC-HN-8 and Hep-2 cells co-transfected with WT-STYK1/MUT-STYK1 and NC/pcDNA-TGF-β1. AMC-HN-8 cells were transfected with NC or pcDNA-STYK1. Hep-2 cells were transfected with anti-NC or anti-STYK1. **B-C,** Protein **(B)** and mRNA levels **(C)** of TGF-β1 in AMC-HN-8 and Hep-2 cells. **D,** TGF-β1 levels in LSCC tissues and adjacent normal tissues. **E,** Overall survival in LSCC patients expressing high or low level of TGF-β1. Data were expressed as mean ± SD. *$p<0.05$, ***$p<0.001$.

small cell lung cancer, STYK1 is up-regulated and correlated with poor prognosis. STYK1 level is associated with poor prognosis of ovarian cancer patients. In this study, our results demonstrated that STYK1 was upregulated in LSCC tissues and cells. STYK1 level was positively correlated with T stage and poor prognosis of LSCC patients. Hence, it is believed that STYK1 exerts an oncogenic role in the progression of LSCC.

Subsequently, *in vitro* experiments were conducted to uncover the influence of STYK1 on regulating the proliferative ability of LSCC cells. CCK-8 and colony formation assays demonstrated that STYK1 significantly promoted the proliferative ability of LSCC. Furthermore, TGF-β1 was predicted and verified to be the target binding STYK1 through bioinformatics and dual-luciferase reporter gene assay, respectively. TGF-β signaling is responsible for various aspects of cell phenotypes[24-26]. Our findings uncovered that TGF-β1 was downregulated in LSCC tissues, and low level of TGF-β1 predicted poor prognosis of LSCC patients. Through rescue experiments, TGF-β1 was involved in STYK1-regulated progression of LSCC. Collectively, STYK1 promoted the proliferative ability of LSCC through targeting and negatively regulating TGF-β1 level, thus aggravating the progression of LSCC.

Conclusions

The novelty of this study was that STYK1 is upregulated in LSCC and is closely linked to T stage and poor prognosis of LSCC patients.

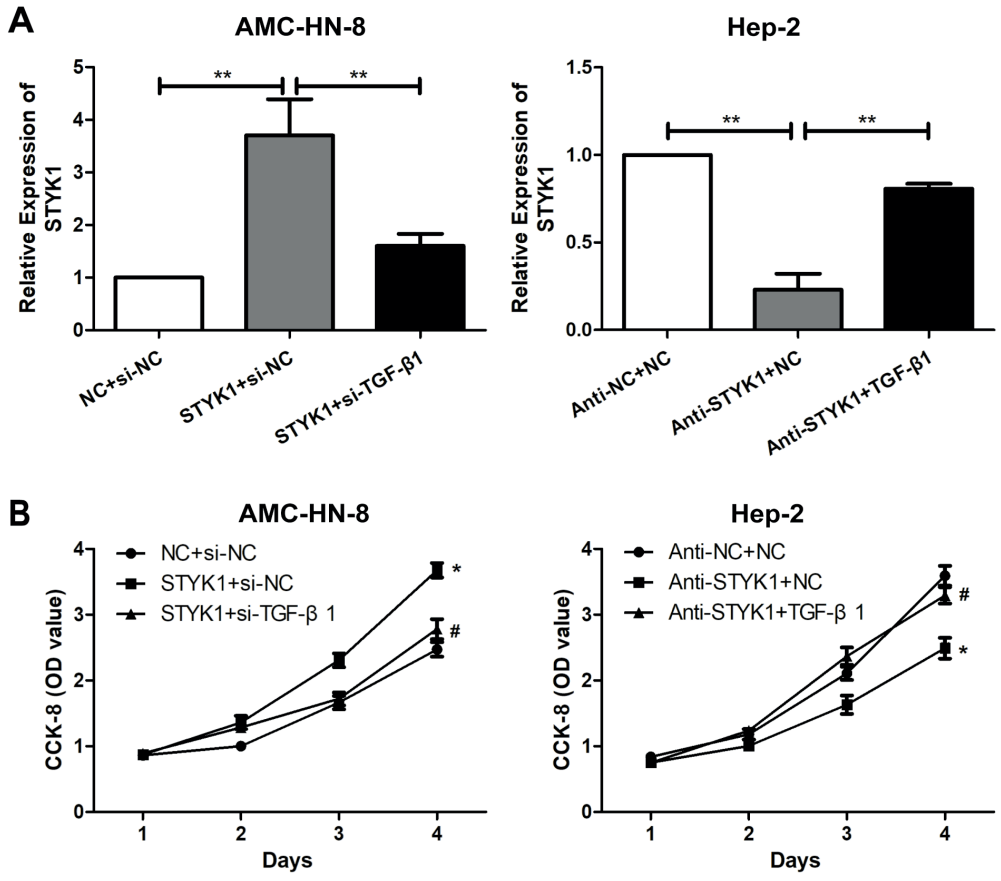

Figure 4. STYK1/TGF-β1 axis in LSCC progression. AMC-HN-8 cells were transfected with NC+si-NC, pcDNA-STYK1+-si-NC or pcDNA-STYK1+si-TGF-β1. Hep-2 cells were transfected with anti-NC+NC, anti-STYK1+NC or anti-STYK1+pcDNA-TGF-β1. **A,** STYK1 level in AMC-HN-8 and Hep-2 cells. **B,** Viability in AMC-HN-8 and Hep-2 cells. **C,** Colony number in AMC-HN-8 and Hep-2 cells. Data were expressed as mean ± SD (magnification: 10×). *#$p<0.05$, **$p<0.01$.

Figure continued

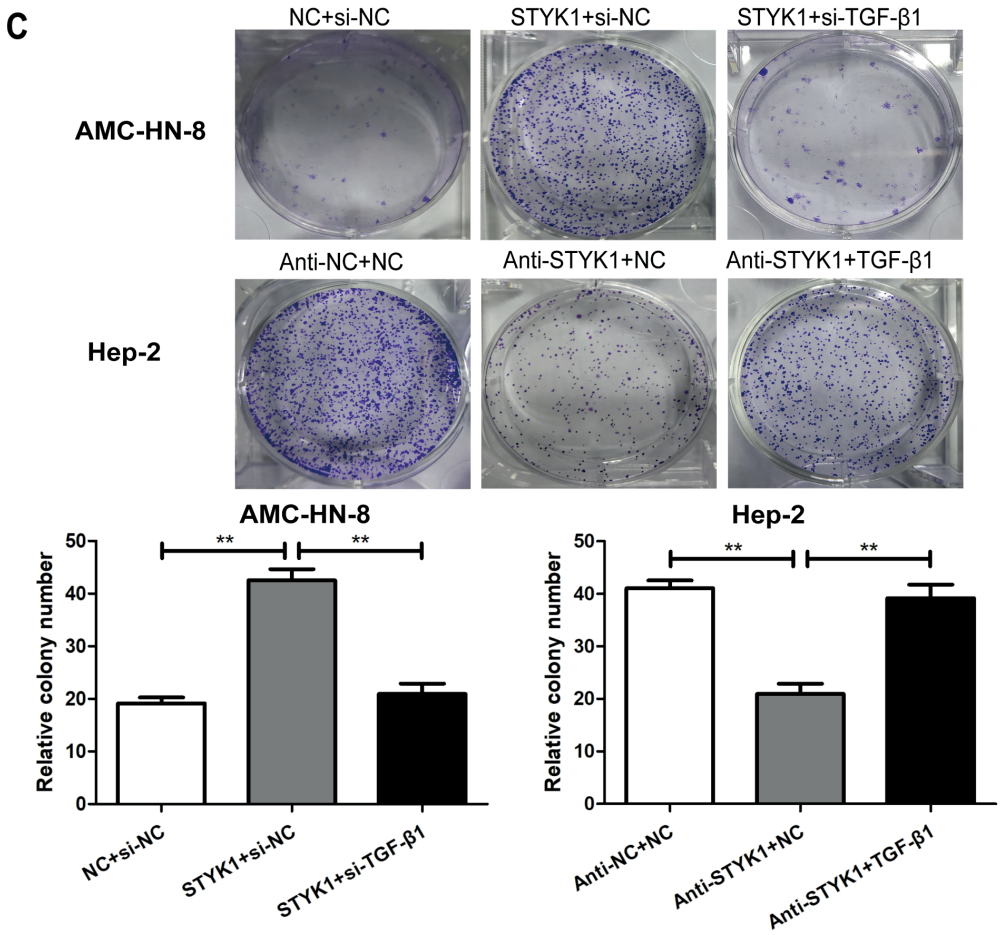

Figure 4 ***(Continued)*****. C,** Colony number in AMC-HN-8 and Hep-2 cells. Data were expressed as mean ± SD (magnification: 10×). *#$p<0.05$, **$p<0.01$.

Furthermore, STYK1 promotes the proliferative ability of LSCC cells through targeting TGF-β1, aggravating the malignant progression of LSCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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