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LINC01605 promotes the proliferation of laryngeal squamous cell carcinoma through targeting miR-493-3p

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Abstract. – OBJECTIVE: The purpose of this study was to elucidate the potential influence of LINC01605 on the progression of laryngeal squamous cell carcinoma (LSCC) and the underlying mechanism.

PATIENTS AND METHODS: LINC01605 and microRNA-493-3p (miR-493-3p) levels in normal laryngeal tissues, LSCC tissues, and paired paracancerous tissues were detected. Regulatory effects of LINC01605 on proliferative ability and apoptosis in HEp-2 and AMC-HN-8 cells were assessed. Besides, the interaction between LINC01605 and miR-493-3p was evaluated by Dual-Luciferase reporter gene assay and Spearman's rank correlation analysis. Finally, rescue experiments were conducted to clarify the role of LINC01605/miR-493-3p axis in the progression of LSCC.

RESULTS: LINC01605 was upregulated and miR-493-3p was downregulated in LSCC tissues. Knockdown of LINC01605 inhibited proliferative ability, and stimulated apoptosis in HEp-2 and AMC-HN-8 cells. Moreover, LINC01605 directly bound to miR-493-3p, and the former negatively regulated the level of the latter. In addition, miR-493-3p was able to reverse the regulatory effect of LINC01605 on proliferative ability in LSCC.

CONCLUSIONS: LINC01605 is upregulated in LSCC tissues, and it promotes the malignant progression of LSCC via targeting miR-493-3p.

Key Words:
LSCC, LINC01605, MiR-493-3p, Proliferation.

Introduction

Laryngeal carcinoma (LC) is an invasive malignancy originating in the head and neck. Pathologically, laryngeal squamous cell carcinoma (LSCC) accounts for over 95% of LC cases[1]. The incidence of LSCC ranks second in head and neck cancer[2]. Recently, the incidence of LSCC presents an increasing trend, and its mortality remains high[3]. The 5-year survival of LSCC, especially advanced or metastatic LSCC, is relatively low[4,5]. It is necessary to clarify molecular mechanism of LSCC, thus improving the clinical outcomes of LSCC patients.

Long non-coding RNAs (lncRNAs) are non-coding RNAs without protein-encoding ability. Their transcription length is over 200 nucleotides[6]. Accumulating evidence[7,8] has demonstrated critical functions of lncRNAs in influencing cell phenotypes. Besides, many lncRNAs have been reported to exert a certain impact on tumor progression[9-12]. For example, in kidney cancer, lncRNA MALAT1 stimulates the malignant invasion[13]. Overexpression of lncRNA OIP5-as1 increases proliferative rate in lung cancer cells, leading to poor prognosis[14]. In addition, the upregulation of lncRNA PTTG3P triggers growth and metastasis of hepatocellular carcinoma[15].

MicroRNAs (miRNAs) are conversely, short non-coding RNAs with about 22 nucleotides in length. A previous study has uncovered the negative regulation of miRNA on the expression of target mRNA by binding 3'UTR mRNA[16]. Moreover, differentially expressed miRNAs are extensively involved in cell proliferation[17], invasiveness[18], metastasis[19], angiogenesis[20], and drug resistance[21]. Potential influences of miRNAs on tumor progression have been highlighted[22-24].

Competitive binding of lncRNAs and miRNAs exerts an important role in the development of

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LSCC. For instance, RP11-169D4.1/miR-205-5p axis inhibits proliferative and metastatic abilities, as well as stimulates apoptosis in LSCC[25]. NEAT1 triggers invasiveness and metastasis of LSCC *via* miR-107/CDK6 axis[26]. Li et al[27] have shown that lncRNA H19/miR-148a-3p/DNMT1 axis plays an important role in LSCC tissues. The purpose of this research was to uncover the biological functions of LINC01605 in the progression of LSCC.

Patients and Methods

Patients and Samples

Normal laryngeal tissues, LSCC tissues, and paired paracancerous tissues were collected from 34 LSCC patients and stored at -80°C. Tumor staging of each patient was recorded (early stage or advanced stage). Patients and their families in this study have been fully informed, and this investigation was approved by the Ethics Committee of The Affiliated Hospital of Jiangnan University.

Cell Culture

Human LSCC cell lines (HEp-2 and AMC-HN-8) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 1% penicillin-streptomycin in a 5% CO_2 incubator at 37°C.

Cell Transfection

LINC01605 siRNAs, LINC01605 overexpression plasmid, miR-493-3p mimics and negative control were provided by British Columbia (Richmond, Canada). Cells were inoculated in 6-well plates and cultured to 60% confluence. Then, they were transfected with corresponding plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 h later, the cells were harvested for functional experiments.

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

TRIzol (Invitrogen, Carlsbad, CA, USA) was applied for isolating cellular RNA, and RNA concentration was quantified using a spectrometer. RNA was reversely transcribed into cDNA using the PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan), and SYBR Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) was utilized for qRT-PCR. Next, the relative level of the target was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences were listed as follows: LINC01605 Forward: 5′-TGTACCGCTATGGTTACACTCG-3′, Reverse: 5′-GGCAGGGACAGTTGCTTCT-3′, MiR-493-3p Forward: 5′-UGAAGGUCUACUGUGUGCCAGG-3′, Reverse: 5′-CCUGGCACACAGUAGACCUUCA-3′, GAPDH Forward: 5′-CGGAGTCAACGGATTTGGTCGTAT-3′, Reverse: 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′, and U6 Forward: 5′-GCTGAGGTGACGGTCTCAAA-3′, Reverse: 5′-GCCTCCCAGTTTCATGGACA-3′.

Cell Counting Kit-8 (CCK-8) Assay

Cells were inoculated into 96-well plates with 1×10^3 cells per well. At the appointed time points, 10 μL of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added in each well. Then, the absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

Cells were seeded in a 96-well plate with 1×10^5 cells per well. They were labeled with EdU solution in the dark for 30 min, and stained with 4',6-diamidino-2-phenylindole (DAPI) for another 30 min. Images of EdU-labeled cells, DAPI-labeled nuclei, and merged one were taken under a fluorescence microscope.

Colony Formation Assay

Cells were inoculated in 6-well plates with 800 cells per well and cultured for 10 days. Afterwards, colonies were fixed in methanol for 20 min and dyed in 1% crystal violet for 30 min. Visible colonies were finally captured.

Flow Cytometry

Cells were cultured in serum-free medium and dyed in Annexin V and prpidium iodide (PI) in the dark at room temperature for 20 min. Then, apoptosis was determined using flow cytometry (FACSCalibur; BD Biosciences, Detroit, MI, USA).

Dual-Luciferase Reporter Gene Assay

Luciferase vectors were constructed based on the binding sequences between miR-493-3p and LINC01605. Cells were co-transfected with LINC01605-wt/LINC01605-mut and miR-493-3p mimics/NC, respectively. After cell culture for 48 h, cells were lysed for mea-

suring relative luciferase activity (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) was used for data analysis. Data were expressed as mean ± SD (standard deviation). Differences between two groups were compared using the *t*-test. Correlation between expression levels of LINC01605 and miR-493-3p in LSCC tissues was analyzed by Spearman's rank correlation analysis. $p<0.05$ considered the difference was statistically significant.

Results

LINC01605 Was Upregulated in LSCC

Compared with that in 34 normal laryngeal tissues, LINC01605 was highly expressed in LSCC tissues (Figure 1A). In addition, LINC01605 was upregulated in 27/34 LSCC tissues (79.4%) compared to the paracancerous ones (Figure 1B). Tumor staging showed that LINC01605 level remained higher in 16 advanced LSCC patients relative to 18 LSCC patients in early stage (Figure 1C).

Knockdown of LINC01605 Suppressed Proliferative Ability and Stimulated Apoptosis in LSCC

Three LINC01605 siRNAs were constructed and their transfection efficacy was tested in HEp-2 and AMC-HN-8 cells (Figure 2A). Transfection of either one could downregulate LINC01605 in LSCC cells. Among them, LINC01605 siRNA-1 presented the best efficacy and was utilized for the following experiments. Subsequently, CCK-8 assay uncovered the decreased viability in HEp-2 and AMC-HN-8 cells transfected with si-LINC01605 than those of controls (Figure 2B). EdU assay consistently proved the reduced EdU-positive ratio after knockdown of LINC01605 (Figure 2C). In addition, the relative colony number was decreased in LSCC cells transfected with si-LINC01605 compared with those of controls (Figure 2D). Furthermore, flow cytometry revealed the elevated apoptotic rate in LSCC cells with LINC01605 knockdown (Figure 2E).

LINC01605 Sponged MiR-493-3p

Through bioinformatics prediction, binding sequences between LINC01605 and miR-493-3p were identified (Figure 3A). Declined luciferase activity was observed after co-transfection of LINC01605-wt and miR-493-3p mimics, verifying the binding between LINC01605 and miR-493-3p (Figure 3B). In LSCC tissues, miR-493-3p was lowly expressed compared to those of normal laryngeal tissues (Figure 3C). Meanwhile, it was downregulated in 29/34 LSCC tissues (85.3%) compared with paracancerous tissues (Figure 3D). Besides, higher abundance of miR-493-3p was observed in advanced LSCC tissues relative to those in early stage (Figure 3E). In LSCC tissues, a negative correlation was identified between expression levels of LINC01605 and miR-493-3p (R=-0.6201, $p<0.001$; Figure 3F). Lastly, transfection of si-LINC01605 markedly upregulated miR-493-3p in LSCC cells (Figure 3G).

Overexpression of LINC01605 Reversed Regulatory Effects of MiR-493-3p on Proliferative Ability in LSCC

Transfection of miR-493-3p mimics markedly reduced EdU-positive ratio and colony number in HEp-2 and AMC-HN-8 cells, and the decreased

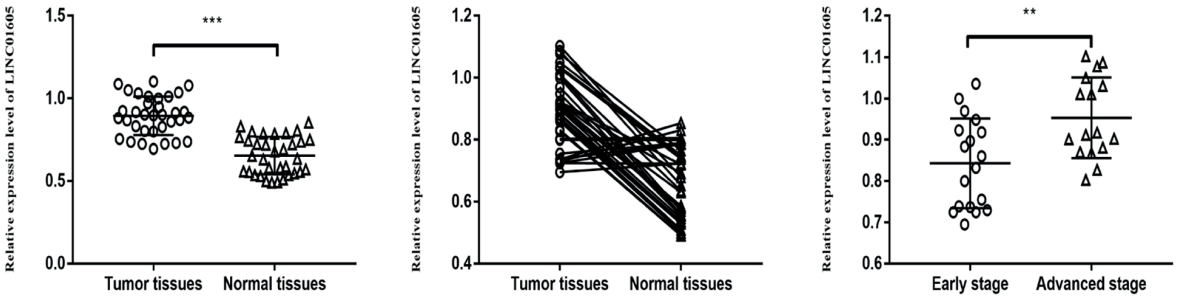

Figure 1. LINC01605 is upregulated in LSCC. **A,** LINC01605 is highly expressed in LSCC tissues compared with that of normal laryngeal tissues. **B,** LINC01605 is highly expressed in 27/34 LSCC tissues (79.4%) compared with that of paracancerous ones. **C,** LINC01605 is highly expressed in advanced LSCC tissues relative to those in early stage. $^{**}p<0.01$, $^{***}p<0.001$.

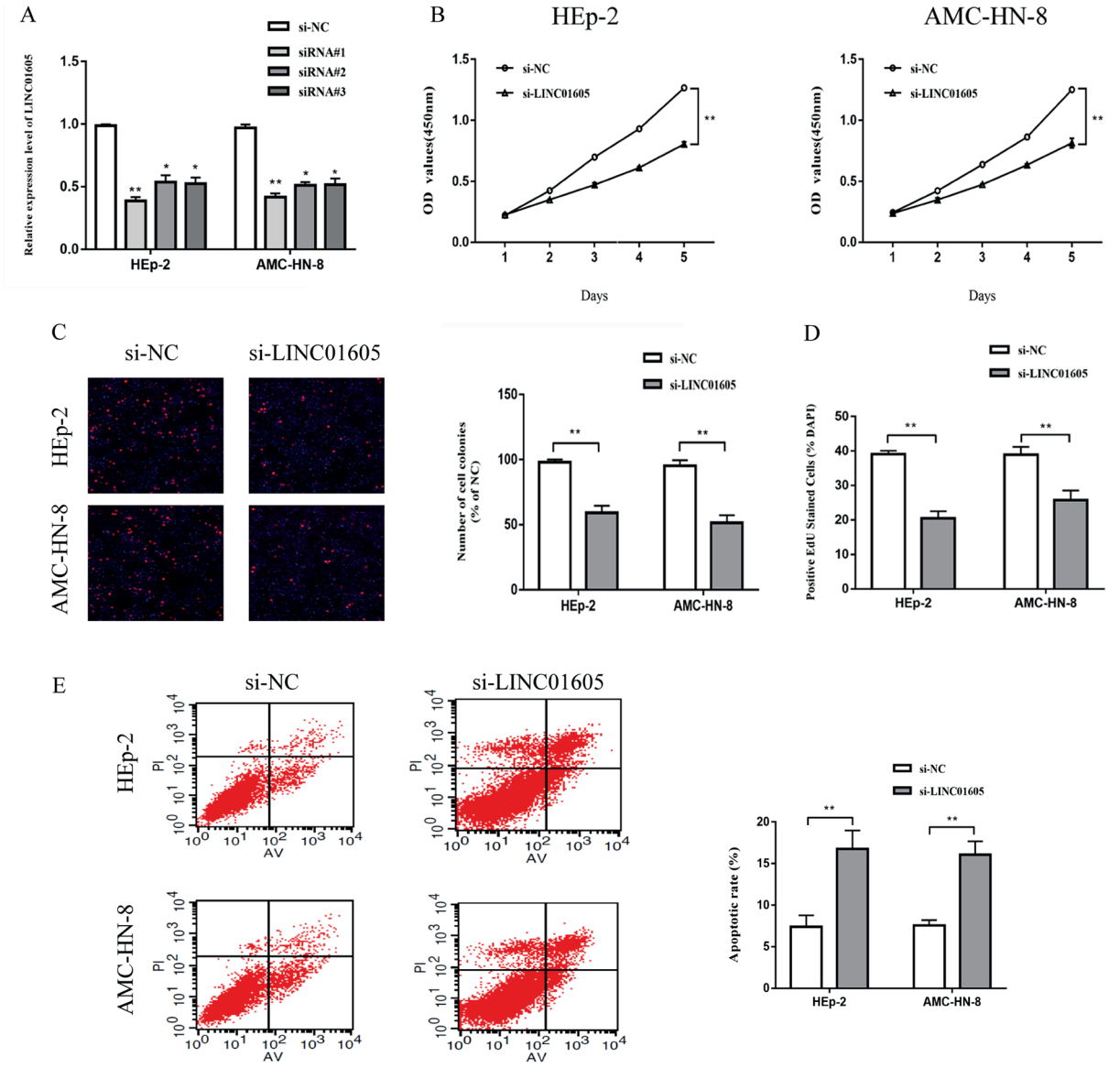

Figure 2. Knockdown of LINC01605 suppresses proliferative ability and stimulates apoptosis in LSCC. **A,** Transfection efficacy of LINC01605 siRNA (siRNA#1, siRNA#2 and siRNA#3) in HEp-2 and AMC-HN-8 cells. **B,** Regulatory effects of LINC01605 on viability in HEp-2 and AMC-HN-8 cells detected by CCK-8. **C,** Regulatory effects of LINC01605 on EdU-positive ratio in HEp-2 and AMC-HN-8 cells detected by EdU assay (magnification 10×). **D,** Regulatory effects of LINC01605 on clonality in HEp-2 and AMC-HN-8 cells detected by colony formation assay. **E,** Regulatory effects of LINC01605 on apoptosis in HEp-2 and AMC-HN-8 cells detected by flow cytometry. *p<0.05, **p<0.01

trends were partially reversed by co-transfection of LINC01605 overexpression plasmid (Figure 4A, 4B). In addition, the elevated apoptotic rate in LSCC cells overexpressing miR-493-3p was partially reversed by co-overexpression of LINC01605 (Figure 4C). Collectively, LINC01605 promoted proliferation and inhibited apoptosis in LSCC *via* negatively regulating miR-493-3p.

Discussion

Head and neck squamous cell carcinoma (HNSCC) is the sixth leading malignancy throughout the world, and LSCC takes up 30-40% of HNSCC cases[28]. LSCC is a heterogeneous disorder with a high mortality[3]. Survival rate of LSCC has been sharply improved with the progression achieved

on diagnostic and therapeutic approaches[29]. However, LSCC is a fatal and highly invasive disease[28]. Pathogenic factors of LSCC are diverse, including smoking, drinking, as well as HPV and EBV infection. Development of sensitive hallmarks for LSCC is of significance.

LncRNAs have been well concerned because of their potentials as tumor biomarkers[30]. LncRNAs are competing endogenous RNAs (ceRNAs) containing miRNA response elements (MERs), which can inhibit the regulation of miRNAs on downstream genes by competitively binding miRNA sites. For example, H19 is upregulated in liver cancer, serving as an oncogene[31]. LINC01605, also known as LINC DUSP, locates on chromosome 8p11.23, and it is found to be upregulated in bladder cancer tissues[32]. In this paper, LINC01605 was upregulated in LSCC tissues, and its level was higher in LSCC patients in advanced stage compared with those in early

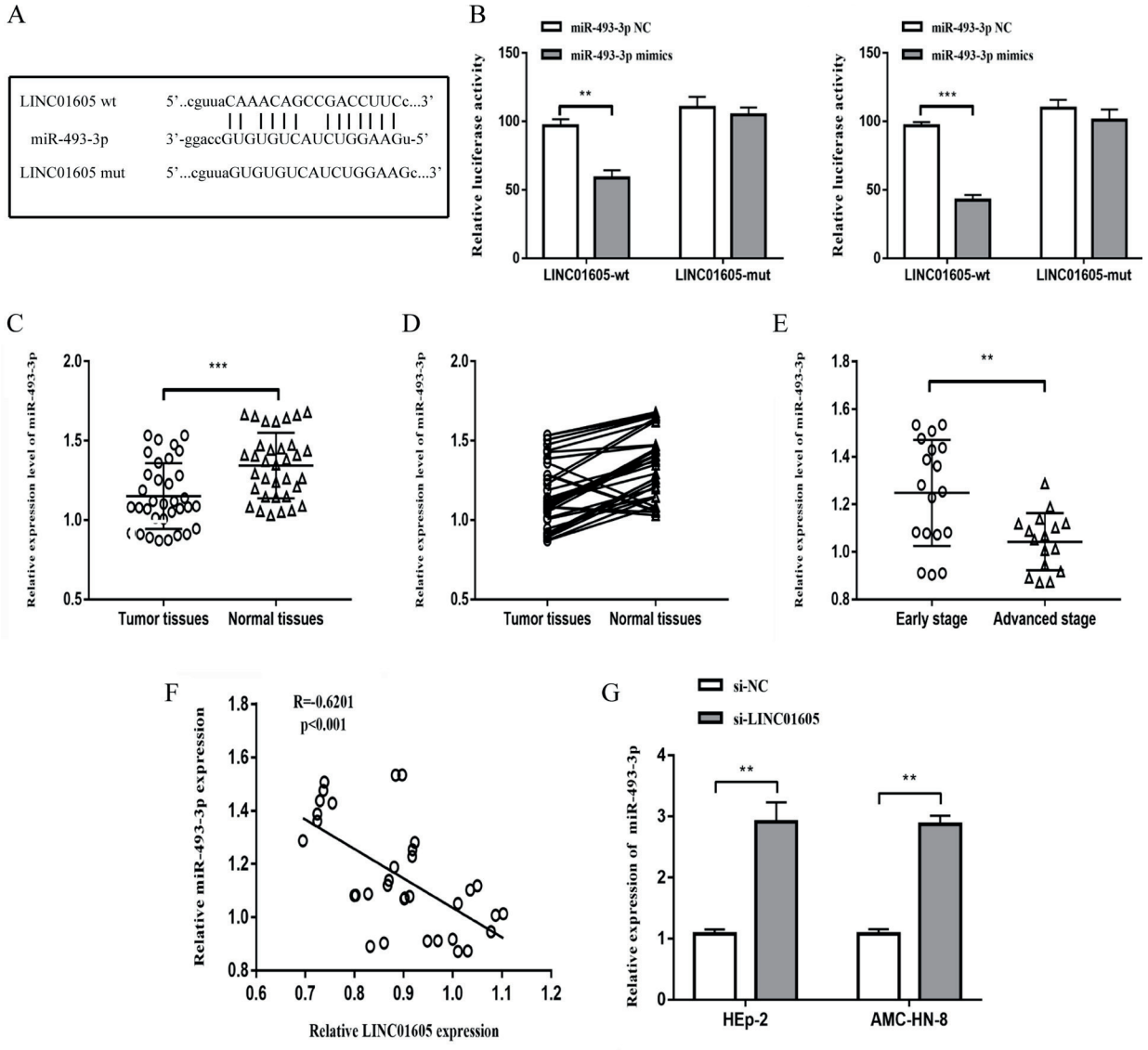

Figure 3. LINC01605 sponges miR-493-3p. **A,** Binding sequences between LINC01605 and miR-493-3p predicted by bioinformatics method. **B,** Binding relationship between LINC01605 and miR-493-3p verified by dual-luciferase reporter gene assay. **C,** MiR-493-3p is lowly expressed in LSCC tissues relative to that of normal laryngeal tissues. **D,** MiR-493-3p is lowly expressed in 29/34 LSCC tissues (85.3%) relative to that of paracancerous ones. **E,** MiR-493-3p is lowly expressed in advanced LSCC tissues relative to those in early stage. **F,** A negative correlation between expression levels of LINC01605 and miR-493-3p in LSCC tissues is assessed by Spearman's rank correlation analysis. **G,** MiR-493-3p level in HEp-2 and AMC-HN-8 cells transfected with si-NC or si-LINC01605. $^{**}p<0.01$, $^{***}p<0.001$.

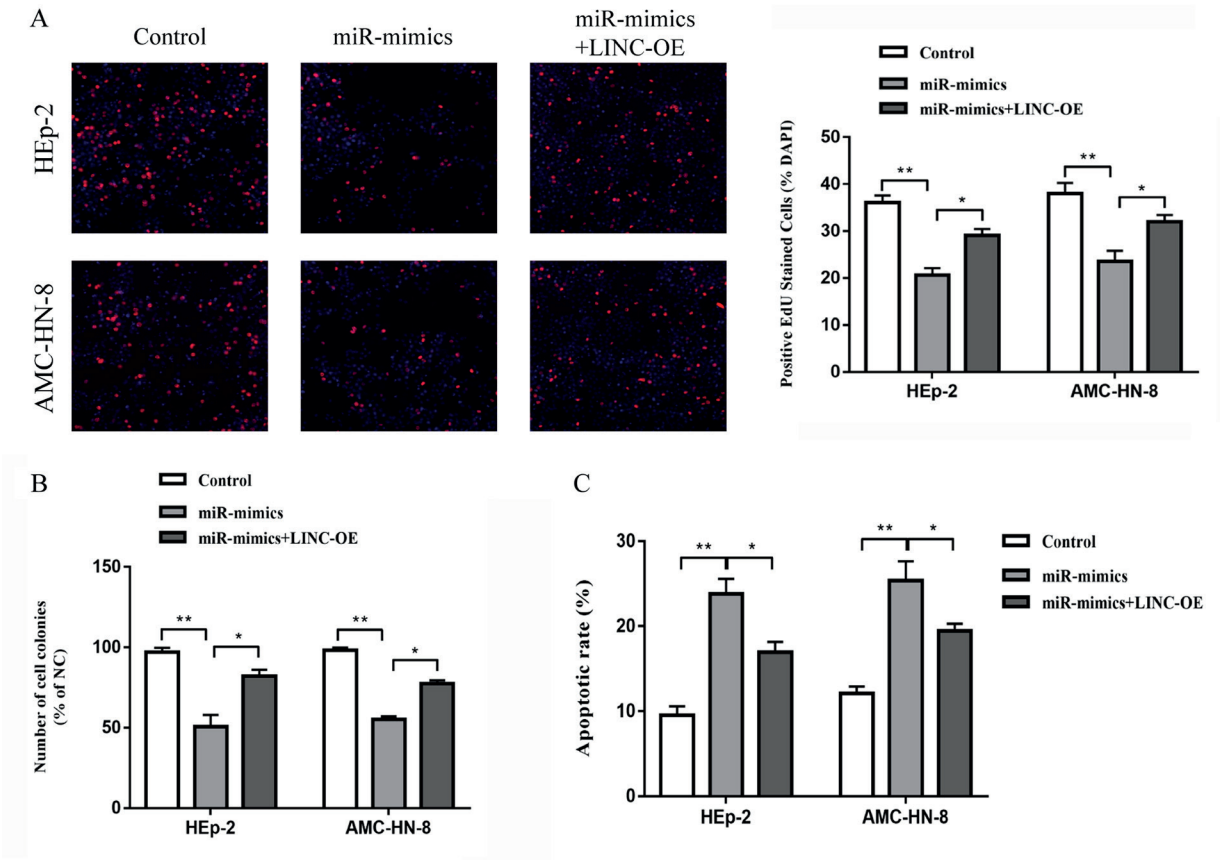

Figure 4. Overexpression of LINC01605 reverses the regulatory effects of miR-493-3p on proliferative ability in LSCC. **A,** EdU-positive ratio in HEp-2 and AMC-HN-8 cells transfected with NC, miR-493-3p mimics or miR-493-3p mimics+LINC01605 overexpression plasmid detected by EdU assay (magnification 10×). **B,** Colony number in HEp-2 and AMC-HN-8 cells transfected with NC, miR-493-3p mimics or miR-493-3p mimics+LINC01605 overexpression plasmid detected by colony formation assay. **C,** Apoptotic rate in HEp-2 and AMC-HN-8 cells transfected with NC, miR-493-3p mimics or miR-493-3p mimics+LINC01605 overexpression plasmid detected by flow cytometry. *p<0.05, **p<0.01.

stage. *In vitro* experiments suggested that knockdown of LINC01605 inhibited proliferative ability, and stimulated apoptosis in LSCC cells.

Through base pairing complementation, miRNAs degrade or inhibit translation of target mRNAs[33]. It is reported that a certain miRNA could bind up to 200 target genes with various functions involving transcription, secretory factors, receptors, and transporters[34]. In nasopharyngeal carcinoma, miR-519 is capable of suppressing the disease development. LncRNA H19 stimulates the development of LSCC *via* binding microRNA-148a-3p and DNMT1[27]. MiR-1297 promotes the progression of LSCC by binding PTEN[35]. Moreover, miR-618, miR-29a, and miR-548a are verified to be sensitive and effective indicators for predicting lymphatic metastasis of LSCC[36,37]. The findings of this study predicted that miR-493-3p was the direct gene binding LINC01605. MiR-493-3p was downregulated in LSCC and its level was negatively regulated by LINC01605. Notably, miR-493-3p was responsible for LSCC progression regulated by LINC01605.

Conclusions

LINC01605 is upregulated in LSCC tissues and it promotes the malignant progression of LSCC *via* targeting miR-493-3p. The findings of this study indicate that LINC01605 can be utilized as a therapeutic target for LSCC.

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Conflicts of interest

The authors declare no conflicts of interest.

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