TGF-β-induced long non-coding RNA *MIR155HG* promotes the progression and EMT of laryngeal squamous cell carcinoma by regulating the *miR-155-5p/SOX10* axis

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Abstract. Non-coding RNAs, particularly long non-coding RNAs (lncRNAs), play important roles in tumorigenesis. The *miR-155* host gene (*MIR155HG*) lncRNA has been found to play a crucial role in tumor progression. However, the role of *MIR155HG* in laryngeal squamous cell carcinoma (LSCC) remains unclear. Thus, the aim of the present study was to explore the roles and underlying molecular mechanisms of action of *MIR155HG* and *miR-155-5p* in LSCC, in an effort to provide novel approaches for the antitumor therapy for LSCC. In the present study, the expression levels of *miR-155-5p* and *MIR155HG* were detected by reverse transcription-quantitative polymerase chain reaction. In addition, the biological functions of *MIR155HG* and *miR-155-5p* on LSCC were evaluated *in vitro* by MTS assay, colony formation assay and Transwell assays, and *in vivo* by tumorigenesis assays. It was revealed that *MIR155HG* and *miR-155-5p* were significantly upregulated in LSCC tissues, and were associated with the TNM stage, pathological differentiation and lymph node metastasis. Moreover, the knockdown of *MIR155HG* and *miR-155-5p* inhibited the proliferation, migration and invasion of LSCC cells, whereas their overexpression exerted the opposite effects *in vitro* and *MIR155HG* overexpression promoted tumorigenesis *in vivo*. Furthermore, *MIR155HG* downregulation reduced the expression level of *miR-155-5p*. The inhibitory effect of *MIR155HG* knockdown on malignant behavior was abrogated by *miR-155-5p* overexpression. Bioinformatics analysis and luciferase reporter assay confirmed that *miR-155-5p* contributed to the progression of LSCC by directly binding to the 3' untranslated region of SRY-related-HMG-box 10 (*SOX10*). In addition, *MIR155HG* and *miR-155-5p* were upregulated by the induction of transforming growth factor-β (*TGF-β*) and promoted the expression of mesenchymal markers synergistically. On the whole, the findings of the present study indicate a novel role of *MIR155HG* in the *TGF-β*-induced EMT of LSCC cells by regulating EMT markers through the *miR-155/SOX10* axis. The *MIR155HG/miR-155-5p/SOX10* axis plays an important role in promoting the progression of LSCC and may thus serve as a potential therapeutic target for LSCC treatment.

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Abbreviations: LSCC, laryngeal squamous cell carcinoma; lncRNA, long non-coding RNA; *MIR155HG*, *miR-155* host gene; *TGF-β*, transforming growth factor-β; EMT, epithelial-to-mesenchymal transition; *SOX10*, SRY-related-HMG-box 10; 3'UTR, 3' untranslated region

Introduction

Laryngeal carcinoma is one of the most common malignant tumors among head and neck carcinomas, and >95% of laryngeal carcinomas are of the squamous cell type [laryngeal squamous cell carcinoma (LSCC)]. In 2018, there were ~177,422 new cases and ~94,771 LSCC-related deaths worldwide (1). The incidence of LSCC is increasing annually worldwide. Despite major advances in LSCC diagnosis and treatment, the prognosis of patients with advanced LSCC remains dismal. Treatment failure is most commonly attributed to recurrence and metastasis (2). Approximately 60% of patients with LSCC are already at an advanced stage (III or IV) at the time of treatment (3). Therefore, the identification of specific molecular biomarkers for early diagnosis and effective targeted therapy is crucial for the early detection, treatment and follow-up of LSCC.

With the development of high-throughput sequencing technologies, only <2% of the transcripts were found to encode proteins, whereas the vast majority were transcribed into non-coding RNAs, including microRNAs (miRNAs or miRs), small interfering RNAs (siRNAs) and long non-coding RNAs (lncRNAs) (4). lncRNAs are a class of non-coding RNAs with a length of >200 nucleotides which, although they

cannot encode proteins themselves, regulate gene expression at the transcriptional, as well as the post-transcriptional level. Furthermore, lncRNAs are involved in a number of cellular functions, such as chromatin remodeling, RNA decay, epigenetic regulation and chromatin modification (5,6), and are closely associated with tumor formation, invasion and metastasis (7,8). However, the role of lncRNAs in LSCC has not yet been fully determined. Certain lncRNAs, such as *H19* (9), *HOTAIR* (10,11), *NEAT1* (12) and *TUG1* (13), have been shown to be upregulated in laryngeal cancer cells and tissues, and may promote cancer by participating in various biological processes. The differential expression of lncRNAs was detected by microarray assays on four pairs of LSCC and adjacent normal tissues. The lncRNA, *miR-155* host gene (*MIR155HG*), was found to be upregulated in LSCC tissues. By searching biological information websites (UCSC, http://genome.ucsc.edu/), *miR-155-5p* was found to be located in the third exon of *MIR155HG*. In recent years, the inter-relationship between miRNAs and lncRNAs has become a main focus of research. lncRNAs can form the precursors of miRNAs through intracellular shear action, and certain genes can be transcribed to lncRNAs and miRNAs at the same time (14). For example, lncRNA *MIR100HG*-derived *miR-100* and *miR-125b* have been shown to participate in the resistance of colorectal cancer to cetuximab through Wnt/β-catenin signaling (15). *MIR31HG*, the host gene of *miR-31*, has been shown to contribute to the pathogenesis of Hirschsprung's disease through the *MIR31HG-miR-31/31*-ITIH5/PIK3CG* pathway (16). *MIR155HG*-derived *miR-155-5p* and *miR-155-3p* have been shown to suppress the transcription and translation of protocadherin (*PCDH*)9 and *PCDH7*, thereby promoting glioma cell migration and invasion (17). However, the roles of *miR-155-5p* and *MIR155HG* and their interaction in laryngeal cancer have not yet been fully elucidated.

Epithelial-to-mesenchymal transition (EMT) is associated with distant metastasis and tumor dissemination. Multiple growth factors and cytokines may induce EMT, and transforming growth factor (*TGF*)-β is a key factor in the induction of EMT (18). EMT has been reported to be involved in the development of LSCC. Non-coding RNAs, such as *miR-203* (19), *miR-205* and *miR-375* (20), may regulate the progression of LSCC by regulating EMT. The coding gene enhancer of zeste homolog 2 (*EZH2*) may also promote the invasion and migration of LSCC cells through EMT (21). However, whether lncRNAs are involved in the EMT of laryngeal cancer cells remains unclear.

Therefore, the aim of the present study was to detect the expression of *MIR155HG* and its exon miRNA, *miR-155-5p*, in LSCC, and to explore the potential functional roles and downstream regulatory mechanisms of the *MIR155HG/miR-155* axis in the development and progression of LSCC, as well as its role in *TGF-β*-induced EMT. The aim of this study was also to determine whether *MIR155HG* plays a carcinogenic role in LSCC, and whether it may be used as a biomarker and as a target in novel therapeutic strategies for patients with LSCC.

Materials and methods

Patients and tissue specimens. LSCC tissue samples and adjacent normal tissues were collected from 45 patients with LSCC at the Otorhinolaryngology Head and Neck Surgery Biobank of Hebei Medical University (Shijiazhuang, China) from October, 2016 to March, 2018. Informed consent was obtained from all patients, none of whom had received chemotherapy or radiotherapy prior to surgery. The use of human tissues specimens was approved by and carried out according to the guidelines of the Ethics Committee of the Second Hospital of Hebei Medical University (Shijiazhuang, China). One part of the tissue specimens was placed into RNAlater solution (CoWin Biosciences, Beijing, China) and stored at -80°C for RNA extraction. The other part of the tissue specimens was fixed in 10% neutral formaldehyde solution, and paraffin blocks were routinely prepared and preserved. Tumor and normal adjacent tissues were confirmed by routine pathological diagnosis. Agilent SBC Human (4*180K) lncRNA Microarray (ID: 74348) was used to test the transcript expression profiles in 4 pairs of LSCC and normal tissues. The clinicopathological characteristics of the 45 paired specimens are presented in Table SI.

Cell culture. Three human LSCC cell lines (TU686, TU177 and AMC-HN-8) and 293T cells were purchased from BNBIO (Beijing, China) and preserved at the Otorhinolaryngology Head and Neck Surgery Biobank of Hebei Medical University. The TU686 and TU177 cells were cultured in RPMI-1640 medium (Gibco/Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (FBS; Gibco/Thermo Fisher Scientific, Inc.). The AMC-HN-8 and 293T cells were cultured in Dulbecco's modified Eagle's medium (Gibco/Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. The TU177 cells were treated with 10 ng/ml recombinant *TGF-β* (R&D Systems, Inc., Minneapolis, MN, USA) for 7 days and the medium was replenished every 2 days. All the cells were cultured at 37°C in a humidified 5% CO_2 incubator (Thermo Fisher Scientific, Inc.).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. Total RNA was extracted from the tissues and cells using the the Eastep®Super Total RNA Extraction kit (Promega, Madison, WI, USA), and the RNA integrity was evaluated by 1% agarose gel electrophoresis (containing DEPC; Bio-Rad Laboratories, Inc., Hercules, CA, USA). cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis kit (Roche, Basel, Switzerland) following the manufacturer's protocol. RT-qPCR was performed with GoTap®qPCRMaster Mix (Promega) according to the manufacturer's instructions using CFX96™ Real-Time PCR Detection Systems. The reaction conditions were as follows (two-step method): One cycle of pre-denaturation for 2 min at 95°C, followed by 40 cycles of 15 sec at 95°C for denaturation, and for annealing to extension, selecting the most suitable annealing temperature according to different primers for 60 sec. The relative expression levels were estimated with the $2^{-\Delta\Delta Cq}$ method. Each specimen was tested 3 times. The specific primer sequences are listed in Table SII.

Cell cytoplasm and nuclear fraction isolation. Subcellular fractionation was performed with the PARIS™ Kit Protein and RNA Isolation System (Invitrogen/Thermo Fisher Scientific, Inc.) from LSCC cell lines according to the manufacturer's instructions.

Cell transfection. The 4 shRNAs specifically targeting *MIR155HG* and the control sh-NC were synthesized by Vigene Biosciences (Shandong, China). The pcDNA3.1-*MIR155HG* vector was synthesized by Sangon Biotech (Beijing, China). hsa-*miRNA-155-5p* mimic/inhibitor/negative controls were purchased from GenePharma (Shanghai, China) (Table SII). The cells were grown on 6-well plates and transfected using Lipofectamine 2000 (Invitrogen/Thermo Fisher Scientific, Inc.) when they reached 80% confluence, according to the manufacturer's instructions. At 48 h following transfection, the cells were collected for RT-qPCR verification and further experiments.

Cell proliferation assay. Cell proliferation was detected by MTS assay. Cells were seeded in 96-well plates at 2×10^3 per well following transfection for 24 h. Cell proliferation was evaluated using the CellTiter96®AQ$_{ueous}$One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. MTS reagent (20μl) was added to 100μl culture medium after seeding for 0, 24, 48, 72 and 96 h, and incubated in CO_2 incubator at 37°C for 2.5 h each time. The absorbance at 490 nm was measured using a Spark® multimode microplate reader (Tecan, Männedorf, Switzerland).

Colony formation assay. The cells were seeded in 6-well plates at a density of 2×10^3 per well following transfection for 24 h. After 12-14 days, the cells were washed twice with phosphate-buffered saline, fixed with paraformaldehyde and stained with 0.5% crystal violet solution at room temperature for 20 min. (Generay, Shanghai, China). The number of colonies (≥ 50 cells) was counted under a microscope (Olympus, Tokyo, Japan).

Transwell migration and invasion assays. For migration assays, non-Matrigel-coated chambers (Corning Costar, Corning, NY, USA) with 8-μm pore membranes were used. A total of 1×10^5 cells per well were seeded into the upper chamber and 650μl medium (10% FBS) was added to the lower chamber. Following incubation in CO_2 incubator (37°C, 5% CO_2) (Thermo Fisher Scientific, Inc.) for 24 h, the residual cells on the upper surface of the membrane were removed, the membrane was fixed in 4% paraformaldehyde for 20 min, stained with 0.5% crystal violet solution for 20 min and examined under a microscope (CKX53; Olympus, Tokyo, Japan) in 5 randomly selected fields. For invasion assays, the upper chamber was pre-coated with 50μl 1X Matrigel® Basement Membrane Matrix (Corning Costar); the remaining steps were as described above for the migration assay. For the Transwell assays of *TGF-β*-treated and untreated cells, 5×10^4 cells were seeded into the upper chamber.

Tumor xenograft model. A total of 16 male nude (BALB/c-nude) mice (age, 5 weeks; weight, 20-25 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Beijing, China; animal permit no.: [SCXY (Jing) 2016-0006]. The maintenance conditions for the mice were as follows: Temperature, 23-27°C; humidity, 40-60%; ventilation, 15 times/h; 12 h light/12 h dark cycle. The mice were fed standard laboratory food and water. The health and maintenance condition of the mice were monitored every 2 days. The mice were randomly divided into 2 groups (pcDNA3.1, and pcDNA3.1-MIR155HG; n=8 per group). The TU177 cells (5×10^6) stably transfected with pcDNA 3.1 and pcDNA3.1-*MIR155HG* in 100μl PBS were subcutaneously injected into the right flanks of the mice. Tumor volume was measured using a caliper at indicated time points and calculated using the following formula: $V = 0.5 \times$ length $\times$ width^2. After 4 weeks, the mice were sacrificed by cervical dislocation and the tumors were removed and weighed. The maximum diameter of a single tumor found was 17 mm and no mouse developed multiple tumors. All mice were in well body condition throughout the experiment. All animal experiments were performed at the Experimental Animal Center of the Fourth Hospital of Hebei Medical University according to the NIH guidelines, and were approved by the Institutional Animal Care and Use Committee of the Fourth Hospital of Hebei Medical University.

Prediction of miR-155-5p target genes. The potential downstream targets of *miR-155-5p* were predicted using TargetScan (http://www.targetscan.org/vert_72/) and miRwark (http://mirwalk.umm.uni-heidelberg.de/). *SOX10* was predicted by the two databases and considered a potential target of *miR-155-5p*. The 3'-UTR of *SOX10* mRNA containing the intact *miRNA-155-5p* recognition sequences was PCR-amplified and subcloned into the *Nhe*l and *Xba*l sites of the pmirGLO vector (Youbio, Changsha, China). Mutation sites were promoted using the Site-Directed Mutagenesis kit (New England Biolabs, Inc., Ipswich, MA, USA). Mutation primers were designed in NEBase changer (http://nebasechanger.neb.com/) and synthesized by Sangon Biotech (Table SII).

Western blot analysis. Cell protein lysates were prepared using RIPA buffer (Solarbio, Beijing, China) followed by the addition of protease inhibitor cocktail (Promega) and PMSF (Solarbio), according to the manufacturers' instructions. The protein concentration was measured using the BCA Protein Assay kit (Generay). Proteins were separated by 10% SDS-PAGE and then transferred to PVDF membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were incubated with 5% non-fat milk (Becton-Dickinson and Company, Suzhou, China) for 2 h at room temperature and incubated overnight at 4°C with rabbit-anti-human *SOX10* (molecular weight: 60 kDa) (1:2,000; cat. no. DF8009; Affinity, Cincinnati, OH, USA) and rabbit-anti-human *GAPDH* (molecular weight: 37 kDa) (1:5,000; cat. no. 10494-1-AP; Protein Tech Group, Inc., Chicago, IL, USA) antibodies. Subsequently, the membranes were incubated at room temperature with IgG (H+L) HRP (1:10,000; cat. no. RS0002; Ruiying Bio, Suzhou, China) for 1 h. Proteins were visualized with an enhanced ECL reagent (Vazyme, Nanjing, China) by ChemiDoc™ XRS+ (Bio-Rad Laboratories, Inc.).

Dual luciferase reporter assay. The TU177 and 293T cells were seeded at a density of 3×10^4 cells per well in 24-well plates. After 24 h, the cells were co-transfected with pmirGLO-SOX10-WT or pmirGLO-SOX10-MUT reporter plasmids, and *miR-155-5p* mimic or inhibitor or NC. Following transfection for 48 h, the relative luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. The luciferase activity of each well was normalized to *Renilla* luciferase activity.

Statistical analysis. The results were analyzed using SPSS version 21.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism version 7 (GraphPad Software, Inc., La Jolla, CA, USA). The differences between 2 groups were analyzed with the Student's t-test. Differences between >2 groups were determined by one-way ANOVA followed by Tukey's post hoc test. Differences between the growth of different groups, two-way ANOVA was performed and followed by Tukey's post hoc test. Pearson's correlation analysis was used to analyze the correlation between the expression of *MIR155HG* and that of *miR-155-5p*, and between the expression of *miR-155-5p* and *SOX10*. Each experiment was performed at least 3 times. All data are presented as the means ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

MIR155HG is upregulated in LSCC and is associated with advanced clinicopathological characteristics. To determine the potential role of lncRNAs in LSCC, microarray analysis was used to compare lncRNA expression levels between LSCC tissues and corresponding normal tissues. There were 3,073 differentially expressed lncRNAs in total (fold change ≥2, $P<0.05$), of which 1,967 were upregulated and 1,106 were downregulated in LSCC tissues compared with corresponding normal tissues (Fig. 1A). A miRNA host gene, *MIR155HG*, which was found to be upregulated in the microarray analysis, was selected for further analysis. To investigate the functional role of *MIR155HG* in LSCC, the expression of *MIR155HG* was initially analyzed in 45 pairs of LSCC tissues and corresponding adjacent non-tumor tissues by RT-qPCR. The expression level of *MIR155HG* was found to be significantly increased in the LSCC tissues compared with the adjacent normal tissues (Fig. 1B). The expression levels of *MIR155HG* in 3 LSCC cell lines (TU686, TU177 and AMC-HN-8) are shown in Fig. 1C. The association between *MIR155HG* expression and different clinicopathological characteristics in LSCC was further analyzed. High expression levels of *MIR155HG* were found to be closely associated with poor differentiation, lymph node metastasis and a more advanced TNM stage. However, there was no observed association between the expression of *MIR155HG* and age, alcohol consumption or smoking (Fig. 1D).

MIR155HG is located on chromosome 21q21.2, and contains 3 exons and 2 introns. *miR-155-5p* is located within the third exon of *MIR155HG* (Fig. 1E). The coding potential of *MIR155HG* was analyzed by using several prediction software programs, and the results revealed that the open reading frame (ORF) of *MIR155HG* was very short, containing only 82 amino acids (https://www.ncbi.nlm.nih.gov/orffinder/), and had no coding potential (http://lilab.research.bcm.edu/cpat/) (Fig. 1F and G). These results indicated that *MIR155HG* is a non-coding RNA. Furthermore, *MIR155HG* was found to be predominantly located in the nucleus of LSCC cells by subcellular fractionation assay (Fig. 1H).

Knockdown of MIR155HG suppresses LSCC cell proliferation, migration and invasion. To explore the biological function of *MIR155HG*, shRNAs against *MIR155HG* (sh-*MIR155HG*) were transfected into the LSCC cells to knock down endogenous *MIR155HG* expression. Due to the poor transfection efficiency,

the TU686 cells, which had a relatively higher expression level of *MIR155HG*, were not selected for the *in vitro* assay. The TU177 and AMC-HN-8 cells, with a relatively higher transfection efficiency, were used as model cells for the follow-up experiments. sh-MIR155HG-1, which exhibited the most evident knockdown efficacy, was selected for use in further experiments (Fig. 2A).

MTS and colony formation assays were used to measure cell proliferation. *MIR155HG* knockdown suppressed TU177 and AMC-HN-8 cell proliferation (Fig. 2B). Consistent with the results of MTS assay, the results of colony formation assay also revealed that the downregulation of *MIR155HG* suppressed the colony-forming ability of the TU177 and AMC-HN-8 cells (Fig. 2C). These data provide evidence to suggest the tumor growth-promoting role of *MIR155HG in vitro*.

In order to examine the effects of *MIR155HG* on the malignant biological properties of LSCC cells, the cell migratory and invasive abilities were evaluated using Transwell Matrigel migration and invasion assays. The migration and invasion capacities of the TU177 and AMC-HN-8 cells were found to be markedly decreased when *MIR155HG* was knocked down (Fig. 2D and E). Taken together, these data demonstrated that *MIR155HG* suppressed the proliferation, migration and invasion of LSCC cells.

Overexpression of MIR155HG promotes LSCC cell proliferation, migration and invasion in vitro and promotes tumorigenesis in vivo. Subsequently, in order to assess the biological effects of *MIR155HG* overexpression, the pcDNA3.1-*MIR155HG* expression plasmid was transfected into the TU177 and AMC-HN-8 cells with relatively low expression levels of *MIR155HG*. pcDNA3.1 was used as a control. The relative expression level of *MIR155HG* in the pcDNA3.1-*MIR155HG*-transfected TU177 and AMC-HN-8 cells was markedly increased (Fig. 3A). MTS (Fig. 3B) and colony formation assays (Fig. 3C) demonstrated that *MIR155HG* upregulation promoted TU177 and AMC-HN-8 cell proliferation. Moreover, *MIR155HG* overexpression enhanced the migratory and invasive capacity of both cell lines (Fig. 3D and E). All these results indicated that the overexpression of *MIR155HG* increased the tumorigenicity of LSCC cells. To further verify the results of the *in vitro* experiments, animal experiments were conducted. A TU177 cell line stably transfected with pcDNA3.1 and pcDNA3.1-*MIR155HG* was constructed, and 5x10^{6} cells were subcutaneously injected into nude mice. The results demonstrated that the overexpression of *MIR155HG* significantly promoted tumor growth compared with the pcDNA3.1 group (Fig. 3F and G), whereas the volume and weight of the tumors were higher in the *MIR155HG* overexpression group (Fig. 3H and I).

miR-155-5p is upregulated in LSCC and promotes cancer cell proliferation, migration and invasion. As *MIR155HG* is the host gene of *miR-155-5p*, we further investigated whether *miR-155-5p* exerts the same effects as *MIR155HG* on LSCC. The expression of *miR-155-5p* was found to be significantly increased in the LSCC tissues compared with the adjacent normal tissues (Fig. 4A). The high expression level of *miR-155-5p* was found to be closely associated with the TNM
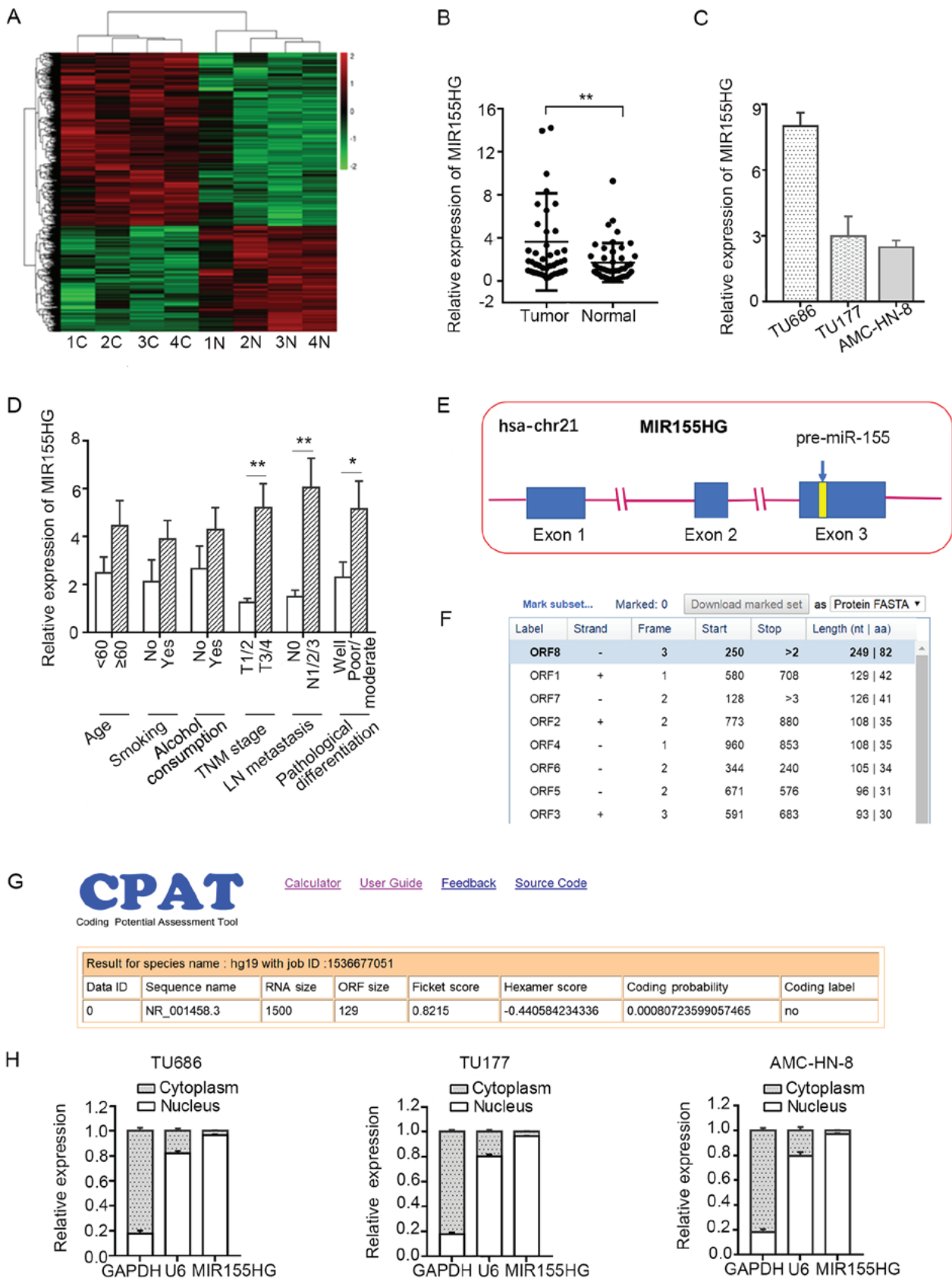
Figure 1. *MIR155HG* is upregulated in LSCC and is associated with advanced clinicopathological characteristics. (A) Heatmap of differentially expressed lncRNAs in 4 pairs of LSCC tissues and corresponding normal tissues. (B) The relative expression of *MIR155HG* in 45 pairs of LSCC tissues and the corresponding adjacent non-tumor tissues by RT-qPCR. (C) The expression of *MIR155HG* in 3 LSCC cell lines (TU686, TU177 and AMC-HN-8). (D) The association between *MIR155HG* expression and different clinicopathological characteristics. (E) The genetic structure schematic map of MIR155HG. *Homo sapiens* MIR155 host gene (*MIR155HG*) (NR_001458) is located on chromosome 21q21.2, contains 3 exons and 2 introns. MiR-155-5p is located within the third exon of *MIR155HG*. (F and G) The coding potential of *MIR155HG* was analyzed using several prediction software, and the results showed *MIR155HG* did not have any coding potential. (H) The location of *MIR155HG* in LSCC cells. *U6* and GAPDH were used as nuclear and cytoplasm control respectively. Data are shown as mean ± SD; *P<0.05 and **P<0.01. LSCC, laryngeal squamous cell carcinoma; *MIR155HG*, MIR155 host gene; miR, microRNA; C, cancer; N, normal.
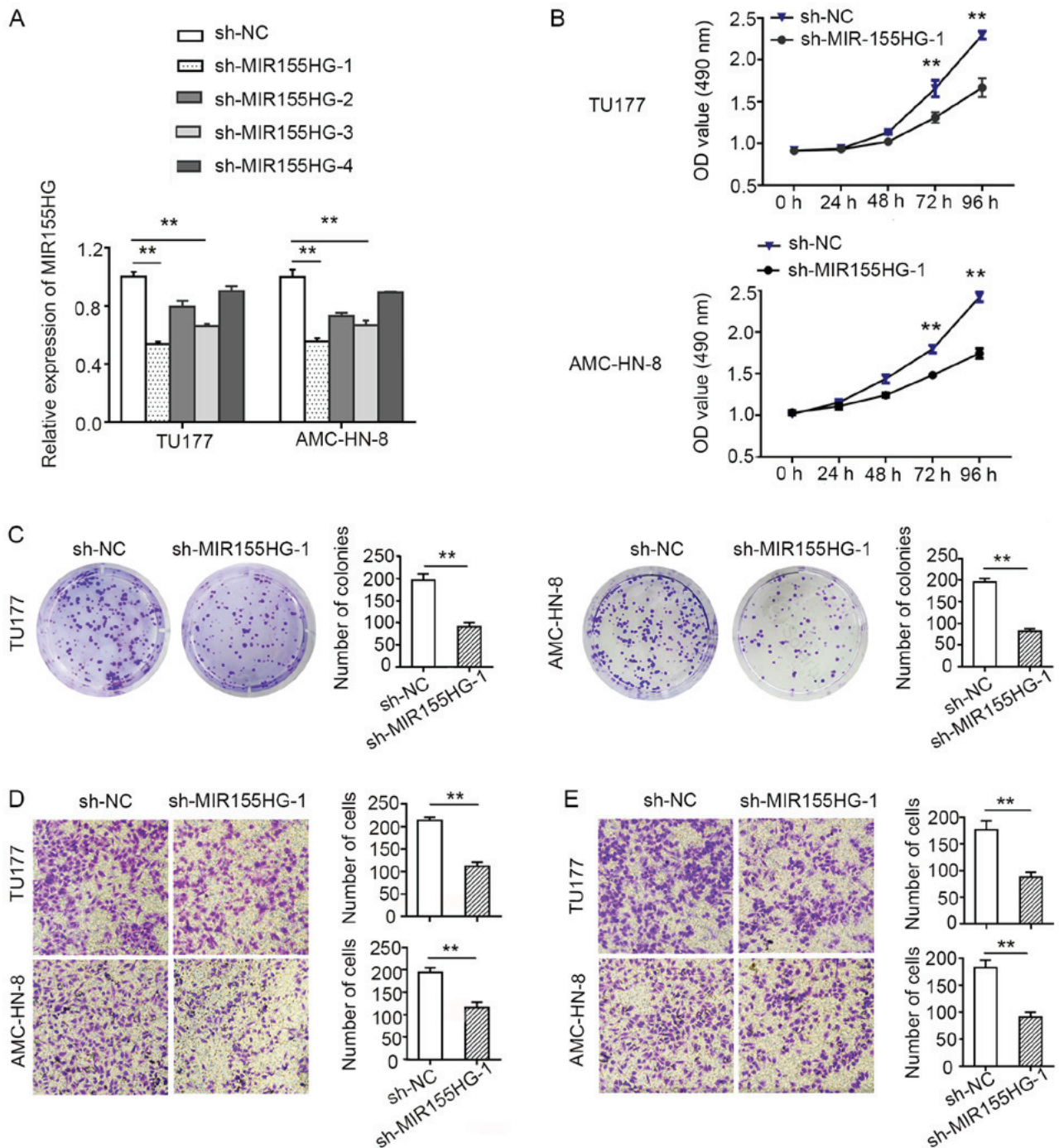
Figure 2. Knockdown *MIR155HG* suppresses LSCC cell proliferation, migration and invasion. (A) RT-qPCR was carried out to analyze the expression of *MIR155HG* in TU177 and AMC-HN-8 cells after transfected with four shRNAs against *MIR155HG* and one negative control. (B) Growth curves of TU177 and AMC-HN-8 cells following transfection with sh-MIR155HG or NC were determined by MTS assays. (C) Colony formation assay following the downregulation of *MIR155HG* in TU177 and AMC-HN-8 cells. (D) Transwell migration assays of TU177 and AMC-HN-8 cells with *MIR155HG* knockdown (magnification, x200). (E) Transwell invasion assays of TU177 and AMC-HN-8 cells with *MIR155HG* knockdown (magnification, x200). Data are shown as the means ± SD; **P<0.01. LSCC, laryngeal squamous cell carcinoma; *MIR155HG*, MIR155 host gene; NC, negative control; shRNA, short hairpin RNA.

stage, lymph node metastasis and poor differentiation. However, there was no association between the expression of *miR-155-5p* and age, alcohol consumption, or smoking (Fig. 4B).

To further determine whether *miR-155-5p* is associated with the progression of LSCC, *miR-155-5p* mimics and inhibitor were transfected into the TU177 and AMC-HN-8 cells (Fig. 4C). As shown in Fig. 4D-F, the overexpression of *miR-155-5p* markedly promoted the proliferation, migration and invasion of TU177 and AMC-HN-8 cells, whereas the

downregulation of *miR-155-5p* markedly inhibited cell proliferation, migration and invasion. Collectively, these data suggest that *miR-155-5p* acts as an oncogenic miRNA by promoting LSCC cell proliferation, migration and invasion.

Overexpression of miR-155-5p reverses the inhibitory effects of MIR155HG knockdown on LSCC cell proliferation, migration and invasion. The expression level of *miR-155-5p* was found to highly correlate with the expression of *MIR155HG* (Fig. 5A).
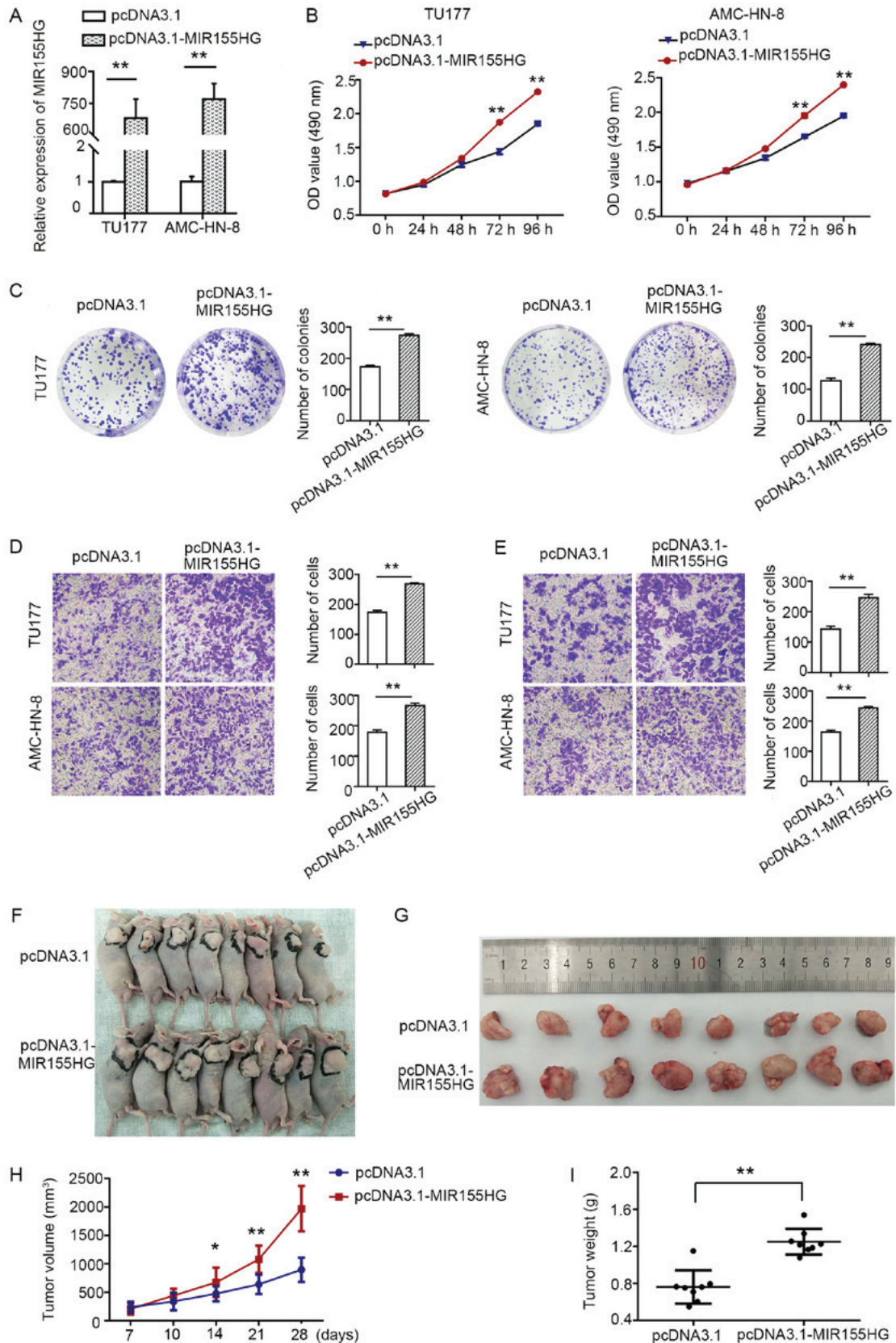
Figure 3. Overexpression of MIR155HG promotes LSCC cell proliferation, migration, and invasion *in vitro* and promotes tumorgenesis *in vivo*. (A) RT-qPCR was carried out to analyze the expression of *MIR155HG* in TU177 and AMC-HN-8 cells following transfection with pcDNA3.1-MIR155HG and pcDNA3.1. (B) Growth curves of TU177 and AMC-HN-8 cells after transfected with pcDNA3.1-MIR155HG and pcDNA3.1 were determined by MTS assay. (C) Colony formation assay following the upregulation of *MIR155HG* in TU177 and AMC-HN-8 cells. (D) Transwell migration assays of TU177 and AMC-HN-8 cells with *MIR155HG* overexpression. (magnification, x200). (E) Transwell invasion assays of TU177 and AMC-HN-8 cells with *MIR155HG* overexpression. (magnification, x200). (F) External whole-body images of mice 4 weeks after subcutaneous injection. (G) Representative images of tumors collected from mice 4 weeks after subcutaneous injection. (H) Growth curves of xenograft tumors following the subcutaneous injection of TU177 cells stably overexpressing *MIR155HG* or negative control. The tumor volumes were measured each week after inoculation. (I) Tumor weights of xenograft tumors after subcutaneous injection of TU177 cells for 4 weeks. Data are shown as the means ± SD; **P<0.01. LSCC, laryngeal squamous cell carcinoma; MIR155HG, MIR155 host gene.
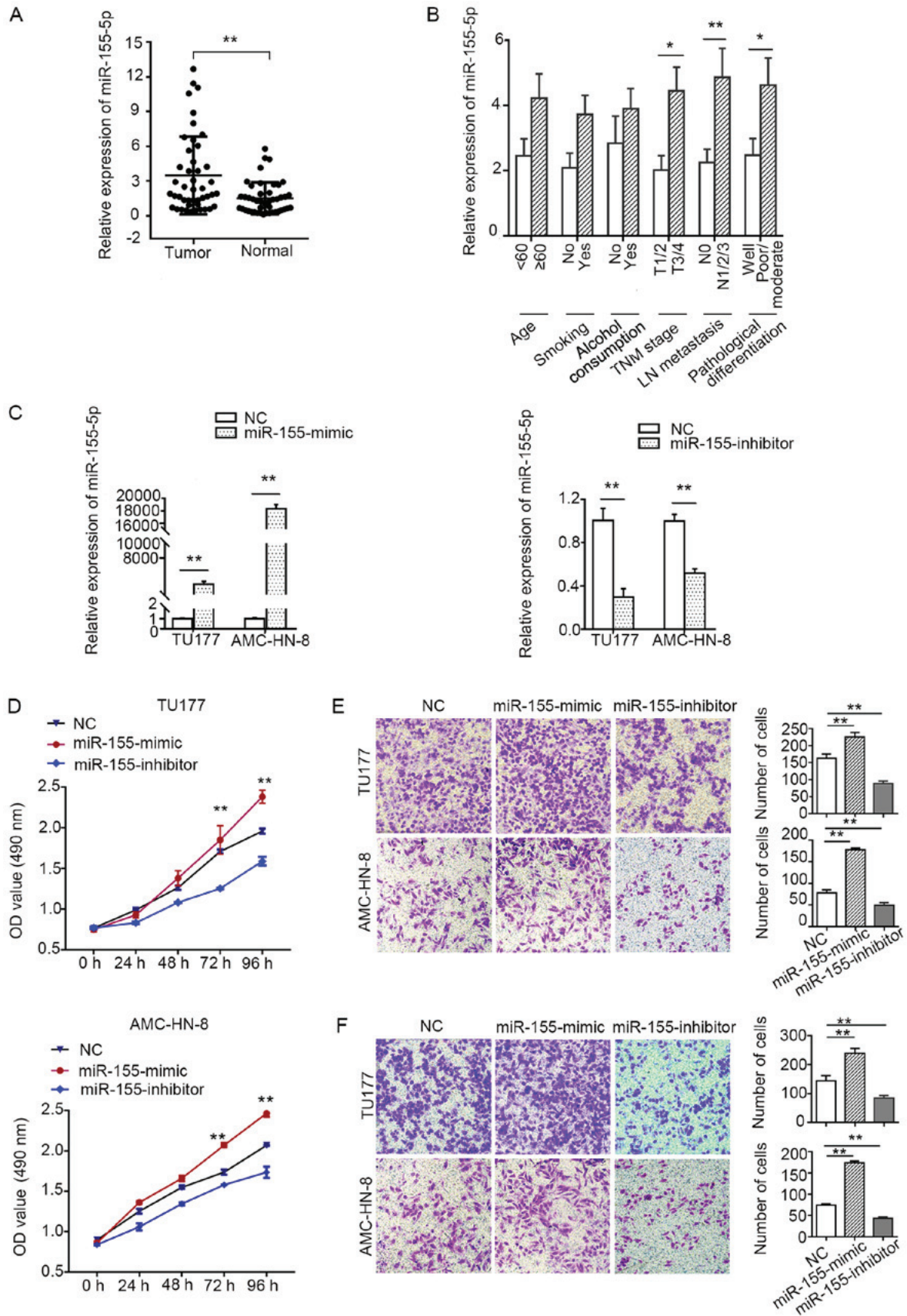
Figure 4. *miR-155-5p* is upregulated in LSCC and promotes LSCC cell proliferation, migration and invasion. (A) The relative expression of *miR-155-5p* in 45 pairs of LSCC tissues and the corresponding adjacent non-tumor tissues examined by RT-qPCR. (B) The association between relative expression of *miR-155-5p* and different clinicopathological characteristics. (C) The relative of expression of *miR-155-5p* in TU177 and AMC-HN-8 cells following transfection with *miR-155-5p* mimic and inhibitor by RT-qPCR. (D) Growth curves of TU177 and AMC-HN-8 cells following transfection with *miR-155-5p* mimic and inhibitor determined by MTS assays. (E) Migration assays and (F) invasion assays of TU177 and AMC-HN-8 cells with *miR-155-5p* knockdown and overexpression. (magnification, x200). Data are shown as the means ± SD; *P<0.05 and **P<0.01 vs. NC group. LSCC, laryngeal squamous cell carcinoma; NC, negative control; miR, microRNA.
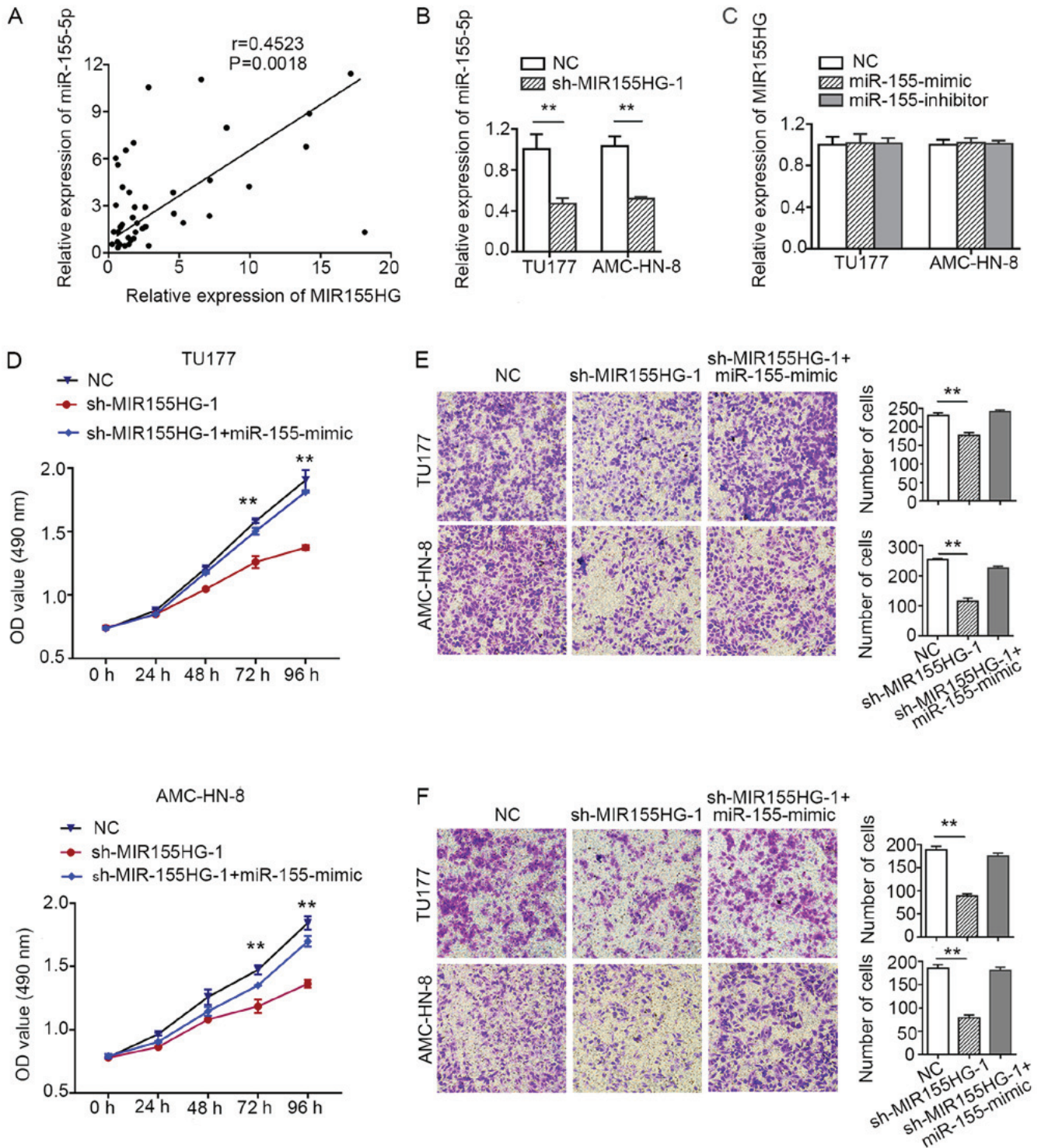
Figure 5. Overexpression of *miR-155-5p* reverses the inhibition of LSCC cell proliferation, migration and invasion induced by *MIR155HG* knockdown. (A) The correlation of the relative expression level of *miR-155-5p* and *MIR155HG* (r=0.4523, P=0.0018). (B) The relative expression of *miR-155-5p* following transfection with *MIR155HG* shRNA in TU177 and AMC-HN-8 cells. (C) The relative expression of *MIR155HG* following transfection with *miR-155-5p* mimic and inhibitor in TU177 and AMC-HN-8 cells. (D) Growth curves of TU177 and AMC-HN-8 cells following co-transfection with *miR-155-5p* mimic and *MIR155HG* shRNA determined by MTS assays. (E) Migration assays and (F) invasion assays of TU177 and AMC-HN-8 cells following co-transfection with *miR-155-5p* mimic and *MIR155HG* shRNA (magnification, x200). Data are shown as the means ± SD; **P<0.01 vs. NC group. LSCC, laryngeal squamous cell carcinoma; *MIR155HG*, MIR155 host gene; NC, negative control; shRNA, short hairpin RNA; miR, microRNA.

These findings suggested that *miR-155-5p* and its host gene, *MIR155HG*, may be co-expressed in LSCC. The downregulation of *MIR155HG* reduced the expression levels of *miR-155-5p* in the TU177 and AMC-HN-8 cells (Fig. 5B). However, the expression of *MIR155HG* was not affected by *miR-155-5p* knockdown or overexpression in LSCC cells (Fig. 5C). Overexpression of *miR-155-5p* may abrogate the inhibitory effect of *MIR155HG* knockdown on cell proliferation, migration and invasion (Fig. 5D-F). These results indicated that *miR-155-5p* may act synergistically with *MIR155HG* to promote LSCC progression.
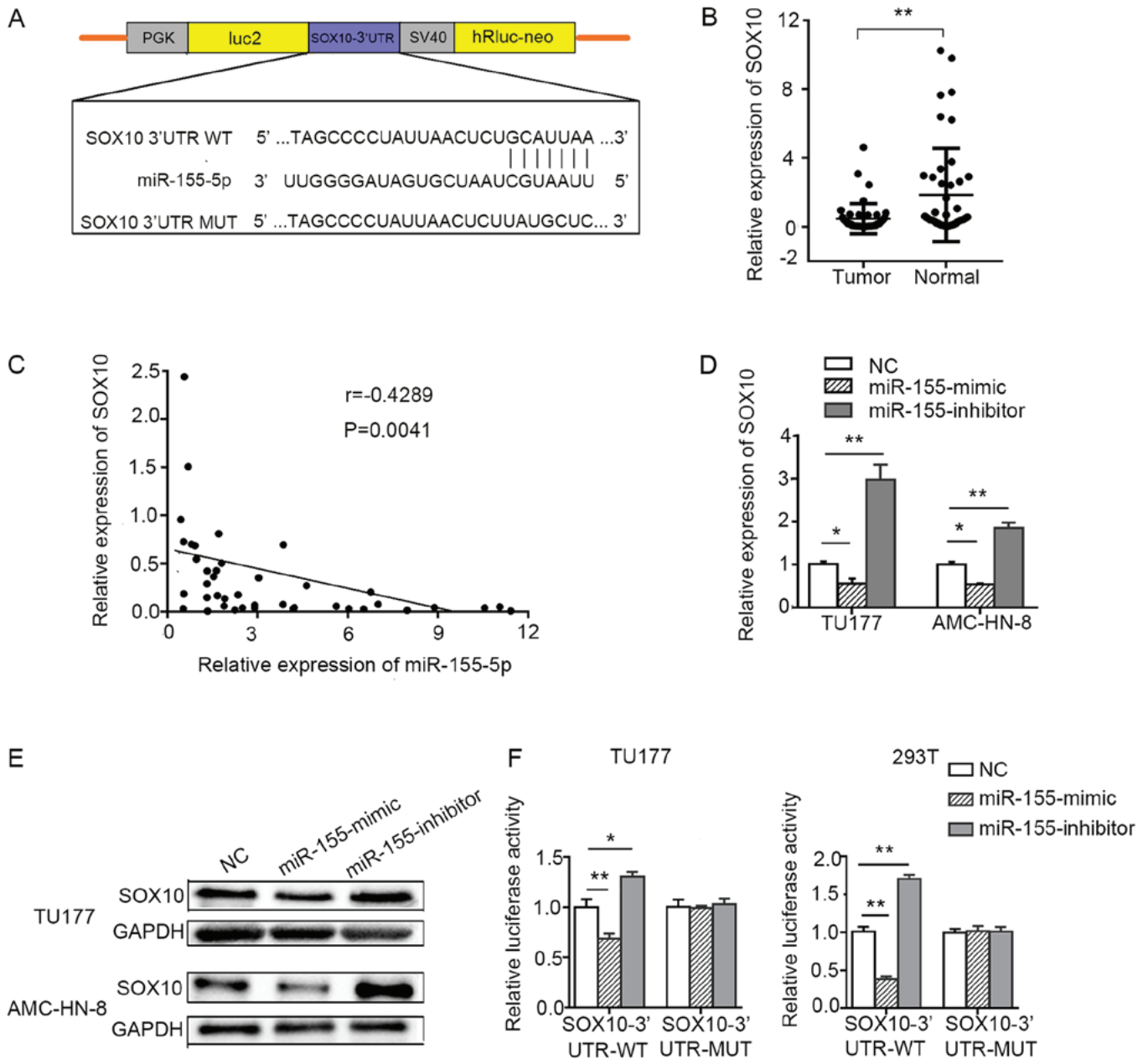
Figure 6. miR-155-5p directly targets SOX10 in LSCC cells. (A) Schematic of the potential binding sites of *miR-155-5p* on SOX10 3'UTR predicted by TargetScan and miRwark databases. (B) The relative expression of *SOX10* in 45 pairs of LSCC tissues and the corresponding adjacent non-tumor tissues by RT-qPCR. (C) The correlation between *miR-155-5p* and *SOX10* mRNA level in LSCC tissues. (D) The relative expression level of *SOX10* mRNA following transfection with *miR-155-5p* mimic and inhibitor in TU177 and AMC-HN-8 cells by RT-qPCR. (E) The relative protein level of *SOX10* following transfection with *miR-155-5p* mimic and inhibitor in TU177 and AMC-HN-8 cells by western blot analysis. (F) Luciferase assays of TU177 cell and 293T cell transfected with pmirGLO-SOX10-WT or pmirGLO-MIR31HG-MUT and NC or *miR-155-5p* mimic or *miR-155-5p* inhibitor. Data are shown as the means ± SD; *P<0.05 and **P<0.01 vs. NC group. LSCC, laryngeal squamous cell carcinoma; NC, negative control; *MIR155HG*, MIR155 host gene; miR, microRNA; 3' untranslated regions, 3'UTR; *SOX10*, SRY related-HMG-box 10; WT, wild-type; Mut, mutant type.

miR-155-5p directly targets SOX10 in LSCC cells. To further explore the mechanisms underlying the effects of *miR-155-5p* on the proliferation, migration and invasion of LSCC cells, bioinformatics tools, such as TargetScan and miRwalk, were used to search for potential target genes of *miR-155-5p*. Among these target genes, we focused on *SOX10*, a tumor suppressor gene that is involved in suppressing tumorigenicity (22) and EMT (23). TargetScan prediction revealed that the 3'UTR of *SOX10* contains a conserved binding site for *miR-155-5p* (Fig. 6A). The expression level of *SOX10* was markedly decreased in the LSCC tissues compared with the corresponding adjacent non-tumor tissues (Fig. 6B). The expression level of *miR-155-5p* negatively correlated with the expression of *SOX10* in LSCC (Fig. 6C). The downregulation of *miR-155-5p* by transfection with *miR-155-5p* inhibitor markedly increased the transcriptional level and protein expression of *SOX10*, while the overexpression of *miR-155-5p* significantly decreased the transcriptional level and protein expression of *SOX10* in LSCC cell lines (Fig. 6D and E). To confirm whether the *SOX10* mRNA expression is regulated by *miR-155-5p* through direct binding to the 3'UTR of *SOX10*, a dual luciferase reporter assay was performed in the TU177 and
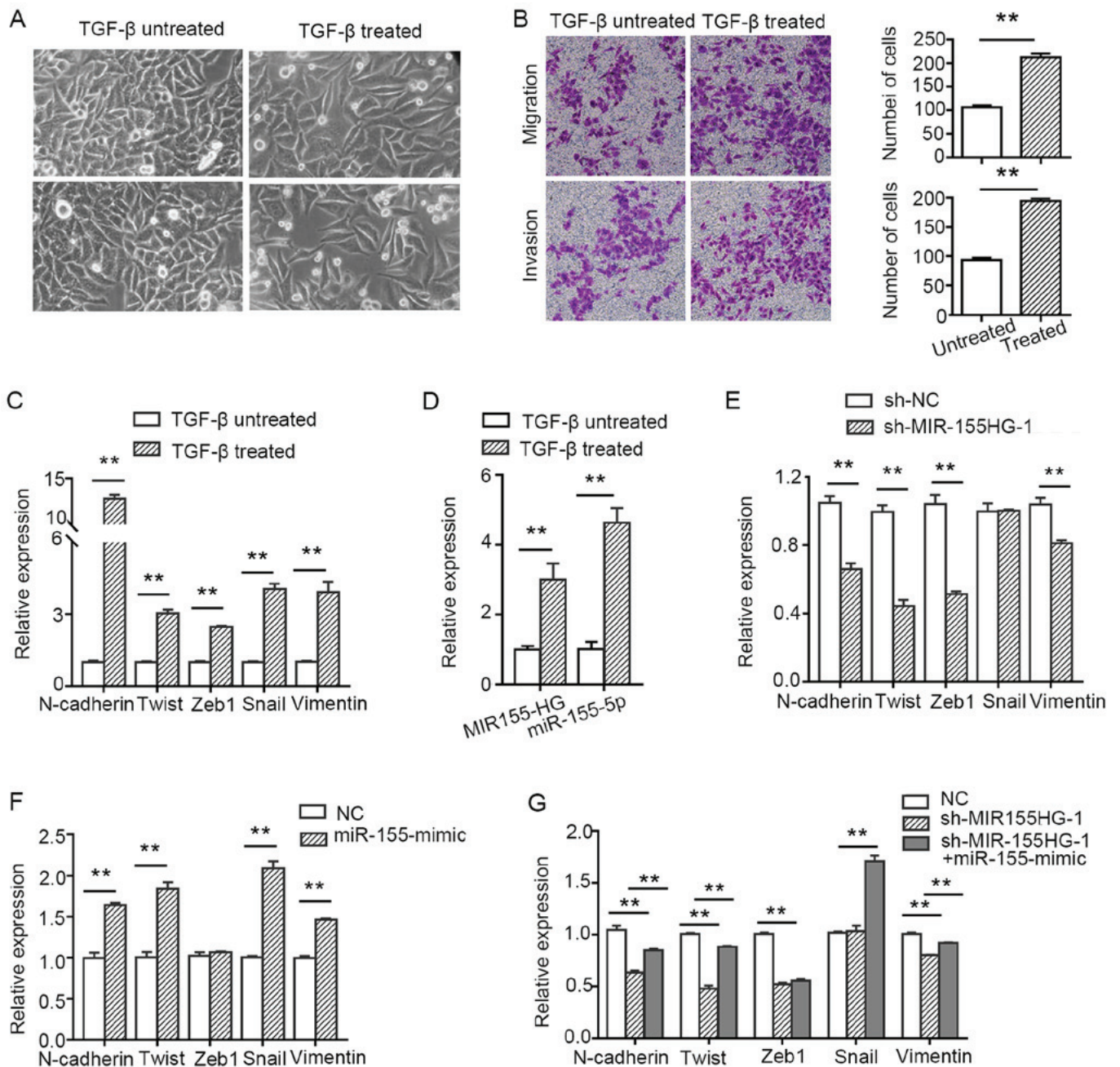
Figure 7. *MIR155HG* and *miR-155-5p* are upregulated in *TGF-β*-induced TU177 cell and contribute to the EMT process synergistically. (A) Cell morphology of TU177 cell treated or untreated with *TGF-β* for 7 days. (B) The migration and invasion assays of TU177 cell treated or untreated with *TGF-β* (magnification, x200). **P<0.01 vs. untreated group (C) The relative expression of mesenchymal related markers following induction by *TGF-β* in TU177 cells by RT-qPCR. **P<0.01 vs. untreated group. (D) The relative expression of *MIR155HG* and *miR-155-5p* following induction by *TGF-β* in TU177 cells by RT-qPCR. **P<0.01 vs. untreated group. (E) The expression of mesenchymal-related markers following transfection with sh-MIR155HG by RT-qPCR. **P<0.01 vs. NC group. (F) The relative expression of mesenchymal-related markers following transfection with *miR-155-5p* mimic by RT-qPCR. **P<0.01 vs. NC group. (G) The relative expression of mesenchymal-related markers by RT-qPCR following co-transfection with sh-MIR155HG and miR-155-mimic. (sh-MIR155HG-1 group vs. NC group; co-transfected group vs. sh-MIR155HG-1 group). Data are shown as the means ± SD; *MIR155HG*, MIR155 host gene; miR, microRNA; NC, negative control; shRNA, short hairpin RNA; *TGF-β*, transforming growth factor-β; EMT, epithelial-mesenchymal transition.

293T cells. The luciferase signal was reduced in the *miR-155-5p* mimic and pmirGLO-SOX10-3'UTR-wild-type co-transfected cells compared with the control group. However, co-transfection of *miR-155* mimic and pmirGLO-SOX10-3'UTR-mutant-type did not reduce the luciferase signal compared with the control group. The luciferase signal was increased in the *miR-155-5p* inhibitor and pmirGLO-SOX10-3'UTR-wild-type, but not the mutant-type, co-transfected cells compared with the control group (Fig. 6F).

MIR155HG and miR-155-5p are upregulated in TGF-β-induced TU177 cells and synergistically contribute to the process of EMT. To investigate whether *MIR155HG* and *miR-155-5p* regulate the migration and invasion of LSCC cells via EMT, the TU177 cells were treated with 10 ng/ml of *TGF-β* for 7 days. The cells treated with *TGF-β* exhibited a change in morphology and acquired a spindle-shaped morphology (Fig. 7A). Furthermore, their migratory and invasive abilities were enhanced compared with the untreated cells (Fig. 7B). Subsequently, the molecular

markers of EMT were analyzed by RT-qPCR. The levels of the mesenchymal markers, N-cadherin, Twist, Zeb1, Snail and vimentin, were found to be upregulated. However, we did not detect the expression of the epithelial marker, E-cadherin (after using several common primers of E-cadherin), possibly due to the fact that the expression abundance was too low to be monitored (Fig. 7C). These results suggested that the TU177 cells treated with *TGF-β* exhibited EMT-related characteristics. Moreover, to further determine whether *MIR155HG* and *miR-155-5p* were involved in *TGF-β*-induced EMT, their expression was detected by RT-qPCR analysis. The results indicated that the levels of *MIR155HG* and *miR-155-5p* were upregulated after the TU177 cells were treated with *TGF-β* (Fig. 7D). The knockdown of *MIR155HG* inhibited the expression of the mesenchymal markers, N-cadherin, vimentin, Twist and Zeb1, but did not affect the expression of Snail (Fig. 7E). *miR-155-5p* overexpression promoted the expression of the mesenchymal markers, N-cadherin, vimentin, Twist and Snail, but did not increase the expression of Zeb1 (Fig. 7F), whereas it partially reversed the inhibitory effect exerted by *MIR155HG* knockdown (Fig. 7G). Taken together, the above-mentioned results indicate that *MIR155HG* and *miR-155-5p* are EMT-related non-coding RNAs, and they synergistically promote EMT in LSCC cells.

Discussion

In recent years, non-coding RNAs, particularly lncRNAs and miRNAs, have been found to play important roles in the development and progression of tumors, including LSCC. In our study, the differential expression of lncRNAs was detected by microarray assays, and *MIR155HG* was found to be highly expressed in LSCC tissues. *MIR155HG*, also referred to as B-cell integration cluster (*BIC*), was first identified as a novel gene that was transcriptionally activated in avian leukosis virus-induced lymphomas by the insertion of retrovirus integration sites in the promoter region (24). *MIR155HG* has also been found to be upregulated in pediatric Hodgkin's lymphoma and Burkitt's lymphoma (25-27). The transcription of *MIR155HG* is regulated by multiple transcription factors, such as *MYB*, *NF-κB* and *AP-1* (28-30). A recent study reported that *MIR155HG* acts as an oncogene in glioma, and is associated with prognosis and tumor progression (17). In the present study, the expression of *MIR155HG* was found to be upregulated in LSCC tissues, suggesting that *MIR155HG* is a potential oncogene in LSCC. Moreover, the high expression level of *MIR155HG* was associated with a poor differentiation, lymph node metastasis and a higher TNM stage, suggesting that *MIR155HG* may be of clinical value in the assessment of invasion and metastasis, malignant behavior and prognosis of laryngeal carcinoma. Furthermore, *in vitro* and *in vivo* assays verified the oncogenic role of *MIR155HG* in LSCC.

It is well known that lncRNAs exert their effects through a variety of mechanisms, including serving as the host genes and regulating the expression of miRNAs (14). *miR-155* is located in the third exon of *MIR155HG*, and has been reported to play important roles in a number of solid malignancies (31-33). The overexpression of *miR-155* has been found to promote cell proliferation and migration through targeting *TGFβR2* in gastric cancer (31). *miR-155-5p* has also been found to inhibit the

migration and invasion of colorectal cancer cells by targeting *CTHRC1* (32). *miR-155-5p* has been found to be upregulated in oral squamous cell carcinoma and to be associated with metastasis, poor prognosis and EMT progression (34,35). The aberrant expression of *miR-155* has also been previously observed in LSCC (36-38). *miR-155* acts as an oncogene in LSCC, promoting the growth, migration and invasion of LSCC cells by regulating *SOSC1* and *STAT3* (37). Another study demonstrated that *miR-155* was highly expressed in the plasma and tissues of patients with LSCC (38). However, the correlation between *miR-155* and its host gene, *MIR155HG*, in LSCC has not yet been elucidated, and the other target genes of *miR-155* require further investigation. The present study demonstrated that the expression of *miR-155-5p* was upregulated in LSCC tissues, and that the overexpression of *miR-155-5p* significantly promoted the growth, migration and invasion of LSCC cells. Furthermore, the expression level of *miR-155-5p* was found to be associated with the malignant phenotype of LSCC, which was consistent with the findings on *MIR155HG*. Although a positive correlation between the expression level of *MIR155HG* and *miR-155-5p* was detected, and *MIR155HG* may regulate the expression of *miR-155-5p*, *miR-155-5p* did not affect the expression of *MIR155HG*, suggesting that the transcriptional activity of *miR-155* is under the control of *MIR155HG*, which is consistent with the findings of other studies on Hodgkin's lymphoma, Burkitt's lymphoma and glioma (17,25,26). Furthermore, the upregulation of *miR-155-5p* reversed the inhibitory effects of *MIR155HG* knockdown on cell malignant biological properties, suggesting that these two non-coding RNAs synergistically control the same biological processes in LSCC.

SOX10, a member of the *SOX* family, has been found to be a target gene of *miR-155-5p*. *SOX10* plays an important role in the formation of the neural crest and peripheral nervous system, the maturation and differentiation of Schwann and oligodendrocyte lineage cells, and the occurrence and development of tumors (39,40). In recent years, the bidirectional role of *SOX10* in regulating tumor progression has been gradually revealed. For example, *SOX10* has been reported to inhibit the growth and metastasis of digestive tumors via inhibiting the Wnt/β-catenin pathway (22). However, *SOX10* has been found to be highly expressed in nasopharyngeal carcinoma, whereas *SOX10* knockdown has been shown to markedly inhibit nasopharyngeal carcinoma cell proliferation, migration, invasion and EMT (23,41). *SOX10* acts as an oncogene in hepatocellular carcinoma by activating Wnt/β-catenin signaling (42). In the present study, *SOX10* was found to be downregulated in LSCC tissues. The overexpression of *miR-155-5p* reduced the transcriptional and translational levels of *SOX10*, whereas *miR-155-5p* downregulation exerted the opposite effects. Moreover, the direct target association between *miR-155-5p* and *SOX10* was proven by a dual-luciferase reporter assay. These results indicate that *SOX10* is a target gene of *miR-155-5p*, and that *miR-155-5p* exerts oncogenic effects partly via the regulation of *SOX10*.

EMT is an important biological process for malignant tumor cells, characterized by epithelial cells acquiring mesenchymal characteristics and, thereby, the ability of migration and invasion, which is a prerequisite for the initiation of tumor invasion and metastasis (43). Numerous studies have

demonstrated that lncRNAs induced by *TGF-β* play important roles in EMT in different types of cancer (44-46). lncRNA ATB (lncRNA-activated by *TGF-β*), a regulator and mediator of the *TGF-β* signaling pathway, has been found to play a key role in inducing EMT and promoting invasion and metastasis in hepatocellular carcinoma (44). *LINC01186*, a downregulated lncRNA in *TGF-β*-treated lung cancer cells, has been shown to be regulated by *TGF-β/SMAD3* and to inhibit the malignant biological behavior of lung cancer by regulating EMT (46). However, the involvement of lncRNAs in the process of EMT in LSCC is poorly understood. For this purpose, in this study, the TU177 cells were treated with *TGF-β* for the indicated number of days. The treated cells exhibited a change in morphology to a spindle-shaped one, and their migratory and invasive abilities were enhanced compared with the untreated cells. Moreover, the expression levels of mesenchymal markers were upregulated. However, we could not detect the expression of the epithelial marker, E-cadherin, possibly since the increase in its expression was too low to be monitored. The expression levels of *MIR155HG* and *miR-155-5p* were also upregulated in the *TGF-β*-treated group, which indicated that *MIR155HG* and *miR-155-5p* are *TGF-β*-induced non-coding RNAs. Consistent with our conclusions, *MIR155HG* has been reported to be associated with mesenchymal transition in glioma (17), and *miR-155* is an EMT-related onco-miRNA (47,48) that has been found to be induced by stimulation of *TGF-β* in hepatocellular carcinoma cells (49). Further experiments indicated that *MIR155HG* and *miR-155-5p* may synergistically affect the expression of EMT-related markers, thereby promoting EMT. As stated above, *SOX10* contributes to EMT in nasopharyngeal carcinoma. Therefore, we hypothesized that *SOX10* may promote EMT in LSCC. All the above-mentioned results indicate that *MIR155HG* and *miR-155-5p* are EMT-related non-coding RNAs, and that *MIR155HG* may promote EMT in LSCC cells by regulating the *miR-155-5p/SOX10* axis.

In conclusion, the present study is, to the best of our knowledge, the first to confirm the functional promoting role of *MIR155HG* and its co-expression with *miR-155-5p* in LSCC tumorigenesis. *miR-155-5p* acts synergistically with *MIR155HG* to promote the progression of LSCC, partly by regulating the downstream target gene, *SOX10*. Moreover, the findings of the present study indicate a novel role of *MIR155HG* in *TGF-β*-induced EMT of LSCC cells by regulating EMT marker expression through the *miR-155/SOX10* axis. In addition, these findings indicate a novel mechanism underlying the aggressive biological behavior of LSCC, and the *MIR155HG/miR-155-5p/SOX10* axis may represent a promising therapeutic target for patients with LSCC.

Acknowledgements

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BW conceived and designed the study, modified the manuscript; WCu collected and analysed the data, designed and perform the experiments, drafted the manuscript; WM, LZ, WCh and HC performed the experiments and were involved in the revise of manuscript; All authors participant in revising manuscript, and agree with the final manuscript.

Ethics approval and consent to participate

The use of human tissues specimen was approved by and carried out according to the guidelines of the Ethics Committee of the Second Hospital of Hebei Medical University (Hebei, China) and written informed consent was obtained from all patients. All animal experiments were performed at the Experimental Animal Center of the Fourth Hospital of Hebei Medical University according to the NIH guidelines, and were approved by the Institutional Animal Care and Use Committee of the Fourth Hospital of Hebei Medical University.

Competing interests

The authors declare that they have no competing interests.

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