lncRNA RASSF8-AS1 suppresses the progression of laryngeal squamous cell carcinoma via targeting the miR-664b-3p/TLE1 axis

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Abstract. Long non-coding (lnc)RNAs have been found to play a crucial role in tumor progression. The present study aimed to investigate the association between lncRNA RASSF8-AS1 and laryngeal squamous cell carcinoma (LSCC) and the underlying mechanisms. Reverse transcription-quantitative PCR was used to measure the mRNA expression level of RASSF8-AS1, microRNA(miR)-664b-3p and transducin-like enhancer of split 1 (TLE1) in LSCC. The associations between RASSF8-AS1 and miR-664b-3p, and between miR-664b-3p and TLE1 were investigated using a dual luciferase reporter assay, while the former was further verified using an RNA immunoprecipitation (RIP) assay. The association between RASSF8-AS1 and miR-664b-3p on cell biological functions was investigated *in vitro* using MTS, colony formation and Transwell assays. The RASSF8-AS1 mRNA expression level was decreased in LSCC cell lines and carcinoma tissues, while overexpression of RASSF8-AS1 reduced the migration, invasion and proliferation abilities of LSCC cells. Furthermore, luciferase and RIP assays confirmed that RASSF8-AS1 was a competitive endogenous (ce)RNA by sponging miR-664b-3p to activate TLE1. miR-664b-3p was negatively modulated by RASSF8-AS1; however, TLE1 was positively regulated by RASSF8-AS1. Functionally, RASSF8-AS1 acted as a ceRNA to upregulate TLE1 by sponging miR-664b-3p. In conclusion, the RASSF8-AS1/miR-664b-3p/TLE1 axis acts by suppressing LSCC progression and may provide a novel insight for the molecular mechanism of LSCC.

Introduction

Laryngeal cancer is the second most common type of head and neck cancer and is more prevalent in males compared with females (1). Laryngeal squamous cell carcinoma (LSCC) is the most common type of laryngeal carcinoma. In 2018, there were 177,422 new cases and 94,771 cancer-associated deaths, worldwide, which accounted for 1% of all types of cancer. The incidence rate of LSCC is increasing annually worldwide (2). Approximately 60% of patients with LSCC are diagnosed with advanced disease (stage III or IV), which leads to poor treatment efficacy and worse prognosis (3). There has been an improvement in the treatment of LSCC; however, the survival rate of patients with LSCC has remained low over the past few decades and has shown a downward trend (4). Therefore, it is important to investigate the pathogenesis and molecular mechanism of LSCC, to identify novel prevention and treatment strategies.

A study of the human genome reported that ~80% of DNA are transcribed into RNA; however, only ~2% of RNA are translated into proteins. RNAs that do not encode proteins are termed non-coding RNAs (5). Long non-coding (lnc)RNAs are a type of functional RNA and >200 nucleotides in length (6). lncRNAs do not have protein-coding function; however, they can regulate gene expression at both the transcriptional and post-transcriptional levels (7). It was demonstrated that lncRNAs may play key roles in the occurrence and development of tumors and they could be potential biomarkers for early diagnosis of multiple types of tumor and potential therapeutic targets (8-10). However, the specific roles and molecular mechanisms of lncRNAs in LSCC are limited. In our previous study, the differential expression level of lncRNAs in four LSCC and adjacent normal tissues was identified using a microarray (11). It was verified that the expression level of RASSF8-AS1 in tumor tissues was significantly lower compared with that in paired normal tissues, which was further validated using reverse transcription-quantitative

PCR (RT-qPCR). To the best of our knowledge, there are few studies investigating the mechanism of RASSF8-AS1 in other types of tumor. At present, the mechanism of competitive endogenous (ce) RNA has become a hot topic in lncRNA-mediated tumorigenesis. Recent studies have demonstrated that lncRNAs have oncogenic or antitumor activities, by functioning as microRNA (miRNA/miR) sponges, which suggest that they have pivotal roles in the development of tumors (12-14). For example, Song *et al* (12) reported that SPRY4-IT1 increased the expression level of TCF7L2 by targeting miR-6882-3p, promoted the proliferation and stemness of breast cancer cells, and promoted the renewal ability and stemness maintenance of breast cancer stem cells. Chen *et al* (13) demonstrated that SNHG16 promoted the proliferation, migration and invasion of hepatocellular carcinoma cells by negatively regulating the expression level of miR-186, as a ceRNA. However, the mechanism and function of lncRNAs as ceRNAs in LSCC have not been investigated. Therefore, the present study aimed to verify the role of lncRNA RASSF8-AS1 in LSCC through a ceRNA network. To the best of our knowledge, this is the first time RASSF8-AS1 has been investigated in LSCC. The results showed that the mRNA expression level of RASSF8-AS1 was decreased in LSCC tissues and cell lines, while the overexpression of RASSF8-AS1 inhibited the proliferation, invasion and migration of LSCC cells by targeting the miR-664b-3p/transducin-like enhancer of split 1 (TLE1) axis. These findings provide a deeper understanding of the tumorigenic mechanism and suggest a potential target for the treatment of LSCC.

Materials and methods

Patients and tissue samples. A total of 72 pairs of LSCC tumor and adjacent normal tissues were collected from the Second Hospital of Hebei Medical University (Hebei, China) between October 2016 and March 2019. None of the patients with LSCC received radiotherapy and/or chemotherapy prior to surgery. All procedures were conducted following the ethical standards of the Institutional Research Council of Hebei Medical University (Hebei, China) and the Declaration of Helsinki from 2008. The present study was approved by the Ethics Committee of the Second Hospital of Hebei Medical University (Hebei, China). All the tissue specimens were stored at -80˚C at the Otorhinolaryngology Head and Neck Surgery Biobank of Hebei Medical University for RNA extraction. Clinical features and pathological diagnosis were collected from the hospital records (Table I).

Cell lines and cell culture. A total of 4 human LSCC cell lines (TU686, TU177, TU212 and AMC-HN-8) and the 293T cell line were obtained from Beijing Beina Chuanglian Institute of Biotechnology. The TU212 cell line was authenticated using short tandem repeat (STR) profiling by Shanghai Biowing Applied Biotechnology Co., Ltd. DNA was extracted using an Axygen genomic extraction kit and amplified using a 20-STR amplification protocol. STR loci and the gender gene, Amelogenin were detected using an ABI 3730XL genetic analyzer. The AMC-HN-8 and 293T cells were cultured in DMEM, supplemented with 10% FBS, while the TU686, TU177 and TU212 cells were cultured in RPMI-1640 medium, containing 10% FBS. The aforementioned media and reagents

were purchased from Gibco (Thermo Fisher Scientific, Inc.). The cells were cultured at 37˚C in a humidified incubator (Thermo Fisher Scientific, Inc.) with 5% CO_2.

RNA extraction and RT-qPCR assay. Total RNA was extracted from the LSCC tissues and cells using an Eastep® Super Total RNA Extraction Kit (Promega Corp.). The transcriptor First Strand cDNA synthesis kit (Roche Diagnosis GmbH) was used for RT. RT-qPCR was performed using a GoTaq® qPCR Master Mix (Promega Corp.). The following thermocycling conditions were used: Initial denaturation at 95˚C for 2 min, followed by denaturation at 95˚C for 15 sec. For annealing to extension, selecting the suitable annealing temperature according to different primers for 60 sec, for a total of 40 cycles was carried out. lncRNA or mRNA expression was normalized using GAPDH, while U6 was used as the internal control for miRNA. Relative expression was normalized using the $2^{-\Delta\Delta Cq}$ method (15). All primers used are shown in Table II.

Subcellular fractionation. Subcellular fractionation was performed using a PARIS™ Protein and RNA Isolation System (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. U6 and GAPDH were used as the cytoplasmic and nuclear controls, respectively.

Cell transfection. For overexpression of RASSF8-AS1, the pcDNA3.1-RASSF8-AS1 vector was purchased from Sangon Biotech Co., Ltd., while hsa-miR-664b-3p mimic/inhibitor/negative control (NC) was synthesized by Guangzhou RiboBio Co., Ltd. For knockdown of TLE1, the pGenesil-1 plasmid was used to construct the knockdown plasmid to form pGenesil-1-TLE1 [short inhibiting (sh)-TLE1]. A total of 2 primers (Table II) were annealed to form the double-stranded DNA. After restriction digestion (*Bam*HI and *Hin*dIII) and purification of pGenesil-1, double-stranded DNA was ligated into pGenesil-1 to obtain a recombinant plasmid termed sh-TLE1, which was identified by sequencing. The pcDNA3.1 was used as a negative control for overexpression of RASSF8-AS1, and the pGenesil-1 was used as a negative control for sh-TLE1. The TU177 and TU686 cell lines were both seeded in separate 6-well plates and cultured to 70-80% confluence. Then, TU177 and TU686 cells were transfected respectively with pcDNA3.1-RASSF8-AS1 or sh-TLE1 using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instruction. AS control, the pcDNA3.1 or pGenesil-1 empty vector were transfected at the same time. hsa-miR-664b-3p mimic/inhibitor/negative control (NC) were transfected into TU177 and TU686 cells according to the manufacturer's instruction. Transfection efficiency was determined using RT-qPCR.

Dual luciferase reporter assay. The TU177 and TU686 cell lines were seeded in 24-well plates separately, cultured for 24 h, and co-transfected with pmirGLO-RASSF8-AS1-wild-type (WT) or pmirGLO-RASSF8-AS1-mutant (MUT) reporter plasmids (Sangon Biotech, Co., Ltd.) and miR-664b-3p mimic, inhibitor or NC. After 48 h of transfection, luciferase activity was measured using a dual-luciferase reporter assay system (Promega Corp.), while *Renilla* luciferase activity was used

Table I. Information and clinicopathological data of the 72 pairs of tumor and normal tissues obtained from the patients with LSCC.

Characteristics	n (%)
Sex	
Male	72 (100.0)
Female	0 (0.00)
Age (years)	
<60[a]	27 (37.5)
≥60	45 (62.5)
Smoking	
No	9 (12.5)
Yes	63 (87.5)
Alcohol consumption	
No	31 (43.1)
Yes	41 (56.9)
TNM stage	
I+II	30 (41.7)
III+IV	42 (58.3)
Cervical lymph node metastasis	
No	37 (51.4)
Yes	35 (48.6)
Pathological differentiation degree	
Well	38 (52.8)
Moderate/poor	34 (47.2)

[a]Median age of the 72 patients. LSCC, laryngeal squamous cell carcinoma.

for normalization. Using the same method, the TU177 or 293T cell line was co-transfected, using Lipofectamine® 2000, with pmirGLO-TLE1-WT or pmirGLO-TLE1-MUT reporter plasmids (Sangon Biotech, Co., Ltd.) and miR-664b-3p mimic, inhibitor or NC. The subsequent experiments were as aforementioned.

Colony formation assay. A total of $2x10^3$ cells were seeded in each well of a 6-well plate, and cultured at 37˚C in a humidified incubator with 5% CO_2 for 10 days, 24 h following transfection. Then, the LSCC cell lines were washed with PBS, fixed with 4% paraformaldehyde for 20 min, and then stained with 0.5% crystal violet for 20 min. Finally, we used a microscope (CKX53, Olympus Corp.) at x200 magnification to observe cells, colonies of >50 cells per well were calculated.

Cell proliferation assay. The proliferation ability of the transfected LSCC cell lines was detected using an MTS assay. After transfection for 24 h, $2x10^3$ cells/per well were seeded into a 96-well plate. Using a CellTiter 96® AQueous one solution cell proliferation assay kit (Promega Corp.), 20 µl MTS reagent was added into each well, after the cells were seeded for 0, 24, 48, 72 and 96 h and subsequently incubated for 2.5 h, according to the manufacturer's instructions. The optical density (OD) was detected at 490 nm.

Cell migration and invasion assays. A Transwell chamber (Corning, Inc.) was placed into a 24-well plate to detect the migration or invasion ability of the transfected LSCC cells. For the migration assays, the transfected LSCC cell lines were digested with pancreatin, and then suspended in serum-free medium, following which a cell counter was used for cell counting. A total of $1x10^5$ cells were plated into the upper chamber and 650 µl culture medium (including 10% FBS) was added into the bottom chamber. After incubation at 37˚C for 24 h, the Transwell chamber was removed, washed with PBS, and fixed with 4% paraformaldehyde for 20 min, and then stained with 0.5% crystal violet for 20 min. Then, the cells were observed and counted using a microscope (CKX53; Olympus Corp.) at x200 magnification. For the invasion assay, 50 µl Matrigel was added to the upper chamber to form a matrix barrier; the same protocol was then used for the Transwell assay.

Western blot analysis. Total protein was extracted from the LSCC cell linesusing RIPA buffer (Beijing Solarbio Science and Technology, Co., Ltd.), supplemented with a protease inhibitor cocktail (Promega Corp.). Protein samples (20 µg) were separated using 10% SDS-PAGE and then transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Inc.). The membranes were incubated with rabbit anti-human TLE1 (molecular weight, 83 kDa; dilution 1:1,500; Abcam, cat no. ab183742) and rabbitanti-human GAPDH (molecular weight, 37 kDa; dilution 1:5,000; ProteinTech Group, Inc., cat no. 10494-1-AP) overnight. Then, the protein band was visualized and quantified using an enhanced chemiluminescence kit and a ChemiDoc™ XRS + system (Bio-Rad Laboratories, Inc.).

RNA immunoprecipitation (RIP) assay. For the RIP assay, pSL-MS2-12X (Addgene, Inc.) was double digested using *Bam*HI and *Xho*I, and the MS2-12X fragment was inserted to the pcDNA3.1-RASSF8-AS1 vector to form pcDNA3.1-MS2-RASSF8-AS1. Then, pcDNA3.1-MS2-RASSF8-AS1 was mutated using the Q5®Site-Directed Mutagenesis Kit (New England Biolabs, Inc.) into pcDNA3.1-MS2-RASSF8-AS1-MUT. The LSCC cell lines were co-transfected with pMS2-GFP (Addgene, Inc.) and pcDNA3.1-MS2-RASSF8-AS1 or pcDNA3.1-MS2-RASSF8-AS1-MUT. After 48 h, the LSCC cell lines were used for the RIP assay with a green fluorescent protein antibody (Roche Diagnostics, Switzerland) and a Magna RIP™ RNA-binding protein immunoprecipitation kit (EMD Millipore) according to manufacturer's instructions (16).

Bioinformatic analysis. Targets of RASSF8-AS1 were obtained from DIANA-LncBase Predicted v2 (http://carolina. imis.athena-innovation.gr/Diana_tools/web/index.php) and the results showed has-miR-664b-3p might be a potential target for RASSF8-AS1. Targets for has-miR-664b-3p were analyzed at Starbase (http://starbase.sysu.edu.cn/index.php) and revealed TLE1 was a potential target for has-miR-664b-3p.

Statistical analysis. All statistical analyses were performed using SPSS software v21.0 (IBM Corp.) and GraphPad Prism v7 (GraphPad Software Inc.). The data are presented as the mean ± standard deviation. The figures were created

Table II. Oligonucleotide sequences used in this study.

Gene	Sequence
RASSF8-AS1	F: 5'-CAAAGGGTGACACACCAGGA-3'
	R: 5'-TGGTGATCAACACAAACTGGA-3'
GAPDH	F: 5'-AGGTGAAGGTCGGAGTCAACG-3'
	R: 5'-AGGGGTCATTGATGGCAACA-3'
miR-664b-3p (stem-loop reverse transcription)	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCAC
	TGGATACGACTGTAGGCT
miR-664b-3p	F: 5'-GCCGCGTTCATTTGCCTCCCAGCCT-3'
	R: 5'-GTGCAGGGTCCGAGGTATT-3'
U6	F: 5'-GCTTCGGCAGCACATATACTAAAAT-3'
	R: 5'-CGCTTCACGAATTTGCGTGTCAT-3'
TLE1	F: 5'-GAGTCCCTGGACCGGATTAAA-3'
	R: 5'-AATACATCACATAGTGCCTCTGC-3'
pmirGLO RASSF8-AS1-wild	F: 5'-TCAGCTAGCAGTGGTCTCAGGACCATAAG-3'
	R: 5'-CGATCTAGAATACACAGAGTCGGGCAAG-3'
pmirGLO RASSF8-AS1-mut	F: 5'-CAGATTTTTAAACAAATAGGTATGTGGTGC-3'
	R: 5'-CATTCCATAGCCCCCCTAGACT-3'
pmirGLO TLE1-wild	F: 5'-CTACTCGAGGTTGTAACTTTAAAAGAG-3'
	R: 5'-GCAGTCGACCTCACACTTGGGCAAGG-3'
pmirGLO TLE1-mut	F: 5'-CTACATAGACCGACTAGAGCACCAAGG-3'
	R: 5'-ACAGGTGACTTTCTGCTG-3'
miR-664b-3p mimic	5'-UUCAUUUGCCUCCCAGCCUACA-3'
miR-664b-3p inhibitor	5'-UGUAGGCUGGGAGGCAAAUGAA-3'
shRNA-TLE1-1	5'-GATCCGATCTGCACAACCAGACACTATTCAAG
	ACGTAGTGTCTGGTTGTGCAGATCTTTTTTGTCGACA-3'
shRNA-TLE1-2	5'-AGCTTGTCGACAAAAAAGATCTGCACAACCAG
	ACACTACGTCTTGAATAGTGTCTGGTTGTGCAGATCG-3'

TLE1, transducin-like enhancer of split 1; F, forward; R, reverse.

using GraphPad Prism v7. 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. A Pearson's correlation test was performed to determine the correlation between the mRNA expression levels of RASSF8-AS1 and miR-664b-3p, or the correlation between the mRNA expression levels of TLE1 and miR-664b-3p. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Silencing of RASSF8-AS1 in LSCC cell lines and tissues. Microarray analysis was used to compare the lncRNA expression levels between four pairs of LSCC tissues and adjacent normal tissues to investigate the potential role of lncRNAs in LSCC (11) (Fig. 1A). A lncRNA, RASSF8-AS1, which was decreased in the microarray analysis was selected for further experiments. Using National Centre for Biotechnology Information, RASSF8-AS1 was found to be differently expressed in different types of human tissue (Fig. 1B) and subsequently, using RT-qPCR, the mRNA expression level of RASSF8-AS1 was found to be significantly decreased in four

LSCC cell lines and 72 LSCC tissues (Fig. 1C and D). The pool in Fig. 1C represents the average value of lncRNA RASSF8-AS1 relative expression from the normal tissues, and it was used as a control for the laryngeal squamous cell lines, as described previously (17). In the 72 tumor tissues, a low mRNA expression level of RASSF8-AS1 was associated with well-differentiated, lower lymph node metastasis and lower TNM staging; however, no association was observed between RASSF8-AS1 mRNA expression level and age, smoking, or alcohol consumption (Fig. 1E). These results suggest that lncRNA RASSF8-AS1 could be a tumor-inhibiting factor in the progression of LSCC.

Overexpression of RASSF8-AS1 reduces proliferation and colony formation efficiency, and invasion and migration abilities of the LSCC cell lines. To further investigate the effect of RASSF8-AS1 in the LSCC cell lines, pcDNA3.1-RASSF8-AS1 or pcDNA3.1 were transfected into the TU177 and TU686 cell lines, as the expression level of RASSF8-AS1 was lowest out of the LSCC cells investigated. RT-qPCR analysis showed that the mRNA expression level of RASSF8-AS1 was significantly increased by pcDNA3.1-RASSF8-AS1 in both cell lines (Fig. 2A). Using an MTS assay, the cells transfected with pcDNA3.1-RASSF8-AS1 exhibited significantly
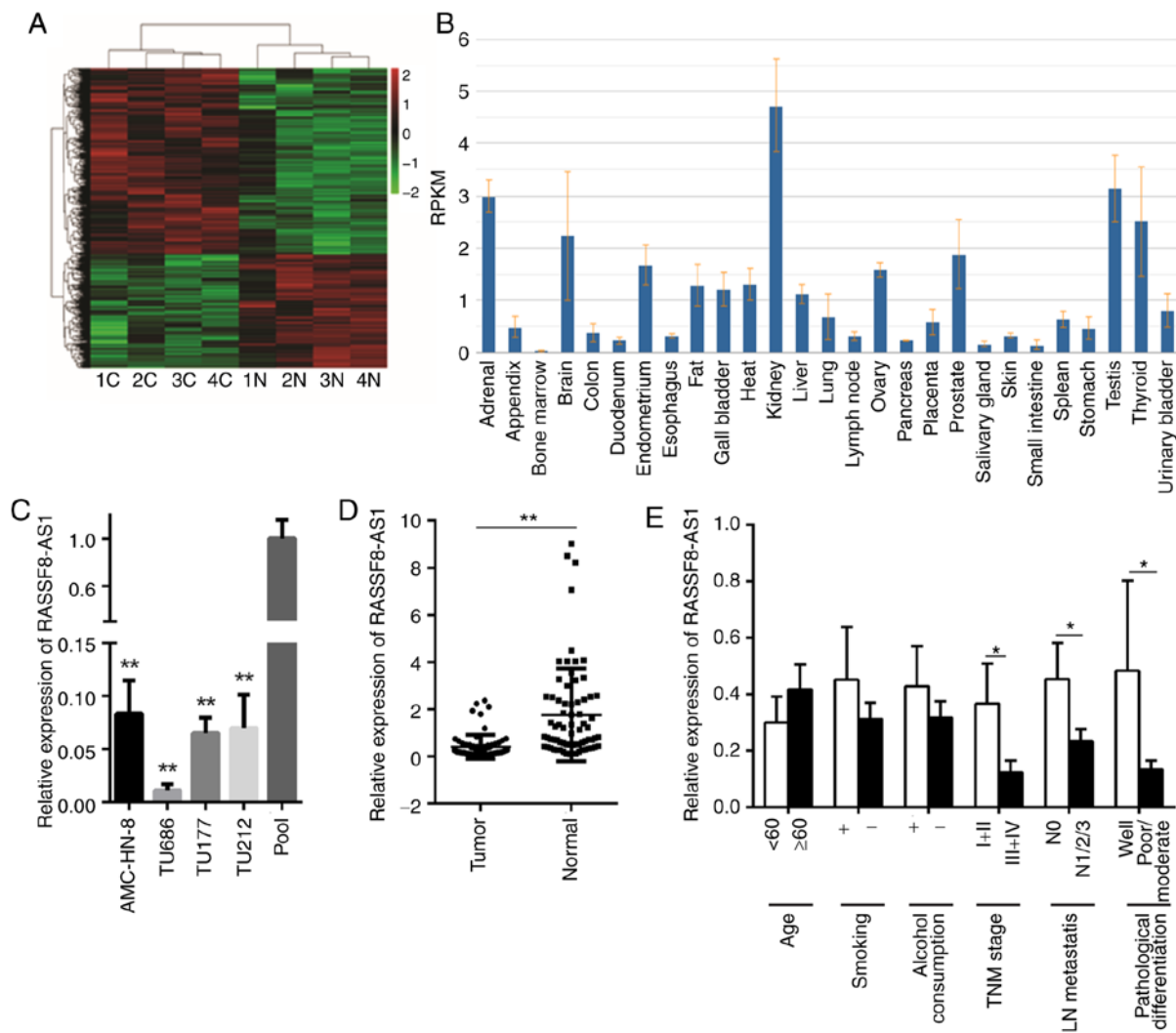
Figure 1. Expression level of RASSF8-AS1 in the LSCC cell lines and tissues. (A) Heatmap of lncRNA expression in 4 pairs of LSCC tissues (1C-4C) and adjacent normal tissues (1N-4N). (B) The expression level of RASSF8-AS1 in different normal tissues from the National Centre for Biotechnology Information. Relative expression level of RASSF8-AS1 in (C) four LSCC cell lines vs. the pool, (D) LSCC tissues and adjacent normal tissues and (E) in various subgroups. Data are presented as the mean ± SD from three independent experiments. *P<0.05 and **P<0.01. lncRNA, long non-coding RNA; LSCC, laryngeal squamous cell carcinoma; LN, lymph node.

reduced proliferative abilities compared with that in the cells transfected with empty vectors (Fig. 2B). Overexpression of RASSF8-AS1 significantly reduced colony formation ability compared with that in cells transfected with empty vectors using a colony formation assay (Fig. 2C), while the cells transfected with overexpression of RASSF8-AS1 had significantly reduced migration and invasion abilities (Fig. 2D and E). Therefore, we hypothesized that RASSF8-AS1 is a tumor inhibitor by suppressing the proliferation, colony formation, migration and invasion abilities of the LSCC cell lines.

lncRNA RASSF8-AS1 acts as a sponge for miR-664b-3p. Recent studies have found that lncRNAs, which are localized in the cytoplasm, are known as ceRNAs to regulate miRNAs (18). The lncRNA, RASSF8-AS1 in the four LSCC cell lines was found in both the cytoplasm and nucleus; however, the expression level was slightly higher in the cytoplasm (Fig. 3A). To discover the ceRNA mechanism of RASSF8-AS1 in LSCC, the miRNA associated with RASSF8-AS1 was investigated and the results showed

that RASSF8-AS1 was found to possess a conserved target site of miR-664b-3p (Fig. 3B) with a high score using bioinformatics miRNA target prediction tools (Starbase v3.0 and DIANA). The RIP assay was then used to validate the binding ability between miR-664b-3p and RASSF8-AS1. miR-664b-3p was found to be markedly enriched in the TU177 cell line, which was transfected with RASSF8-AS1-WT compared with that in cells transfected with RASSF8-AS1-MUT (Fig. 3C). The interaction between miR-664b-3p and RASSF8-AS1 was further confirmed using a dual luciferase reporter assay compared with that in the control group, and the ratio of firefly luciferase to *Renilla* activity was decreased following co-transfection of the TU177 and 293T cell lines with miR-664b-3p mimic and pmirGLO-RASSF8-AS1-WT. However, co-transfection of miR-664b-3p mimic and pmirGLO-RASSF8-AS1-MUT did not decrease the firefly luciferase to *Renilla* activity ratio. In addition, the firefly luciferase to *Renilla* activity ratio was increased in the cells co-transfected with miR-664b-3p inhibitor and pmirGLO-RASSF8-AS1-WT, but not in cells
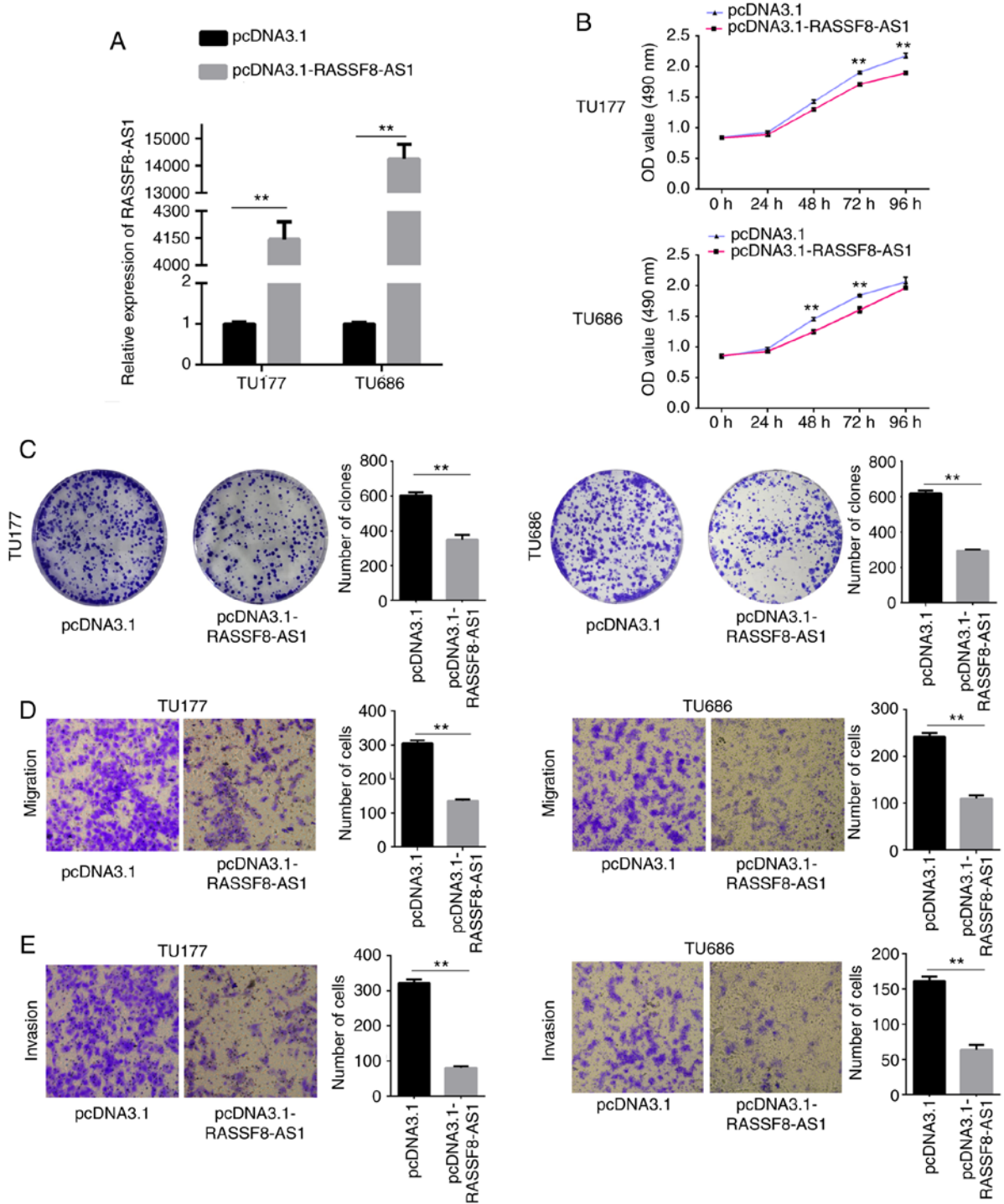
Figure 2. *In vitro* function of the TU177 and TU686 cell lines with overexpression of RASSF8-AS1. (A) The mRNA expression level of RASSF8-AS1 in the TU177 and TU686 cell lines transfected with pcDNA3.1-RASSF8-AS1 and pcDNA3.1. (B) Growth curves of the TU177 and TU686 cell lines, following transfection with pcDNA3.1-RASSF8-AS1 or pcDNA3.1 using an MTS assay. (C) A colony formation assay following the overexpression of RASSF8-AS1 in the TU177 and TU686 cell lines. (D) Migration and (E) invasion abilities of the TU177 and TU686 cell lines, following overexpression of RASSF8-AS1 in the Transwell assay. Data are presented as the mean ± SD from three independent experiments. $^{**}P<0.01$.

transfected with pmirGLO-RASSF8-AS1-MUT (Fig. 3D). miR-664b-3p mRNA expression level was notably decreased in the TU177 and TU686 cell lines transfected with pcDNA3.1-RASSF8-AS1 compared with that in cells transfected with the pcDNA3.1 vector using RT-qPCR (Fig. 3E). Then, further analysis using the LSCC tissues revealed that the RASSF8-AS1 and miR-664b-3p mRNA expression levels were found to be negatively correlated (Fig. 3F). All

the results revealed that RASSF8-AS1 was associated with miR-664b-3p and acts as a ceRNA.

miR-664b-3p is upregulated in LSCC and promotes proliferation, migration and invasion of LSCC cells. The aforementioned results showed that miR-664b-3p is associated with RASSF8-AS1. The mRNA expression level of miR-664b-3p was found to be increased in the LSCC tissues
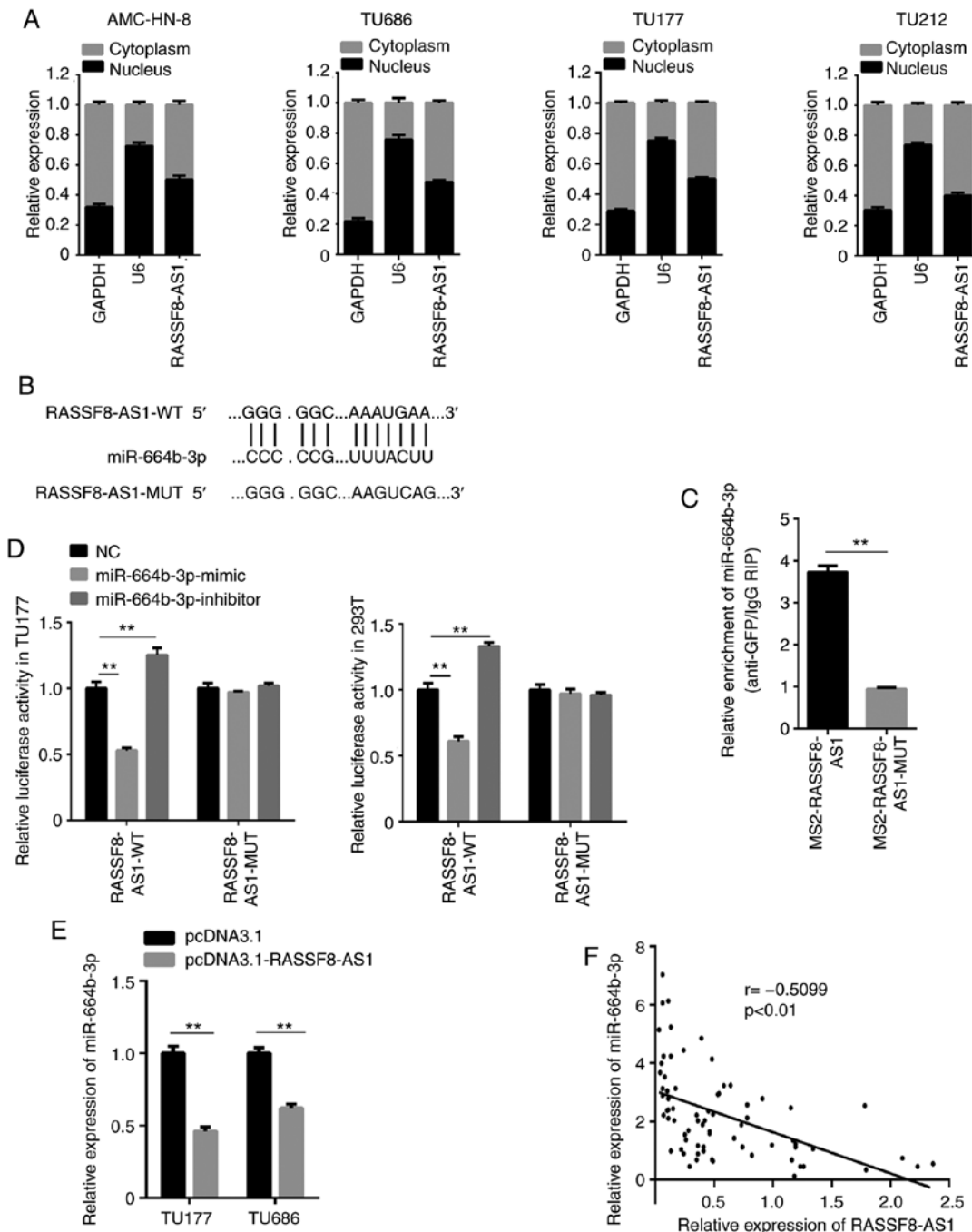
Figure 3. lncRNA RASSF8-AS1 acts as a sponge for miR-664b-3p. (A) The location of RASSF8-AS1 in the LSCC cell lines. U6 and GAPDH were used for nuclear and cytoplasm controls, respectively. (B) Bioinformatic analysis showed the potential binding site of RASSF8-AS1 and miR-664b-3p. (C) RIP assay followed by miRNA reverse transcription-quantitative PCR revealed that the RIP of RASSF8-AS1 in the TU177 cells was significantly enriched with miR-664b-3p compared with that in the mutation type. (D) The ratio of firefly luciferase and *Renilla* activity of the TU177 and 293T cell lines transfected with pmirGLO-RASSF8-AS1-WT or pmirGLO-RASSF8-AS1-MUT and NC or miR-664b-3p mimic or miR-664b-3p inhibitor in the dual-luciferase assays. (E) The mRNA expression level of miR-664b-3p in the TU177 and TU686 cell lines transfected with pcDNA3.1-RASSF8-AS1 or pcDNA3.1. (F) The correlation between RASSF8-AS1 and miR-664b-3p mRNA expression levels in the LSCC tissues. Data are presented as the mean ± SD from three independent experiments. $^{**}P<0.01$. WT, wild-type; MUT, mutant; lncRNA, long non-coding RNA; miR, microRNA; NC, negative control; LSCC, laryngeal squamous cell carcinoma; RIP, RNA immunoprecipitation.

compared with that in the paired normal tissues (Fig. 4A). To further investigate whether miR-664b-3p affects the progression of LSCC, the function of miR-664b-3p was determined. miR-664b-3p mimic, inhibitor and NC were transfected into the TU177 and TU686 cell lines and the transfection efficiency was determined using RT-qPCR (Fig. 4B). The MTS and colony formation assays revealed that upregulation of miR-664b-3p notably increased cell proliferation and colony forming abilities of the TU177 and TU686 cell lines, whereas downregulation of miR-664b-3p had the opposite effect (Fig. 4C and D). The migration and invasion assays revealed the same results (Fig. 5A and B). Taken together, all these results showed that miR-664b-3p promotes the proliferation, migration and invasion abilities of the LSCC cells.
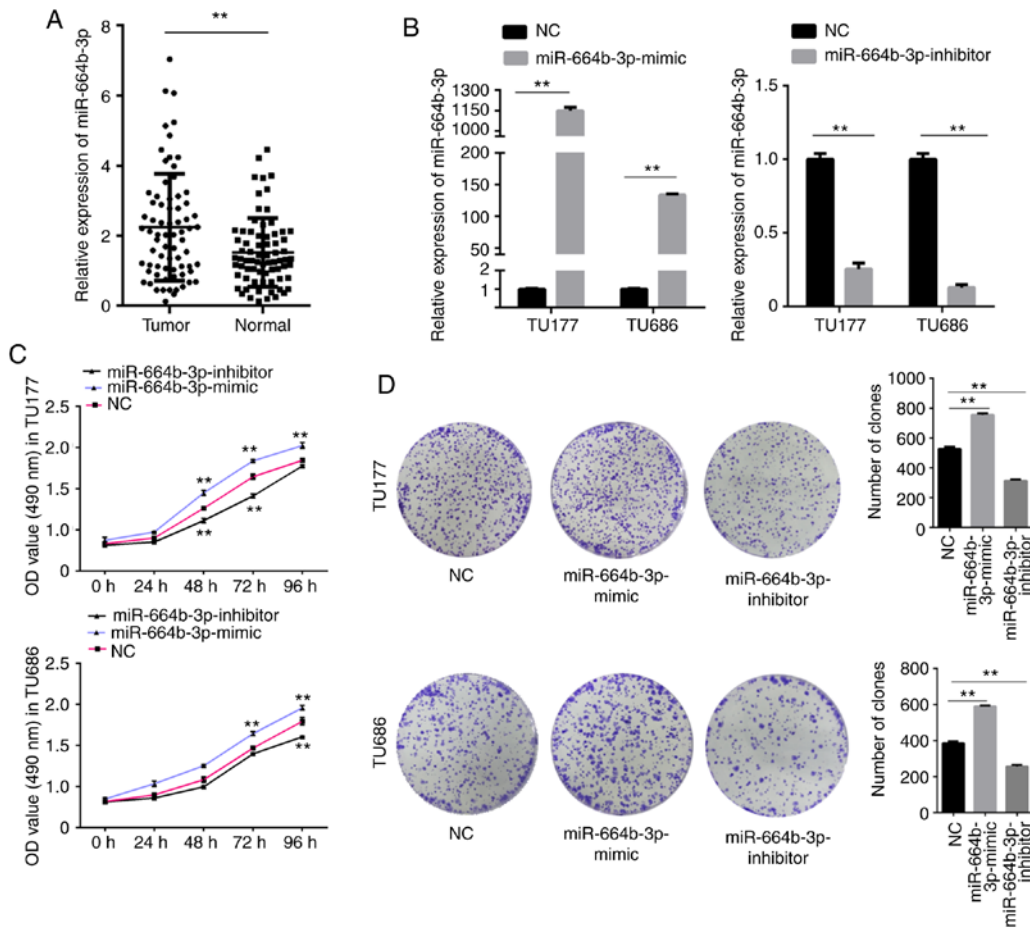
Figure 4. Expression of miR-664b-3p and *in vitro* MTS and colony formation assays of the TU177 and TU686 cell lines with overexpression and knockdown ofmiR-664b-3p. (A) The mRNA relative expression level of miR-664b-3p in the tissues of LSCC and adjacent normal tissues. (B) The mRNA expression level of miR-664b-3p in the TU177 and TU686 cell lines transfected with miR-664b-3p mimic, inhibitor or NC using reverse transcription-quantitative PCR. (C) Growth curves of the TU177 and TU686 cell lines transfected with miR-664b-3p mimic and inhibitor were performed using an MTS assay. (D) Colony formation assay of the TU177 and TU686 cell lines transfected with miR-664b-3p mimic and inhibitor. Data are presented as the mean ± SD from three independent experiments. **$P<0.01$ vs. NC group. LSCC, laryngeal squamous cell carcinoma; NC, negative control; miR, microRNA.
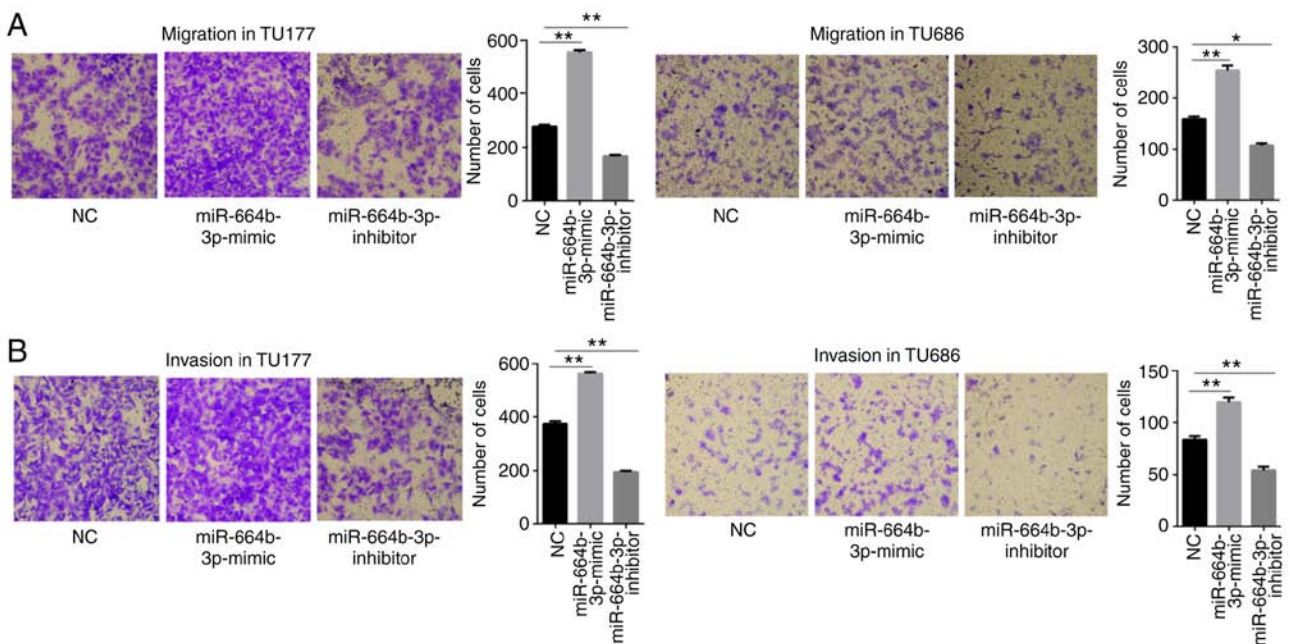

Figure 5. Transwell migration and invasion assays of the TU177 and TU686 cell lines following overexpression and knockdown of miR-664b-3p. (A) Migration and (B) invasion assays of the TU177 and TU686 cell lines following transfection with miR-664b-3p mimic and inhibitor. Data are presented as the mean ± SD from three independent experiments. *$P<0.05$ and **$P<0.01$ vs. NC group. NC, negative control; miR, microRNA.
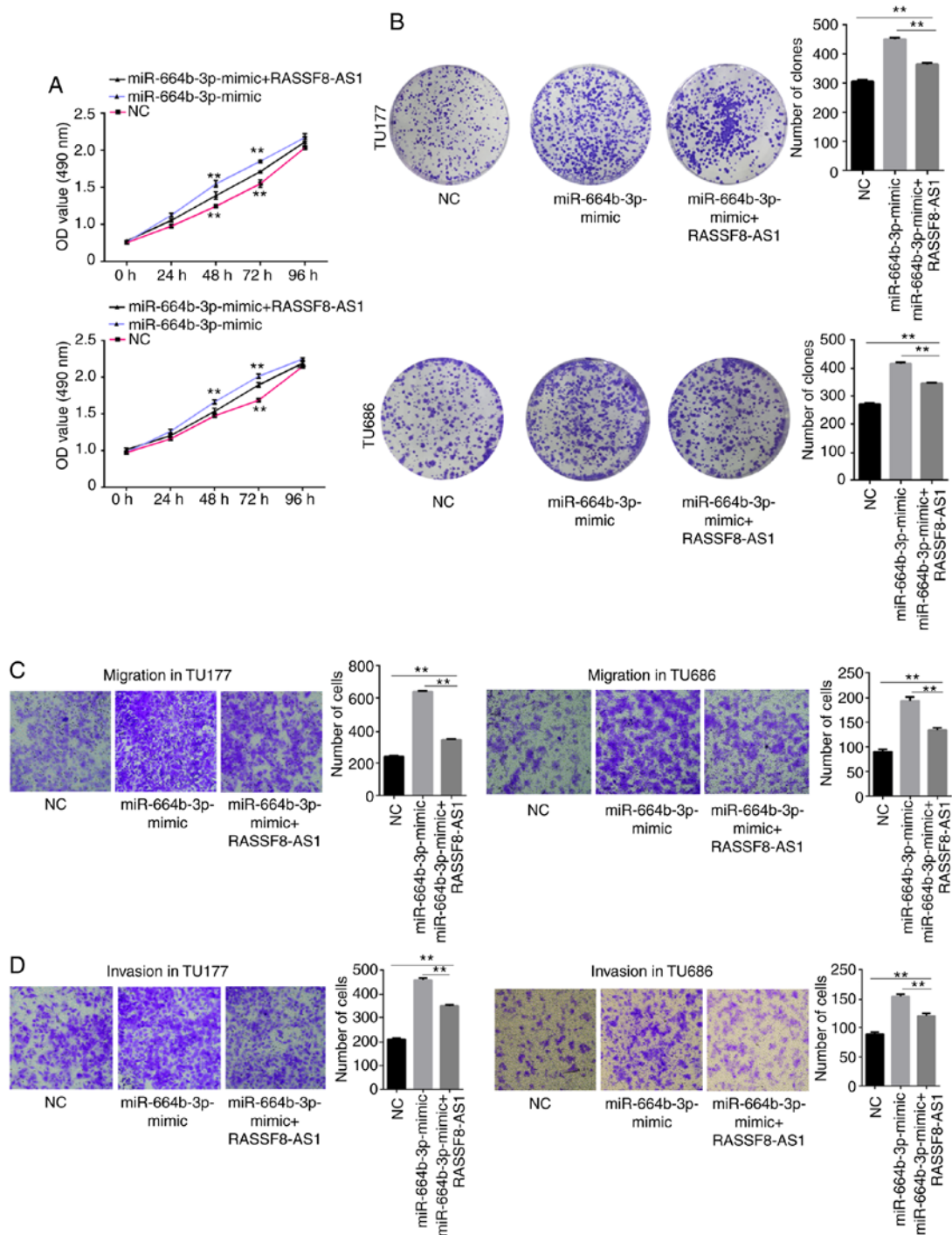
Figure 6. Overexpression of RASSF8-AS1 reverses the stimulatory effect of miR-664b-3p mimic on the LSCC cell lines. Following overexpression of RASSF8-AS1 in the TU177 and TU686 cell lines, notable inhibition of the (A) cell proliferation and (B) colony formation abilities was found, with partial reversal of miR-664b-3p-mimic on cell proliferation and colony formation, using an MTS and colony formation assays, respectively. Following overexpression of RASSF8-AS1 in the TU177 and TU686 cell lines, significant inhibition of cell (C) migration and (D) invasion was found and the partial reversal of the promoting effect of miR-664b-3p mimic on cell migration and invasion. Data are presented as the mean ± SD from three independent experiments. **P<0.01. LSCC, laryngeal squamous cell carcinoma; miR, microRNA.

Overexpression of RASSF8-AS1 partially reverses the promoting effects of miR-664b-3p mimic in regards to LSCC cell proliferation, migration, invasion and colony formation efficiency. From the aforementioned results, it was found that lncRNA RASSF8-AS1 sponges miR-664b-3p and has a negative correlation with miR-664b-3p. Next, the regulatory effect of RASSF8-AS1 on miR-664b-3p was verified using cell functional experiments. NC and pcDNA3.1, miR-664b-3p

mimics and pcDNA3.1 or miR-664b-3p mimic and pcDNA3.1-RASSF8-AS1 were co-transfected into the TU177 and TU686 cell lines. MTS, colony formation, migration and invasion assays were performed and the results showed that the cell proliferation, colony formation, migration and invasion abilities were notably increased with the overexpression of miR-664b-3p, and these were partially reversed by the overexpression of RASSF8-AS1 (Fig. 6A-D). In summary,
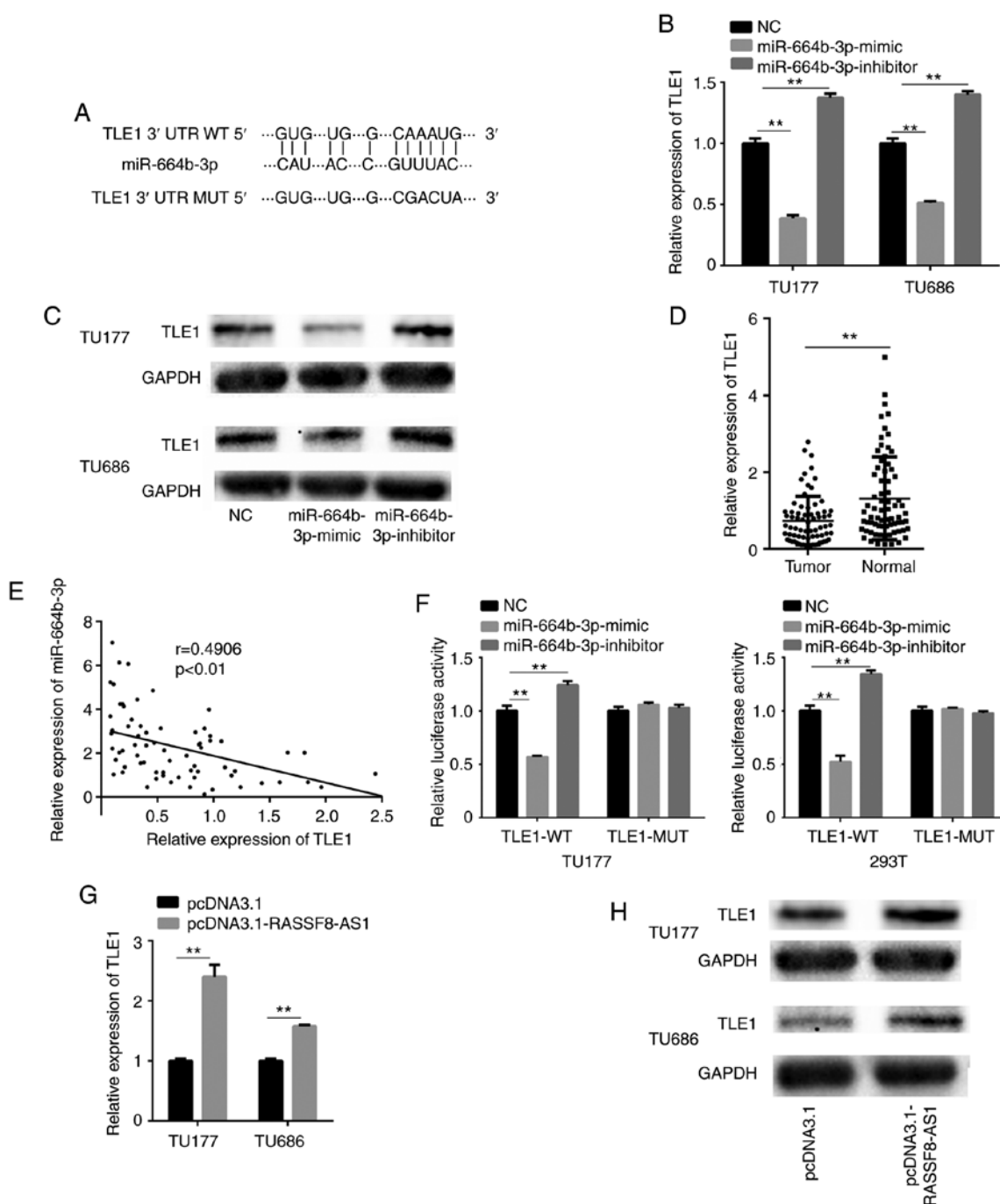
Figure 7. TLE1 is a potential target gene of miR-664b-3p and RASSF8-AS1 regulates TLE1 by binding to miR-664b-3p. (A) The potential binding site of TLE1 and miR-664b-3p predicted using bioinformatic analysis. The TLE1 (B) mRNA and (C) protein expression level in the TU177 and TU686 cell lines transfected with miR-664b-3p mimic and inhibitor performed using RT-qPCR and western blot analysis, respectively. (D) The relative mRNA expression level of TLE1 in LSCC and adjacent normal tissues. (E) Correlation between TLE1 and miR-664b-3p in the LSCC tissues. (F) The firefly luciferase to *Renilla* activity ratio of the TU177 and 293T cell lines transfected with pmirGLO-TLE1-WT or pmirGLO-TLE1-MUT and NC, miR-664b-3p mimic or miR-664b-3p inhibitor in the dual-luciferase assays. The (G) mRNA and (H) protein expression level of TLE1 in the TU177 and TU686 cell lines transfected with pcDNA3.1-RASSF8-AS1 and pcDNA3.1 performed using RT-qPCR and western blot analysis, respectively. Data are presented as the mean ± SD from three independent experiments. **$P<0.01$ vs. NC group. LSCC, laryngeal squamous cell carcinoma; TLE1, transducin-like enhancer of split 1; NC, negative control; WT, wild-type; MUT, mutant; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR.

the results of the cell functional experiments verified that RASSF8-AS1 could inhibit the tumor promotion of LSCC cells by downregulating miR-664b-3p.

TLE1 is a potential target gene of miR-664b-3p. To further investigate the mechanisms underlying the effects of miR-664b-3p on LSCC, a potential target gene for

miR-664b-3p was subsequently determined. The Starbase v3.0 and DIANA tools predicted that the 3'-untranslated region (UTR) of TLE1 aligned with miR-664b-3p (Fig. 7A). To verify their relationship, the mRNA expression level of TLE1 was detected following overexpression and knockdown of miR-664b-3p in the TU177 and TU686 cell lines using RT-qPCR. The results suggested that a significant reduction
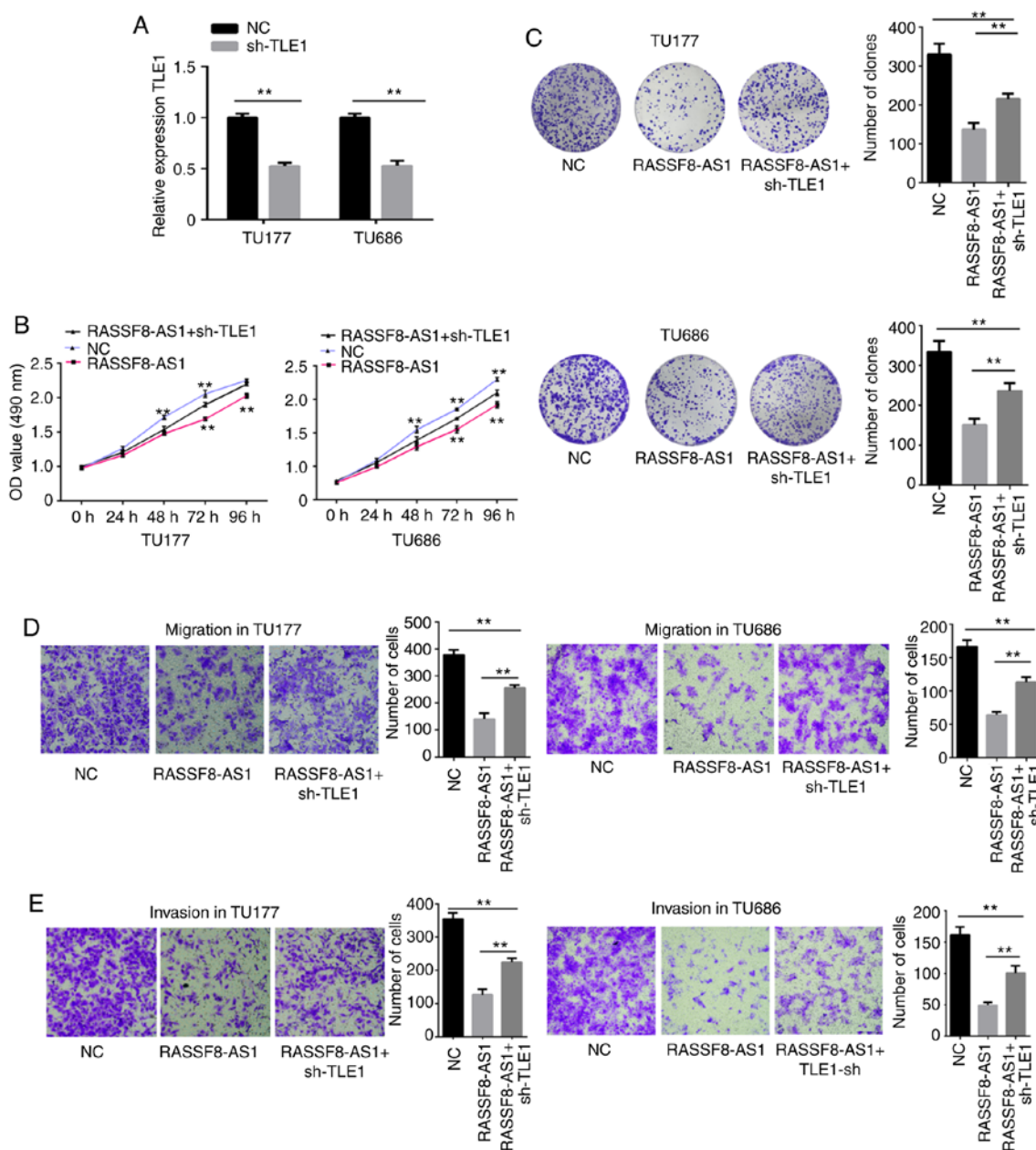
Figure 8. Knockdown of TLE1 reverses the inhibiting effect of pcDNA3.1-RASSF8-AS1 on the LSCC cell lines. (A) The mRNA expression level of TLE1 in the TU177 and TU686 cell lines transfected with sh-TLE1 or pGenesil-1. Following knockdown of TLE1 mRNA expression level in the TU177 and TU686 cell lines, partial reversal of pcDNA3.1-RASSF8-AS1 inhibition was found on the (B) cell proliferation and (C) colony formation abilities in the TU177 and TU686 cell lines, using MTS and colony formation assays, respectively. Following knockdown of TLE1 mRNA expression level in the TU177 and TU686 cell lines, partial reversal of pcDNA3.1-RASSF8-AS1 suppression on (D) cell migration and (E) invasion was found. Data are presented as the mean ± SD from three independent experiments. **P<0.01. sh, short inhibiting; LSCC, laryngeal squamous cell carcinoma; TLE1, transducin-like enhancer of split 1.

in the TLE1 mRNA expression level was induced by over-expressing miR-664b-3p, while the result from miR-664b-3p knockdown had the opposite effect (Fig. 7B). The overexpression of miR-664b-3p in the TU177 and TU686 cell lines transfected with miR-664b-3p mimic significantly decreased the protein expression level of TLE1; however, knockdown of miR-664b-3p notably improved the protein expression level of TLE1 in the LSCC cell lines (Fig. 7C). Detection of the TLE1 mRNA expression level in the LSCC tissues showed a significantly low expression compared with that in the adjacent normal tissues (Fig. 7D) and exhibited a negative correlation with the miR-664b-3p mRNA expression level in LSCC tissues (Fig. 7E). To further investigate whether

miR-664b-3p regulates the expression of TLE1 by binding to the 3'-UTR of TLE1, a luciferase assay was performed. The firefly luciferase activity was reduced in the TU177 and 293T cell lines, co-transfected with miR-664b-3p mimic and pmirGLO-TLE1-WT compared with that in the control group, while co-transfection of miR-664b-3p mimic and pmirGLO-TLE1-MUT did not reduce the firefly luciferase to *Renilla* activity ratio compared with that in the control group. The firefly luciferase to *Renilla* activity ratio was increased in the cell lines, which were co-transfected with the miR-664b-3p inhibitor and pmirGLO-TLE1-WT, but not in cells co-transfected with the MUT vector (Fig. 7F). Taken together, the results suggest that TLE1 is a downstream target

gene of miR-664b-3p, and miR-664b-3p may be involved in downregulating the TLE1 mRNA expression level.

RASSF8-AS1 regulates TLE1 by binding with miR-664b-3p. To verify the regulation of RASSF8-AS1 on TLE1, the transcriptional and protein expression levels were investigated. At the transcriptional level, the TLE1 mRNA expression level was significantly increased by the overexpression of RASSF8-AS1 in the TU177 and TU686 cell lines (Fig. 7G), while the protein expression level was also significantly increased (Fig. 7H). Taken together, these results indicate that RASSF8-AS1 upregulates TLE1 by binding with miR-664b-3p and acts as a ceRNA.

Knockdown of TLE1 partially reverses the suppressive effects of pcDNA3.1-RASSF8-AS1 on LSCC cell proliferation, migration, invasion and colony formation efficiency. The aforementioned results proved that RASSF8-AS1 could regulate TLE1. In the following experiments, the association between RASSF8-AS1 and TLE1 was verified using cell functional experiments. pGenesil-1 and pcDNA3.1, pcDNA3.1-RASSF8-AS1 and pGenesil-1 or sh-TLE1 and pcDNA3.1-RASSF8-AS1 were co-transfected into the TU177 and TU686 cell lines. RT-qPCR analysis showed that TLE1 mRNA expression level was reduced by sh-TLE1 (Fig. 8A). MTS, colony formation, migration and invasion assays were then performed and the results showed that the cell proliferation, colony formation, migration and invasion abilities were notably suppressed following overexpression of RASSF8-AS1, and these were partially reversed by TLE1 knockdown (Fig. 8B-E). In summary, the results of the cell functional experiments verified the association between RASSF8-AS1 and TLE1.

Discussion

The importance of non-coding RNAs (lncRNAs) in regulating tumor progression, as well as other diseases, has become a hot topic, recently, and can be viewed as potential targets for tumor diagnosis, prognosis and treatment targets (19). Recently, lncRNAs have been verified to regulate laryngeal squamous cell carcinoma (LSCC) progression. Gao *et al* (20) found that the low expression of LOC285194 distinguished patients with LSCC from a healthy control group, suggesting that LOC285194 may play an anticancer role in LSCC. In addition, Meng *et al* (21) confirmed that aberrant methylation and low expression of ZNF667-AS1 and ZNF667 may stimulate the progression of LSCC. However, the roles and molecular mechanisms of lncRNAs in LSCC require further elucidation.

In the present study, lncRNA RASSF8-AS1 was found to be expressed at low levels in four LSCC tissues compared with that in paired normal tissues using microarray assays. Then, low mRNA expression level of RASSF8-AS1 was found in the 72 LSCC tissues and 4 LSCC cell lines. In addition, low mRNA expression level of RASSF8-AS1 was associated with well-differentiated, lower lymph node metastasis and lower TNM staging. Furthermore, RASSF8-AS1 was found to play suppressive roles in the progression of LSCC by reducing cell proliferation, colony formation, migration and invasion *in vitro*. The aforementioned results suggest that RASSF8-AS1 may be a biomarker for LSCC invasion and metastasis.

The competitive endogenous RNA (ceRNA) hypothesis is considered to be a novel post-transcriptional approach, which regulates genes by competing with miRNAs (22). The endogenous RNAs, containing mRNAs, long non-coding, pseudogene and circular RNAs, are involved in the development of different types of cancer by competitively binding to miRNAs with miRNA response elements (23,24). For example, Wu *et al* (25) verified that the lncRNA SNHG20 could increasethe expression level of SCGB2A1 to promote prostate cancer migration and invasion by binding with miR-6516-5p. Han *et al* (26) reported that the lncRNA MYOSLID regulated the MCL-1 expression level by sponging miR-29c-3p in gastric cancer and promoted the progression of gastric cancer, as a ceRNA. In the present study, the distribution of RASSF8-AS1 in the 4 LSCC cell lines was found to be in both the cytoplasm and the nucleus, although there was slightly higher expression in the cytoplasm, suggesting that the lncRNA RASSF8-AS1 may act as a ceRNA. Then, bioinformatics analysis revealed that miR-664b-3p was a potential target of RASSF8-AS1, which was confirmed using the dual-luciferase reporter and RIP assays. In addition, correlation analysis using LSCC tissues revealed that RASSF8-AS1 and miR-664b-3p mRNA expression levels were negatively correlated. These results showed that RASSF8-AS1 may act on LSCC by binding with miR-664b-3p as a ceRNA and miR-664b-3p is a downstream target of RASSF8-AS1.

In the present study, it was validated that the expression level of miR-664b-3p was increased in the LSCC tissues. The overexpression of miR-664b-3p promoted the proliferation, migration and invasion abilities of the LSCC cell lines; however, knockdown of miR-664b-3p had the opposite effect. As aforementioned, miR-664b-3p may be a cancer-promoting gene and it could play a carcinogenic role in LSCC. At the same time, cell function rescue assays revealed that overexpression of RASSF8-AS1 partially reversed the increase in proliferation, migration and invasion of the LSCC cell lines by miR-664b-3p mimic. It was also confirmed that RASSF8-AS1 plays a key role in LSCC cells by sponging miR-664b-3p during the progression of LSCC.

In general, lncRNAs play a role on downstream miRNA targets by inhibiting miRNAs in the mechanism of ceRNAs. Therefore, identifying the miRNA target is an important part of the ceRNA network (22). To investigate the downstream target gene of miR-664b-3p, bioinformatics tools were used to reveal that transducin-like enhancer of split 1 (TLE1) is one of the potential miR-664b-3p targets. TLE1 is a member of the Groucho/transducin-like enhancer of split family, acts as a corepressor for numerous transcription factors and is involved in their development. It has been found that abnormal expression of TLE1 may lead to the tumorigenesis and progression of multiple types of tumors (27-29). For example, Brunquell *et al* (30) discovered that TLE1 inhibited Bit1-mediated anoikis and induced Bit1-mediated anoikis might be an effective strategy for breast cancer treatment. Lee *et al* (31) found that in patients with gastric cancer, the TLE1 expression level was also associated with prognosis, indicating that the expression of TLE1 was a prognostic indicator of gastric cancer. In the present study, it was verified that TLE1 mRNA expression level was decreased in LSCC tissues compared with that in the paired normal tissues and there was a negative correlation between TLE1 and miR-664b-3p mRNA

expression levels. Furthermore, to validate that miR-664b-3p targets TLE1, a dual-luciferase reporter assay was used to confirm that the 3'-UTR of TLE1 was the binding site for miR-664b-3p. In addition, it was confirmed that miR-664b-3p regulates TLE1 at the transcription and translation levels in the LSCC cell lines. These results indicate that TLE1 is a downstream target gene of miR-664b-3p. Then, it was confirmed that RASSF8-AS1 could upregulate TLE1, both at the transcription and translation levels in the TU686 and TU177 cell lines. Lastly, cell functional rescue assays revealed that knockdown of TLE1 partially reversed the suppression of proliferation, migration and invasion of the LSCC cell lines by pcDNA3.1-RASSF8-AS1. The aforementioned results confirmed that RASSF8-AS1 regulates TLE1 by reducing miR-664b-3p expression level.

In conclusion, it was verified that lncRNA RASSF8-AS1 is a suppressor gene that reduced the proliferation, migration and invasion abilities of LSCC cell lines by regulating TLE1 via binding with miR-664b-3p, to act as a ceRNA. The present study provides a basis for a further understanding of the role of a ceRNA network in the development of LSCC. RASSF8-AS1 may be a potential important target for the prediction, diagnosis and treatment of LSCC.

Acknowledgements

Funding

Availability of data and materials

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

Author's contributions

BW conceived and designed the experiments. TL performed the experiments, collected the data and wrote the paper. HC and WM recruited the patients and collected the specimens. WCh, LZ, WCu and HY performed experiments and produced the figures. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All procedures performed in this study were in accordance with the ethical standards of the Institutional Research Committee of Hebei Medical University (Hebei, China) and with the Declaration of Helsinki (2008). The present study was approved by the Ethics Committee of Hebei Medical University and the Second Hospital of Hebei Medical University. Informed consent was provided by all the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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