

Original Article

LINC01303 promotes the proliferation and migration of laryngeal carcinoma by regulating miR-200c/TIMP2 axis

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Abstract: Background: It is reported that long non-coding RNA is crucial in many cancer progressions. But the function and regulatory mechanism of LINC01303 in human laryngeal squamous cell carcinoma (LSCC) remains unclear. Hence, this research aims at investigating the biological function and potential mechanism of LINC01303 in LSCC. Methods: Real-time quantitative PCR (qRT-PCR) was applied for the determination of LINC01303, miR-200c and TIMP metalloproteinase inhibitor 2 (TIMP2) expression in LSCC tissues and cell lines. Corresponding experiments were carried out to determine the impacts of LINC01303 on LSCC cell proliferation, apoptosis, migration and invasion. The interaction between LINC01303 and miR-200c was analyzed with bioinformatics analysis and luciferase activity analysis. Results: LINC01303 expression in LSCC tissues was notably higher than that in adjacent normal tissues. High LINC01303 expression was bound up with lymphatic metastasis and advanced clinical stage. In addition, inhibition of LINC01303 by siRNA could evidently block LSCC cell proliferation, induce apoptosis, and inhibit invasion and migration. Mechanically, LINC01303 acted as carcinogenic lncRNA in LSCC by regulating miR-200c/TIMP2 axis. Conclusion: LINC01303 plays a carcinogenic part in LSCC carcinogenesis through regulating miR-200c/TIMP2 axis, which may become a promising target of LSCC therapy.

Keywords: Laryngeal squamous cell carcinoma, long non-coding RNA, LINC01303, TIMP2, miR-200c

Introduction

Laryngeal squamous cell carcinoma (LSCC) is a malignant head and neck tumor, the incidence of which is second only to oral cancer [1]. According to the latest epidemiological statistics of global cancer system, the number of new LSCC cases reached 177,422 by 2018, accounting for 1% of all new cancer cases in the world, and its onset age is gradually become younger [2]. Although the clinical treatment of LSCC has been continuously updated and the prognosis of patients has been improved, some patients will suffer from relapsed disease after treatment, resulting in poor prognosis [3]. Hence, it is urgent to explore the underlying pathogenesis of LSCC to improve its diagnostic and therapeutic molecular mechanisms.

Long-chain non-coding RNA (lncRNA) refers to non-coding RNA with more than 200 nt. In the

early stage of the study, lncRNA was considered as a transcriptional waste product due to its inability to directly encode proteins [4-6]. With the deepening of research, however, more and more studies have found that lncRNA can regulate its downstream target genes to participate in the development of various diseases by acting as microRNA (miR) sponge [7-9]. For example, Jian et al. [10] found that the silence of lncRNA GAS5 can protect sponge H9c2 from hypoxia damage of spongy miR-142-5p, and the interaction between lncRNA NEAT1/miR-204 and lncRNA H19/miR-675 was revealed by Müller et al. [11] in breast cancer. LINC01303 is a newly discovered lncRNA located on human chromosome 14q23.1. It expresses highly in gastric cancer and can mediate miR-101-3p to inhibit the occurrence of gastric cancer [12]. Here, LINC01303 was found to show high expression in LSCC via our analysis on

GSE59652 chip, but the underlying mechanism of LINC01303 in LSCC remains unclear.

Therefore, this research is designed to explore the underlying mechanism of LINC01303 in LSCC and to provide a promising therapeutic target for LSCC treatment.

Materials and methods

Gene Expression Omnibus (GEO) chip analysis

We logged into the GEO (<https://www.ncbi.nlm.nih.gov/gds/>), entered the key words of LSCC and lncRNA, and found the GSE59652 chip after the preliminary search. After downloading the matrix file, we additionally downloaded the platform annotation file GPL13825, annotated the file, and then analyzed the difference of data using limma package of R language, $P=0.05$, $\log FC=0.4$. The relative expressions of LINC01303 in the samples were then extracted and plotted.

Clinical data

From January 2014 to January 2016, a total of 70 patients with LSCC who received treatment in The First Hospital of Jilin University were chosen to be study objects, and all of them underwent surgery. During the surgery, their tumor tissues and adjacent tissues were collected, transported by liquid nitrogen to be sent to the laboratory for testing, and stored at -80°C . In this study, all patients were diagnosed as LSCC by pathological biopsy, and were treated for the first time. No targeted anti-tumor treatment was given to them before this study. Each patient signed the informed consent. This study had been authorized by the Medical Ethics Committee of The First Hospital of Jilin University, and was performed in strict accordance with the *Declaration of Helsinki* [13].

Cell culture and transfection

TU-177, M4E, AMC-HN-8 and TU686LSCC cell lines, and normal human nasopharyngeal epithelial cell line NP69 were purchased from American Type Culture Collection, all of which were then cultured in RPMI-1640 medium (Gibco) comprising 10% FBS (Gibco), with a constant temperature of 37°C with 5% CO_2 . GenePharma (Shanghai, China) designed and synthesized small interfering RNA against

LINC01303 (si-LINC01303) and interfering siRNA (si-NC, as negative control), and constructed overexpressed plasmid LINC01303 (pCDNA3.1-LINC01303). GenePharma (Shanghai, China) was responsible for the synthesis of miR-200c mimics (miR-200c-mimics) and inhibitor (miR-200c-inhibit), and negative control (miR-mimics, miR-inhibit). Transient transfection of cells, and one of the above mimics, plasmid, enzyme inhibitor or siRNA, were transfected by the aid of Lipofectamine® 3000 (Invitrogen, USA). Transfection efficiency was determined using qRT-PCR after 48 hours of transfection.

qRT-PCR

Total RNA of the collected samples (cells, tissues, serum) was extracted with TRIzol reagent and then determined by UV spectrophotometer and agarose gel electrophoresis for the purity, concentration and integrity. Total RNA ($1\text{ }\mu\text{g}$) was reversely transcribed into cDNA (final volume: $20\text{ }\mu\text{L}$) by PrimeScript RT kit (Takara, Dalian, China) under standard conditions with random primers. The SYBR PreMix Ex Taq (Takara) kit and ABI 7500PCR instrument were used for PCR detection. All the operation steps were performed based on kit instructions. U6 was considered as the internal reference of miR, and GAPDH of lncRNA and mRNA. Data collection was conducted based on the cycle threshold (CT) method ($2^{-\Delta\Delta\text{CT}}$) [14].

Cell proliferation detection

In this study, the determination of proliferation adopted Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan), and the procedures were carried out in the light of the kit instructions. Cells were collected after 24 hours of transfection, and the transfected cells were adjusted to 1×10^3 cells per well, and inoculated into 96-well plates. CCK-8 solution ($10\text{ }\mu\text{L}$) was put into each well for 4-hour culture at 37°C at the specified time points (0 h, 24 h, 48 h and 72 h). A microplate reader (Bio-Rad, Hercules, CA, USA) was utilized to measure the absorbance at 450 nm.

Cell invasion and migration detection

A 24-well Transwell chamber (BD Biosciences) with a pore size of $8\text{ }\mu\text{L}$ was used to determine cell invasion and migration capabilities. Ma-

trigel was coated on the upper compartment of Transwell in the invasion experiment, but was not coated on the upper compartment of Transwell in the migration experiment. Methods: the transfected cells (1×10^5 cells/well in 200 μ L serum-free medium) were put in the upper compartment, and a medium comprising 10% FBS (600 μ L) were placed in the lower compartment. The invaded cells were fixed with 70% ethanol for 10 minutes and stained with 0.1% crystal violet (Merck, Darmstadt, Germany) for 15 minutes after 48 hours of incubation. These cells were imaged and counted under a microscope in five randomly selected regions.

Cell apoptosis detection

FACS was utilized to detect apoptosis after transfection. Cells were collected 48 hours after transfection. According to FACS Calibur (BD Biosciences), annexin -V- phycoerythrin (PE) apoptosis detection kit (BD Biosciences, San Jose, California, USA) was applied for the determination of the percentage of apoptotic cells. Flowjo software was applied for data evaluation.

WB detection

Cells in each group were collected after transfection to extract the total protein using RIPA lysis. Protein concentration was determined with BCA, adjusted to 4 μ g/ μ L, isolated by 12% SDS-PAGE, and transferred to PVDF membrane. It underwent Ponceau S staining and was washed after soaking in PBST for 5 min. Then, 5% skimmed milk powder was applied for 2-hour block, and primary antibody (1:1000) was put into it for one-night seal at 4°C. The primary antibody was removed by membrane-washing, and HRP-labeled goat anti-rabbit IgG (1:500) secondary antibody (1:5000) was added for incubation at 37°C for 1 h, and then rinsed with PBS for 3 times, with 5 min each time. Redundant liquid was absorbed from the membrane with filter paper. ECL was used for illumination and development in a dark room. Quantity One software was adopted to scan the protein bands and analyze the grayscale value. Relative expression level of the protein = grayscale value of the target protein band/ β -actin protein band.

Dual luciferase reporter

DNA oligonucleotide and pMiR-Reporter Vector were utilized to construct the report vectors of

LINC01303 wild type/mutant (LINC01303/MUT) and TIMP2 wild type/mutant (TIMP2-WT/MUT). Subsequently, the above two vectors were co-transfected with miR-200c-mimics and negative control (miR-NC), respectively, and then co-transfected into HEK293 cells. After incubation for 24 hours, the collected cells were tested for luciferase activity by the aid of a dual luciferase reporter kit (Promega).

RNA Immunoprecipitation (RIP)

RIP experiments were conducted using EZ-Magna RIP kit (Millipore, USA) in strict accordance with the kit directions. Cells were lysed with RIPA first, and then the whole cell protein extract was incubated with RIP washing buffer comprising magnetic beads bound to anti-AgO2 antibody (Millipool) or mouse immunoglobulin G (IgG) control. Protease K was used to digest the sample protein to extract the immunoprecipitated RNA, and the purified RNA was analyzed using qRT-PCR to prove the existence of binding target.

In vivo study

We purchased ten male athymic BALB/c nude mice (6 weeks old, Vital River, Beijing, China) and put them in a sterile environment with stable humidity and temperature. Totally 3×10^6 AMC-HN-8 cells stably transfected with sh-LINC01303 (n=5) or sh-NC (n=5) were injected into the ventral side of nude mice subcutaneously. The calculation of the tumor volume of nude mice was conducted on the 0th, 7th, 14th, 21st and 28th day of feeding (Volume = $1/2 \times \text{length} \times \text{width}^2$). All mice were executed (euthanized) four weeks later, and the tumors were weighed. The resected tumors were also applied for the detection of miR-200c and TIMP2 expression. The research was conducted with the approval of the medical ethics Committee of The First Hospital of Jilin University and with reference to the Laboratory animal - Guideline for ethical review of animal welfare issued by China in 2018.

Statistical analysis

GraphPad 8 software package was used to draw images required. Data were expressed in the form of mean \pm standard deviation (Means \pm SD), and independent sample t test was applied for intergroup comparison. Comparison among groups adopted one-way analysis of

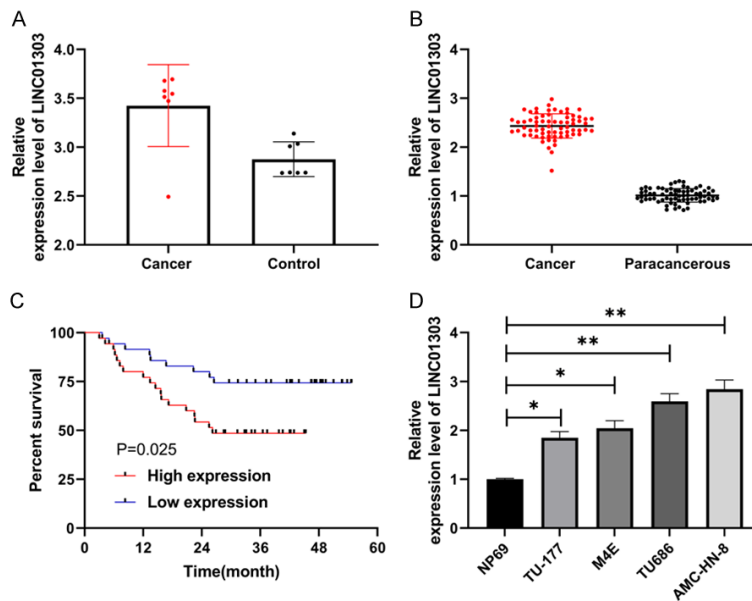


Figure 1. Expression and survival analysis of LINC01303 in LSCC. A. Relative expression of LINC01303 on GSE59652 in GEO database. B. qRT-PCR detected the relative expression of LINC01303 in tumor tissues of LSCC patients. C. K-M survival analysis of the overall survival rate of LSCC patients in high LINC01278 expression group and low LINC01278 expression group. D. qRT-PCR detected the relative expression of LINC01303 in LSCC cells. * denotes $P < 0.05$, ** denotes $P < 0.01$, *** denotes $P < 0.001$.

Table 1. Relationship between LINC01303 and clinical data of LSCC patients

Factors	LINC01303		P value
	High expression (n=35)	Low expression (n=35)	
Gender			0.274
Male (n=52)	28	24	
Female (n=18)	7	11	
Age			0.232
≥ 60 years old (n=56)	26	30	
<60 year old (n=14)	9	5	
Lesion location			0.337
Upper glottis (n=32)	18	14	
Glottis (n=38)	17	21	
Lymphatic metastasis			0.017
Metastasis (n=14)	11	3	
Without metastasis (n=56)	24	32	
Clinical stage			0.029
I+II (n=52)	22	30	
III+IV (n=18)	13	5	

variance, represented with F. LSD-t test was used for afterwards pairwise comparison. Comparison of expression among multiple time

points were analyzed using Repeated Measures, represented by F. Bonferroni was used for back testing. The 5-year survival of patients was plotted using K-M survival curve, and Log-rank test was utilized for analysis. When $P < 0.05$, there was a statistical difference.

Results

LINC01303 is highly expressed in LSCC

At first, we extracted the relative expression data of LINC01303 from GSE59652 chip. Our comparison showed that LINC01303 was highly expressed in LSCC (**Figure 1A**). Then, we detected LINC01303 expression in 70 cases of LSCC patients using qRT-PCR, and found that it was remarkably higher in tumor tissues than in adjacent tissues (**Figure 1B**). In order to further observe the relationship between LINC01303 and pathological data of LSCC patients, patients were grouped (a high expression group and a low expression group) on the basis of the median value of LINC01303. We found that LINC01303 had no obvious correlation with age, gender and tumor location, but was remarkably correlated with lymph node metastasis and cancer stage (**Table 1**). K-M survival analysis indicated that patients with high LINC01303 expression had lower overall survival rate than patients with low expression (**Figure 1C**). Besides, we detected LINC01303 in LSCC cell lines through qRT-PCR

and found its increase (**Figure 1D**), suggesting that LINC01303 might be a potential observation index of LSCC.

Knocking down LINC01303 can inhibit the proliferation, invasion and migration of LSCC cells and induce apoptosis

To verify the mechanism of LINC01303 in LSCC, we first established an inhibitory expression vector of LINC01303 using siRNA (**Figure 2A**), and transfected si-LINC01303#3 into the most significantly different LSCC cells (**Figure 2B**). We further observed cell proliferation, invasion, migration and apoptosis, and found notably inhibited proliferation ability in cells transfected with si-LINC01303#3 in comparison with si-NC through CCK-8 (**Figure 2C, 2D**), and found that the invasion and migration abilities of cells transfected with si-LINC01303#3 were notably weakened compared with si-NC via Transwell test (**Figure 2E, 2F**). In addition, we found through FACS experiment that the apoptosis rate of cells transfected with si-LINC01303 was remarkably higher than that of si-NC (**Figure 2G**). These results indicate that knocking down LINC01303 can effectively promote LSCC cell growth and metastasis, and LINC01303 is expected to become a potential therapeutic target of LSCC.

LINC01303 acts as miR-200c sponge

The above studies have identified the role of LINC01303 in LSCC, but the in-depth mechanism remains unclear. Previous studies supported that lncRNA can participate in tumor occurrence by acting as a sponge of miR. Therefore, we predicted the potential miRs of LINC01303 through starBase online website, and found a targeted relationship between miR-200c and LINC01303 (**Figure 3A**). We applied RIP and dual luciferase activity detection experiments for verification. The results showed that miR-200c and LINC01303 could be precipitated by Ago2 antibody (**Figure 3C**), and miR-200c could inhibit the luciferase activity of LINC01303-wt (**Figure 3B**). Besides, we detected LSCC cells transfected with si-LINC01303#3, and miR-200c relative expression in LSCC cells after transfection of si-LINC01303#3 increased notably (**Figure 3D**). These experiments proved that LINC01303 can act as a sponge for miR-200c. In addition, qRT-PCR indicated that miR-200c was low expressed in tumor tissues of LSCC patients (**Figure 3E**). Furthermore, we found a negative correlation between miR-200c and LINC01303

in tumor tissues of LSCC patients (**Figure 3F**), which also indicated that there might be a regulatory relationship between miR-200c and LINC01303.

miR-200c targets TIMP2

miR participates in LSCC occurrence by mediating downstream target genes [15]. In the above study, we confirmed that LINC01303 acts as miR-200c sponge. However, it is not clear whether miR-200c can regulate the downstream target genes to participate in LSCC. For this reason, we predicted the downstream target genes of miR-200c. Through the joint prediction results of starBase [16], Targetscan [17], miRDB [18], miRDIP [19], TarBase [20], and miRTarBase [21], we found the targeted binding relationship between TIMP2 and miR-200c in all online websites (**Figure 4A**). To verify their relationship, we performed a dual luciferase activity test, which showed that miR-200c-mimics could suppress TIMP2-wt luciferase activity (**Figure 4B, 4C**). We also found through qRT-PCR and WB experiments that TIMP2 mRNA and protein expression in LSCC cells after transfection of miR-200c-mimics was inhibited (**Figure 4D, 4E**). In addition, we detected TIMP2 in tumor tissues of LSCC patients by qRT-PCR and found its remarkable increase (**Figure 4F**). Correlation analysis also suggested that TIMP2 was inversely correlated with miR-200c (**Figure 4H**) and positively correlated with LINC01303 (**Figure 4G**). These experiments also proved that miR-200c regulates TIMP2 in a targeted way. Moreover, co-transfection experiments were conducted to determine the presence of the axis of LINC01303/miR-200c/TIMP2. We found that co-transfection of pcDNA3.1-LINC01303 with miR-200c-mimics or si-TIMP2 inhibits the down-regulation of TIMP2 by miR-200c-mimics or si-TIMP2, suggesting the possible existence of LINC01303/miR-200c/TIMP2 axis.

Up-regulation of LINC01303 mediates miR-200c/TIMP2 axis to promote LSCC

To confirm the presence of LINC01303/miR-200c/TIMP2 axis, we carried out co-transfection experiments, and constructed LSCC cells transfected with pcDNA3.1-NC, pcDNA3.1-LINC01303, miR-200c-mimics+pcDNA3.1-LINC01303, si-TIMP2+pcDNA3.1-LINC01303,

LINC01303 mediates miR-200c/TIMP2 axis to participate in LSCC development

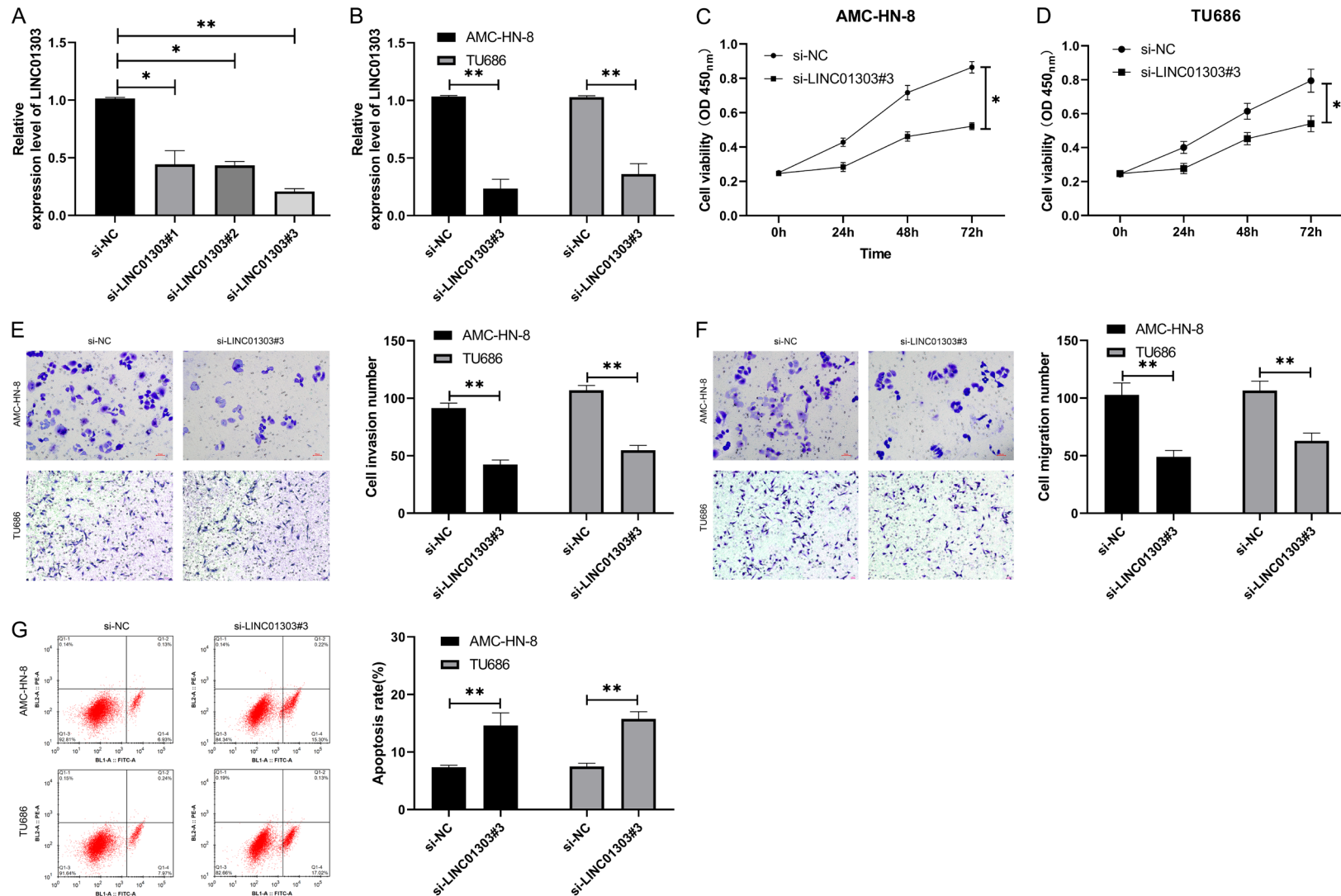


Figure 2. Knocking down LINC01303 can inhibit the development of LSCC. A. qRT-PCR detected the relative expression of LINC01303 in siRNA vector. B. qRT-PCR detected the relative expression of LINC01303 in AMC-HN-8 and TH686 cells after transfection of si-LINC01303#3. C, D. CCK-8 test detected the changes of proliferation ability of AMC-HN-8 and TH 686 cells after transfection of si-LINC01303#3. E, F. Transwell test detected the invasion and migration of AMC-HN-8 and TH 686 cells after transfection of si-LINC01303#3. G. FACS test detected the changes of apoptosis rate of AMC-HN-8 and TH686 cells after transfection of si-LINC01303#3. * denotes $P < 0.05$, ** denotes $P < 0.01$.

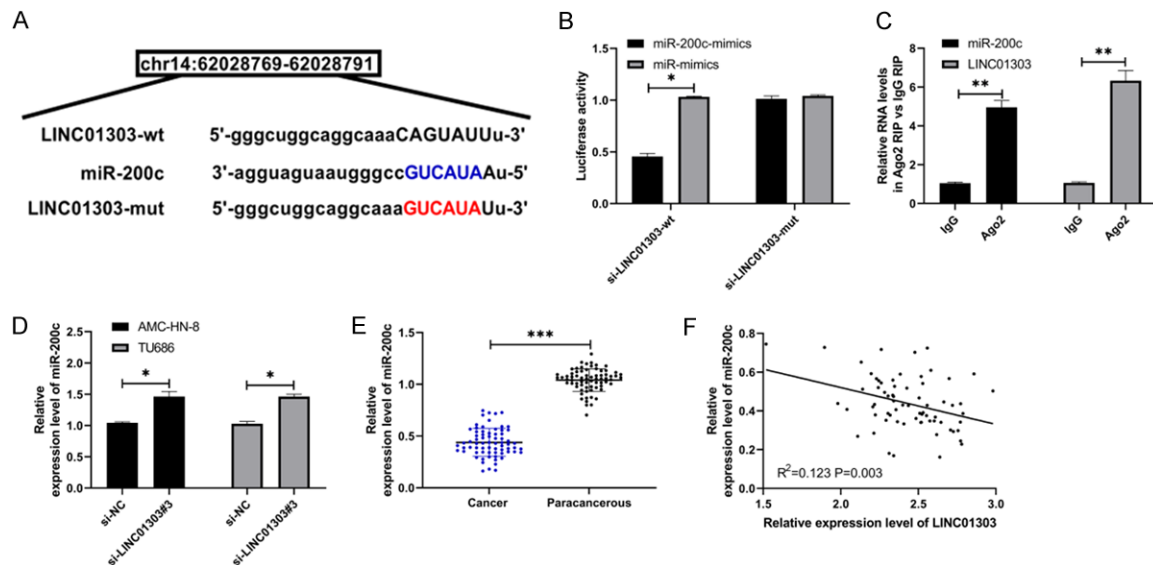


Figure 3. LINC01303 can regulate miR-200c. A. starBase prediction of LINC01303 and miR-200c sites and mutation sites. B. Dual luciferase reporter analysis of the targeted relationship between LINC01303 and miR-200c. C. RIP detected the targeted relationship between LINC01303 and miR-200c. D. qRT-PCR detected the relative expression of miR-200c in LSCC cells transfected with LINC01303#3. E. qRT-PCR detected the relative expression of miR-200c in tumor tissues of LSCC patients. F. Pearson test analyzed the correlation between LINC01303 and miR-200c. * denotes $P<0.05$, ** denotes $P<0.01$, *** denotes $P<0.001$.

respectively, and determined the proliferation, invasion, migration and apoptosis through experiments. According to CCK-8 experiment, LSCC cell proliferation ability was obviously enhanced after transfection of pcDNA3.1-LINC01303 alone (Figure 5A, 5B). Transwell experiment indicated that LSCC cell invasion and migration abilities were evidently enhanced after transfection of pcDNA3.1-LINC01303 alone (Figure 5C, 5D). In FASC experiment, however, the apoptosis rate of LSCC cells decreased notably after the transfection of pcDNA3.1-LINC01303 alone (Figure 5E). Cell proliferation, invasion, migration and apoptosis after co-transfection of miR-200c-mimics or si-TIMP2 with pcDNA3.1-LINC01303 were not significantly different from those of pcDNA3.1-NC, which indicated that LINC01303 mediates miR-200c/TIMP2 axis to promote the occurrence of LSCC.

LINC01303 can inhibit the growth of tumor in nude mice

At the end of the study, in order to determine the situation of LINC01303 *in vivo*, we established an allogeneic tumor model, and injected sh-LINC01303 into nude mice. Compared with sh-NC group, the tumor volume and mass of

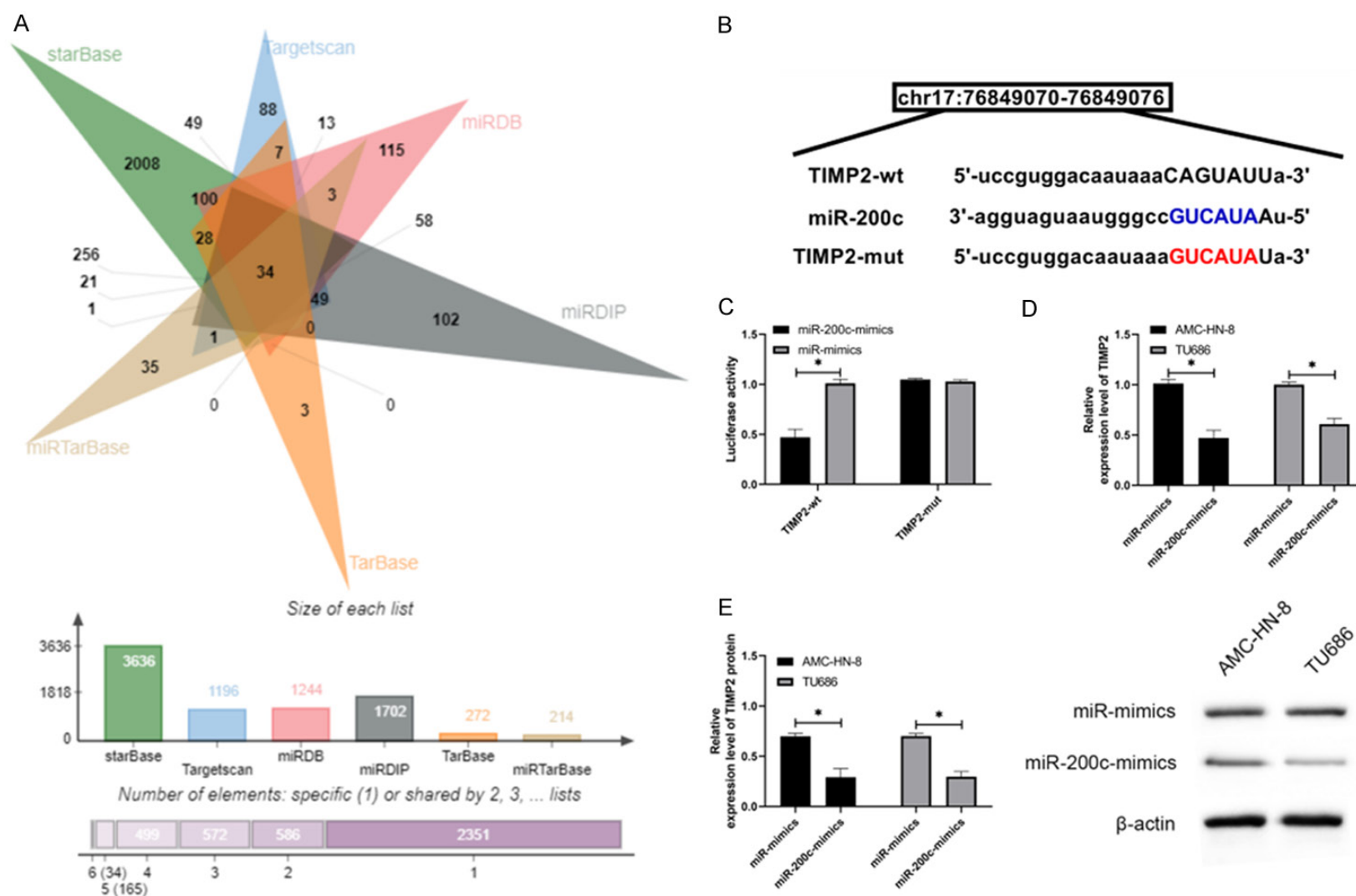
nude mice decreased significantly after the injection of sh-LINC01303 (Figure 6A, 6B). Besides, LINC01303 and TIMP2 relative expression reduced evidently, while miR-200c increased notably, which suggested that LINC01303 can inhibit the growth of LSCC tumor by acting as miR-200c sponge (Figure 6C), and is a potential therapeutic target of LSCC.

Discussion

More and more researches have found that lncRNA plays a vital regulatory part in tumor occurrence and development [22]. Here, we first found the relationship between LINC01303 expression in LSCC patients and clinical indicators. In addition, LINC01303 could regulate LSCC cell growth via mediating miR-200c/TIMP2 axis. These results were consistent with the previously reported progression of LINC01303 in gastric cancer cells.

The mechanism of competing endogenous RNA (ceRNA) has accelerated the research of lncRNA to a certain extent [23]. With the development of research, a growing body of studies supported that lncRNA-miRNA-mRNA plays an important part in regulating the progression of tumors or other diseases [24-26]. In this study,

LINC01303 mediates miR-200c/TIMP2 axis to participate in LSCC development



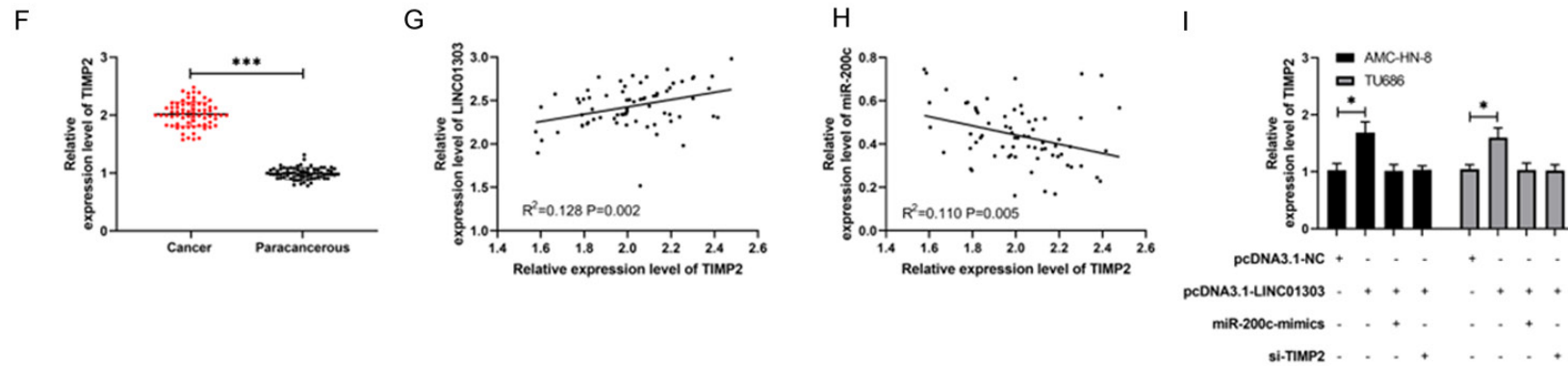
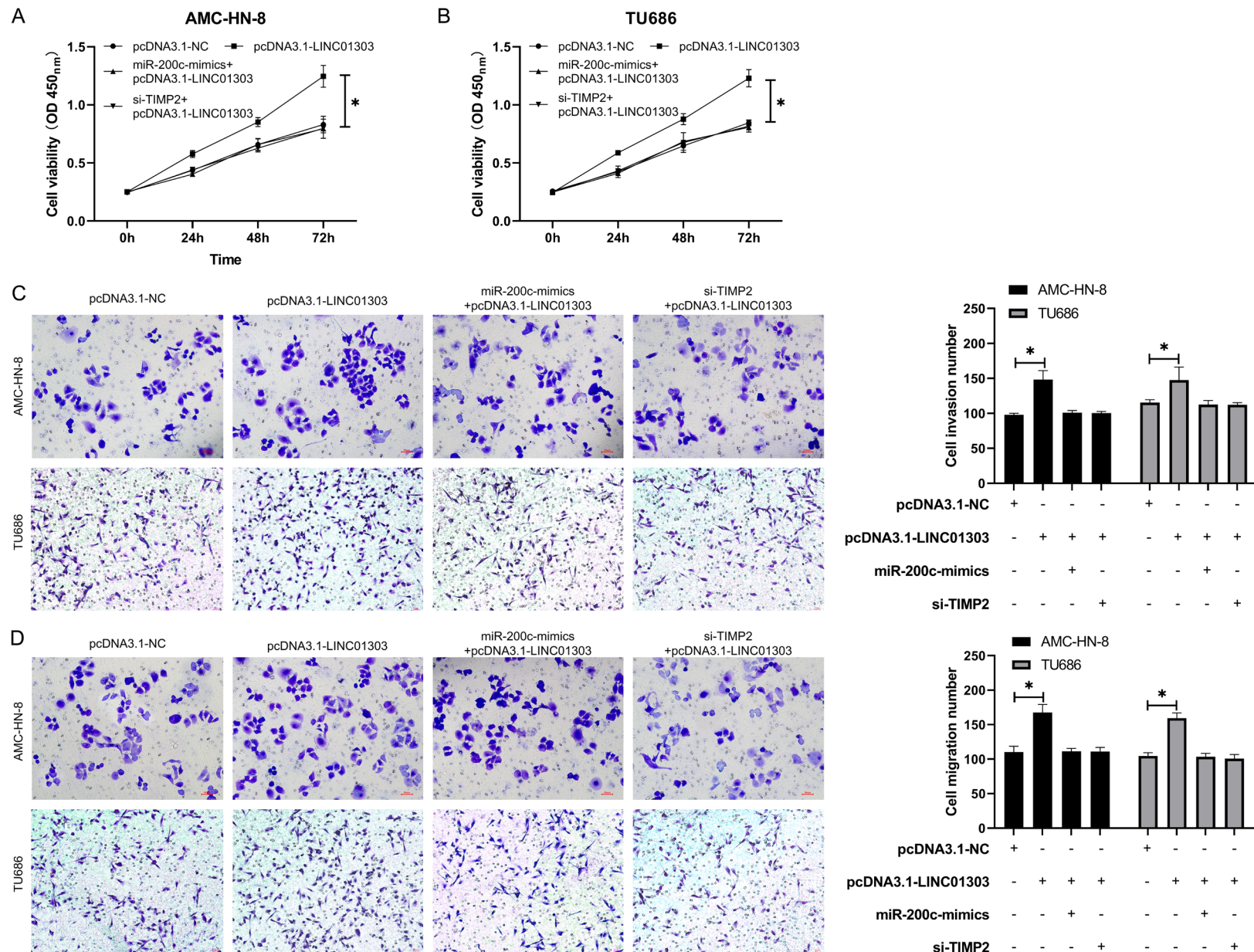


Figure 4. miR-200c can target TIMP2. A. starBase, Targetscan, miRDB, miRDIP, TarBase, miRTarBase predicted the potential binding target genes of miR-200c. B. starBase predicted the targeted binding sites and mutation sites of miR-200c and TIMP2. C. Dual luciferase reporter identified the targeted relationship between miR-200c and TIMP2. D. qRT-PCR detected the relative expression of TIMP2 in LSCC cells transfected with miR-200c-mimics. E. WB detected the relative expression of TIMP2 protein in LSCC cells transfected with miR-200c-mimics. F. qRT-PCR detected the relative expression of TIMP2 in tumor tissues of LSCC patients. G, H. Pearson test analyzed the correlation between TIMP2 with miR-200c and LINC01303. I. qRT-PCR detects the relative expression level of TIMP2 in the cells after co-transfection. * denotes $P<0.05$, *** denotes $P<0.001$.

LINC01303 mediates miR-200c/TIMP2 axis to participate in LSCC development



LINC01303 mediates miR-200c/TIMP2 axis to participate in LSCC development

E

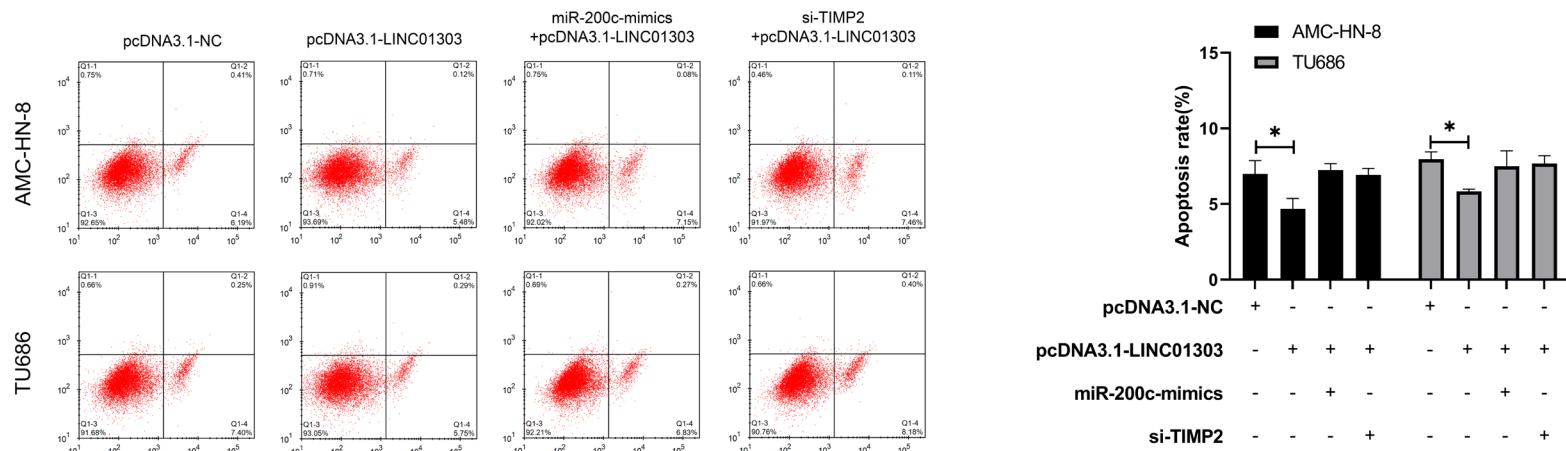
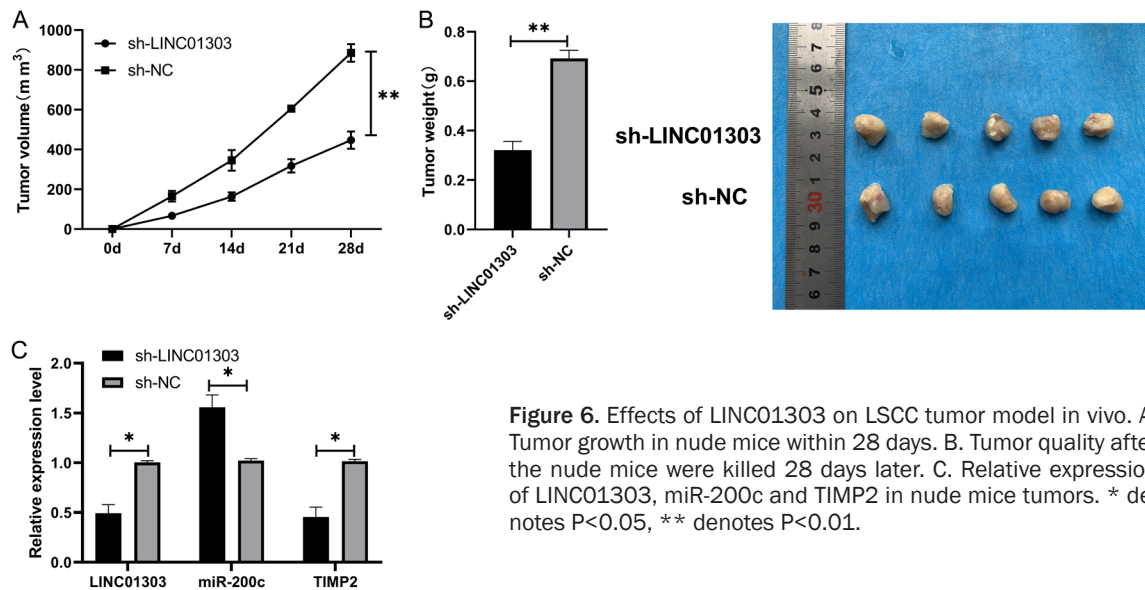


Figure 5. LINC01303 acts as miR-200c sponge to regulate TIMP2 and promote LSCC development. A, B. CCK-8 test detected the changes of proliferation ability of AMC-HN-8 and TH 686 cells after transfection and co-transfection. C, D. Transwell test detected the invasion and migration of AMC-HN-8 and TH686 cells after co-transfection. E. FACS test detected the changes of apoptosis rate of AMC-HN-8 and TH686 cells after co-transfection. * denotes P<0.05.



we found a new lncRNA-miRNA-mRNA model, namely LINC01303-miR-200c-TIMP2 axis. Early studies revealed that miR-200c is a low-expressed tumor suppressor in breast cancer [27], colorectal cancer [28] and gastric cancer [29]. Furthermore, its low expression is involved in the occurrence of LSCC [30]. Here, to further determine the mechanism of miR-200c in LSCC, we predicted its downstream target genes, and found a targeted relationship between TIMP2 and miR-200c through 6 online prediction websites. TIMP2 is a member of the TIMP gene family, which encodes a protein that is a natural inhibitor of matrix metalloproteinases, a group of peptidases involved in the degradation of the extracellular matrix [31]. TIMP2 encoded protein has a unique function of directly inhibiting endothelial cell proliferation in TIMP family members [32]. Therefore, TIMP2 can regulate cell growth. In this study, TIMP2 expression was higher in tumor tissues than in adjacent tissues of LSCC patients, which was in line with the results of an early study [33]. Then, through correlation analysis, we found that TIMP2 was positively correlated with LINC01303 and negatively correlated with miR-200c, which indicated the presence of LINC01303-miR-200c-TIMP2 axis.

At the end of the study, to verify the establishment of LINC01303-miR-200c-TIMP2 axis, we carried out a rescue experiment. By constructing a co-transfected cell model, we found that the malignant phenotypes of LSCC cells were

obviously enhanced after transfection of pcDNA3.1-LINC01303 alone, while the apoptosis was inhibited to some extent. After co-transfection of miR-200c-mimics or si-TIMP2 with pcDNA3.1-LINC01303, the above results were reversed, that is, there was no considerable difference in cell proliferation, invasion, migration and apoptosis compared with pcDNA3.1-NC. Such results indicated that LINC01303-miR-200c-TIMP2 axis is involved in the development of LSCC. In addition, we conducted in vivo experiments. By injecting sh-LINC01303 LSCC cells with stable expression into nude mice, we observed notably smaller tumor volume of nude mice than those of mice injected with sh-NC, and LINC01303 and TIMP2 relative expression in the tumor was remarkably decreased, while the expression of miR-200c was remarkably increased, which further verified our study.

Through the above research, we have determined the relevant mechanism of LINC01303 in LSCC, but there are still some limitations. First, the diagnostic value of LINC01303 in LSCC is unclear, and there are relatively few diagnostic indicators about LSCC at present, we should further explore the diagnostic mechanism of LINC01303 in LSCC. Second, many studies have revealed that lncRNA can participate in the development of tumor by regulating multiple miRs, but it is not clear whether LINC01303 can regulate miRs except miR-200c to participate in the development of LSCC. Therefore, we hope to collect more sam-

ples and conduct bioinformatics analysis in future studies to further investigate the mechanism of LINC01303 in LSCC, so as to supplement our research conclusions.

In conclusion, LINC01303 is highly expressed in LSCC and can participate in the development of LSCC by mediating miR-200c/TIMP2 axis, which is a potential therapeutic target in clinic.

Disclosure of conflict of interest

None.

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References

- [1] Thompson LD. Laryngeal dysplasia, squamous cell carcinoma, and variants. *Surg Pathol Clin* 2017; 10: 15-33.
- [2] Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2018; 68: 394-424.
- [3] Fang R, Huang Y, Xie J, Zhang J and Ji X. Down-regulation of miR-29c-3p is associated with a poor prognosis in patients with laryngeal squamous cell carcinoma. *Diagn Pathol* 2019; 14: 109.
- [4] Zhuo LA, Wen YT, Wang Y, Liang ZF, Wu G, Nong MD and Miao L. LncRNA SNHG8 is identified as a key regulator of acute myocardial infarction by RNA-seq analysis. *Lipids Health Dis* 2019; 18: 201.
- [5] Wu X, Xiao Y, Zhou Y, Zhou Z and Yan W. LncRNA SNHG20 promotes prostate cancer migration and invasion via targeting the miR-6516-5p/SCGB2A1 axis. *Am J Transl Res* 2019; 11: 5162-5169.
- [6] Tu C, Ren X, He J, Zhang C, Chen R, Wang W and Li Z. The value of LncRNA BCAR4 as a prognostic biomarker on clinical outcomes in human cancers. *J Cancer* 2019; 10: 5992-6002.
- [7] Zhang Z, Qian W, Wang S, Ji D, Wang Q, Li J, Peng W, Gu J, Hu T, Ji B, Zhang Y, Wang S and Sun Y. Analysis of LncRNA-associated ceRNA network reveals potential LncRNA biomarkers in human colon adenocarcinoma. *Cell Physiol Biochem* 2018; 49: 1778-1791.
- [8] Yue B, Liu C, Sun H, Liu M, Song C, Cui R, Qiu S and Zhong M. A positive feed-forward loop between LncRNA-CYTOR and Wnt/beta-catenin signaling promotes metastasis of colon cancer. *Mol Ther* 2018; 26: 1287-1298.
- [9] Yang Q, Wan Q, Zhang L, Li Y, Zhang P, Li D, Feng C, Yi F, Zhang L, Ding X, Li H and Du Q. Analysis of LncRNA expression in cell differentiation. *RNA Biol* 2018; 15: 413-422.
- [10] Du J, Yang ST, Liu J, Zhang KX and Leng JY. Silence of LncRNA GAS5 protects cardiomyocytes H9c2 against hypoxic injury via sponging miR-142-5p. *Mol Cells* 2019; 42: 397-405.
- [11] Muller V, Oliveira-Ferrer L, Steinbach B, Pantel K and Schwarzenbach H. Interplay of LncRNA H19/miR-675 and LncRNA NEAT1/miR-204 in breast cancer. *Mol Oncol* 2019; 13: 1137-1149.
- [12] Cao C, Xu Y, Du K, Mi C, Yang C, Xiang L, Xie Y and Liu W. LINC01303 functions as a competing endogenous RNA to regulate EZH2 expression by sponging miR-101-3p in gastric cancer. *J Cell Mol Med* 2019; 23: 7342-7348.
- [13] Issue Information-declaration of Helsinki. *J Bone Miner Res* 2018; 33: BM i-BM ii.
- [14] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) method. *Methods* 2001; 25: 402-408.
- [15] Wang J, Yang S, Ge W, Wang Y, Han C and Li M. MiR-613 suppressed the laryngeal squamous cell carcinoma progression through regulating PDK1. *J Cell Biochem* 2018; 119: 5118-5125.
- [16] Li JH, Liu S, Zhou H, Qu LH and Yang JH. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. *Nucleic Acids Res* 2014; 42: D92-97.
- [17] Agarwal V, Bell GW, Nam JW and Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. *Elife* 2015; 4: e05005.
- [18] Liu W and Wang X. Prediction of functional microRNA targets by integrative modeling of microRNA binding and target expression data. *Genome Biol* 2019; 20: 18.
- [19] Tokar T, Pastrello C, Rossos AEM, Abovsky M, Hauschild AC, Tsay M, Lu R and Jurisica I. mirDIP 4.1-integrative database of human microRNA target predictions. *Nucleic Acids Res* 2018; 46: D360-D370.
- [20] Karagkouni D, Paraskevopoulou MD, Chatzopoulos S, Vlachos IS, Tastsoglou S, Kanellos I, Papadimitriou D, Kavakiotis I, Maniou S, Skoufos G, Vergoulis T, Dalamagas T and Hatzigeorgiou AG. DIANA-TarBase v8: a decade-long collection of experimentally supported miRNA-gene interactions. *Nucleic Acids Res* 2018; 46: D239-D245.
- [21] Chou CH, Shrestha S, Yang CD, Chang NW, Lin YL, Liao KW, Huang WC, Sun TH, Tu SJ, Lee

- WH, Chiew MY, Tai CS, Wei TY, Tsai TR, Huang HT, Wang CY, Wu HY, Ho SY, Chen PR, Chuang CH, Hsieh PJ, Wu YS, Chen WL, Li MJ, Wu YC, Huang XY, Ng FL, Buddhakosai W, Huang PC, Lan KC, Huang CY, Weng SL, Cheng YN, Liang C, Hsu WL and Huang HD. miRTarBase update 2018: a resource for experimentally validated microRNA-target interactions. *Nucleic Acids Res* 2018; 46: D296-D302.
- [22] Chen QN, Wei CC, Wang ZX and Sun M. Long non-coding RNAs in anti-cancer drug resistance. *Oncotarget* 2017; 8: 1925-1936.
- [23] Qi X, Zhang DH, Wu N, Xiao JH, Wang X and Ma W. ceRNA in cancer: possible functions and clinical implications. *J Med Genet* 2015; 52: 710-718.
- [24] Yang J, Qiu Q, Qian X, Yi J, Jiao Y, Yu M, Li X, Li J, Mi C, Zhang J, Lu B, Chen E, Liu P and Lu Y. Long noncoding RNA LCAT1 functions as a ceRNA to regulate RAC1 function by sponging miR-4715-5p in lung cancer. *Mol Cancer* 2019; 18: 171.
- [25] Gong J, Jiang H, Shu C, Hu MQ, Huang Y, Liu Q, Li RF and Wei YZ. Integrated analysis of circular RNA-associated ceRNA network in cervical cancer: observational study. *Medicine (Baltimore)* 2019; 98: e16922.
- [26] Abdollahzadeh R, Daraei A, Mansoori Y, Sepahvand M, Amoli MM and Tavakkoly-Bazzaz J. Competing endogenous RNA (ceRNA) cross talk and language in ceRNA regulatory networks: a new look at hallmarks of breast cancer. *J Cell Physiol* 2019; 234: 10080-10100.
- [27] Damiano V, Brisotto G, Borgna S, di Gennaro A, Armellin M, Perin T, Guardascione M, Maestro R and Santarosa M. Epigenetic silencing of miR-200c in breast cancer is associated with aggressiveness and is modulated by ZEB1. *Genes Chromosomes Cancer* 2017; 56: 147-158.
- [28] Heydari K, Saidijam M, Sharifi MR, Dermani FK, Soleimani Asl S, Shabab N and Najafi R. The effect of miR-200c inhibition on chemosensitivity (5- FluoroUracil) in colorectal cancer. *Pathol Oncol Res* 2018; 24: 145-151.
- [29] Li Y, Nie Y, Tu S, Wang H, Zhou Y, Du Y, Cao J and Ye M. Epigenetically deregulated miR-200c is involved in a negative feedback loop with DNMT3a in gastric cancer cells. *Oncol Rep* 2016; 36: 2108-2116.
- [30] Fu D, Huang Y and Gao M. Hsa_circ_0057481 promotes laryngeal cancer proliferation and migration by modulating the miR-200c/ZEB1 axis. *Int J Clin Exp Pathol* 2019; 12: 4066-4076.
- [31] Ni J, Liu X, Yin Y, Zhang P, Xu YW and Liu Z. Exosomes derived from TIMP2-modified human umbilical cord mesenchymal stem cells enhance the repair effect in rat model with myocardial infarction possibly by the Akt/Sfrp2 pathway. *Oxid Med Cell Longev* 2019; 2019: 1958941.
- [32] Yang JZ, Bian L, Hou JG and Wang HY. MiR-550a-3p promotes non-small cell lung cancer cell proliferation and metastasis through down-regulating TIMP2. *Eur Rev Med Pharmacol Sci* 2018; 22: 4156-4165.
- [33] Wu TY, Zhang TH, Qu LM, Feng JP, Tian LL, Zhang BH, Li DD, Sun YN and Liu M. MiR-19a is correlated with prognosis and apoptosis of laryngeal squamous cell carcinoma by regulating TIMP-2 expression. *Int J Clin Exp Pathol* 2014; 7: 56-63.