

## Original Article

# MicroRNA-708-5p contributes to the malignant behavior of laryngeal squamous cell carcinoma by directly targeting metastasis suppressor-1

Yue Wang<sup>1</sup>, Dan Zhang<sup>1</sup>, Chunhua Yu<sup>1</sup>, Lihua Dong<sup>2</sup>, Junyi Zhang<sup>1</sup>, Peng Wang<sup>1</sup>, Xia Wang<sup>1</sup>, Dongmei Zhao<sup>3</sup>, Linhong Yang<sup>4</sup>

<sup>1</sup>Department of Otolaryngology, Daqing Oilfield General Hospital, Daqing 163001, Heilongjiang Province, China; <sup>2</sup>Intensive Care Unit, The First Hospital of Jilin University, Changchun 130021, Jilin Province, China; <sup>3</sup>Department of Otolaryngology, Changchun Central Hospital, Changchun 130021, Jilin Province, China; <sup>4</sup>Department of Otolaryngology, The First Affiliated Hospital of Jiamusi University, Jiamusi 154000, Heilongjiang Province, China

Received November 26, 2017; Accepted September 5, 2018; Epub January 15, 2019; Published January 30, 2019

**Abstract:** Laryngeal squamous cell carcinoma (LSCC) is one of malignant head and neck squamous cell carcinomas and has been demonstrated to be the second leading cause of mortality. MicroRNAs (miRs) has been reported to be deregulated in many diseases, especially cancers, and demonstrated to be oncogenes or tumor suppressor genes in some cancers. In our research, we explored the role of miR-708-5p in the progression of LSCC. Firstly, by quantitative PCR, we found that compared with non-tumor tissues miR-708-5p is upregulated in LSCC tissues. To clarify the mechanism, we carried out cell proliferation assay, cell wound healing assays. In the further study, we found that the down-regulation of miR-708-5p contributes to the inhibition of cell proliferation and colony formation in LSCC cell line Hep2. On the other hand, miR-708-5p promoted cell migration in cell wound healing assay. One of the tumor suppressor gene, metastasis suppressor-1 (MTSS1) was identified to be a direct target gene of miR-708-5p. Furthermore, MTSS1 was down-regulated in LSCC tissues. The low level of miR-708-5p leads to high level of MTSS1 and decrease in cell migration and proliferation. In conclusion, these results suggested that miR-708-5p promotes tumor growth and migration by directly targeting MTSS1. The identification may provide novel targets for LSCC treatment and improve prognosis.

**Keywords:** microRNA, laryngeal squamous cell carcinoma (LSCC), proliferation, migration, metastasis suppressor-1

## Introduction

As the most common form of laryngeal carcinoma, laryngeal squamous cell carcinoma (LSCC) accounts for a quarter of all head and neck squamous cell carcinomas [1]. Furthermore, among all head and neck squamous cell carcinomas, LSCC is also an aggressive malignancy that has the second highest rate of morbidity [2, 3]. Until now, the pathological mechanism is still poorly understood. The therapeutic treatments include surgery, chemotherapy and radiotherapy, but the 5-year overall survival rate of patients is still low [4]. Therefore, to understand the mechanism of malignant behaviors of laryngeal carcinoma cell is very important for finding novel treatment strategies.

MicroRNAs (MiR) are a group of endogenous, non-coding RNAs with less nucleotides, that mediate mRNA cleavage and translational repression or destabilization [5] by pairing with the 3'-untranslated regions (3'UTRs) of their target genes. Many miRs can regulate about 30% protein-coding genes in humans. MiRs have been found to serve a significant role in many biological processes, including cell proliferation, cell cycle progression, apoptosis, survival, motility, invasion, metastasis, angiogenesis and morphogenesis. In many types of human cancer, deregulation of miRs has been demonstrated as they have been demonstrated to be oncogenes or tumour suppressor genes. These findings suggested that miRs may be novel therapeutic targets for cancer therapy. In LSCC, miRs have been found to be deregulated [6].

**Table 1.** Primers in the research

Name	Sequence
miR-708-5p F	5'-CGG CGG AAG GAG CTT ACA ATC TA-3'
miR-708-5p R	5'-GTG CAG GGT CCG AGG-3'
miR-708-5p mimics	5'-AAG GAG CUU ACA AUC UAG CUG GG-3'
miR-708-5p inhibitor	5'-CCC AGC UAG AUU GUA AGC UCC UU-3'
NC	5'-UUC UCC GAA CGU GUC ACG UTT-3'
U6 F	5'-CTCGCTTCGGCAGCAC-3'
U6 R	5'-AACGCTTCACGAATTTGCGT-3'
MTSS1 F	5'-TAAAGAGGAGTTTGTGGTTGT-3'
MTSS1 R	5'-ACAATCCTAACATCAAAAATAAA-3'
$\beta$ -actin F	5'-TGACGTGGACATCCGCAAAG-3'
$\beta$ -actin R	5'-CTGGAAGGTGGACAGCGAGG-3'

Previous reports have demonstrated that miR-708-5p is deregulated in laryngeal carcinoma patients [7]. Furthermore, miR-708-5p has been reported to be important in invasion of cancer cells [8]. However, the role of miR-708-5p in laryngeal carcinoma has not been well clarified. In our research, we found that miR-708-5p was deregulated in LSCC, which contributes to the malignant behavior of LSCC cells.

## Materials and methods

### *Clinical specimens*

LSCC tissues and their corresponding adjacent nonneoplastic tissues were obtained from 24 patients with LSCC undergoing surgery resection at Daqing Oilfield General Hospital, (Daqing, China). All patients had not received other therapies, including radiotherapy and chemotherapy, prior to surgery. Tissue samples were immediately preserved in liquid nitrogen following excision from patients and subsequently transferred to a -80°C refrigerator until use. The present study was approved by the Human Research Ethics Committee of Daqing Oilfield General Hospital. Written informed consent was obtained from all patients used in the present study, as well as their clinicopathological features.

### *Cell culture and transfection*

The LSCC cell line (HEp2) were obtained from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal

bovine serum (FBS), and 1% (v/v) penicillin/streptomycin (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

24 h before transfection, HEp2 cells were seeded into plates. HEp2 cells were transfected with miR-708-5p mimics, inhibitor and negative control (NC) miRNA, or cotransfected with luciferase reporter plasmid [pGL3-MTSS1-3'UTR-wild-type (Wt) or pGL3-MTSS1-3'UTR-mutated (Mut)] using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to

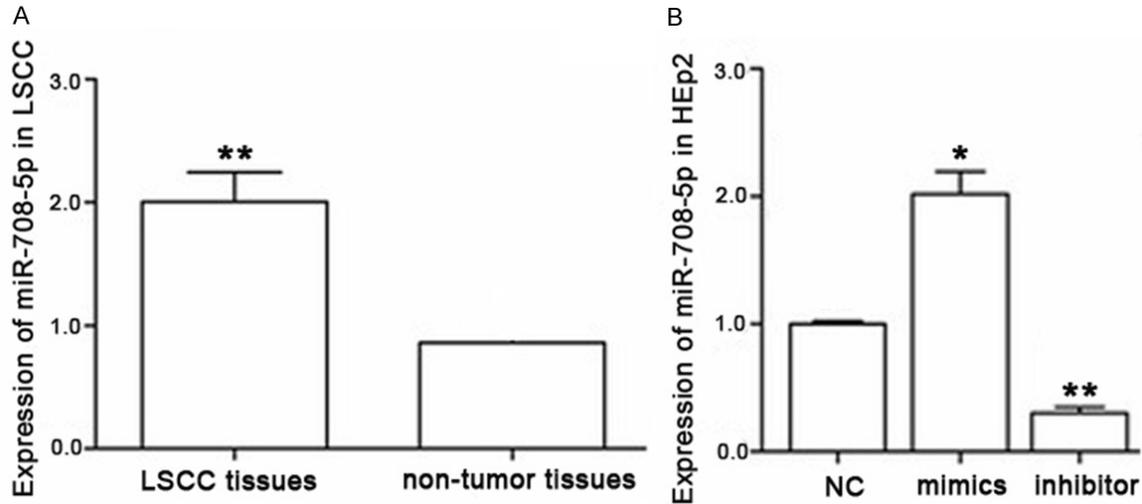
the manufacturer's protocol. The sequence of were listed in **Table 1**. MicroRNA mimics and luciferase report plasmid were synthesized and obtained from Shanghai GenePharma Company (Shanghai, China).

### *RNA isolation and reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis*

Total RNA was isolated from LSCC tissues, adjacent non-neoplastic tissues and cells using Trizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Reverse transcription was performed using the Moloney Murine Leukemia Virus Reverse Transcription system (Promega Corporation, Madison, WI, USA). qPCR was subsequently performed using SYBR® Premix Ex Taq™ II (Takara, China) and the ABI 7300 Real-Time PCR detection system, according to the manufacturer's protocol. The thermocycling conditions for qPCR was as follows: 5 min at 95°C; followed by 40 cycles of 95°C for 30 sec and 65°C for 45 sec. The primer sequences used for qPCR were listed in **Table 1**. Results were quantified using the 2- $\Delta\Delta$ Cq method (21).

### *Western blot analysis*

A total of 72 h following transfection, cells were lysed in cold Radio-immunoprecipitation Assay Buffer (Beyotime Institute of Biotechnology, Haimen, China) containing protease and phosphatase inhibitors. Bicinchoninic Acid Assay kit (Beyotime Institute of Biotechnology) was used to determine the protein concentrations. Equal amounts of protein were subjected to SDS-



**Figure 1.** Expression of miR-708-5p in LSCC tissues. (A) miR-708-5p was upregulated in LSCC tissues compared with their corresponding adjacent non-tumor tissues.  $*P < 0.05$  (B) miR-708-5p was down-regulated in HEP2 cells transfected with miR-708-5p inhibitor, and up-regulated by transfection with mimics compared with cells transfected with the NC.  $*P < 0.05$  vs. the NC-transfected cells. LSCC, laryngeal squamous cell carcinoma; NC, negative control.

PAGE and subsequently transferred to polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk at room temperature for 2 h, and subsequently incubated with primary antibodies against MTSS1 (1:1,000 dilution; catalog: #9139; Cell Signaling Technology, Inc., Danvers, MA, USA) and  $\beta$ -actin (1:1,000 dilution; sc-130065; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4°C overnight. Membranes were washed with TBS containing 0.1% Tween-20 and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (1:5,000).

#### Cell counting Kit-8 (CCK8) assay

Cell Counting Kit-8 (CCK-8) assay (Dojindo; Kumamoto, Japan) was used for evaluating the role of miR-708-5p on LSCC cell proliferation. According to the manufacturer's protocol, about  $1 \times 10^3$  transfected cells were plated in 96-well plates in triplicate and cultured in 90  $\mu$ L medium containing 10% FBS. The ultimate density of CCK-8 is 10%. Cells were incubated for 2 hours at 37°C at each indicated time points. The optical density at 450 nm for each well was measured using an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proliferation rates were determined at 0, 24, 48, and 72 hours after transfection.

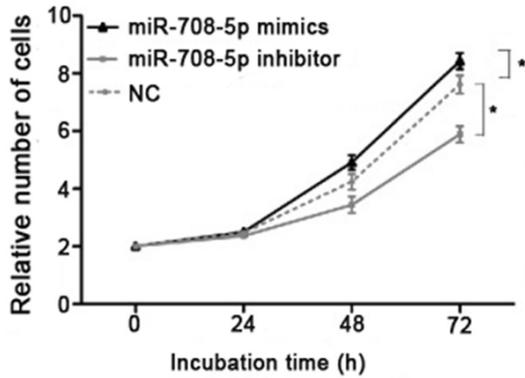
#### Colony formation and cell wound healing assay

Five hundred gastric cancer cells per well were seeded in 6-well plates. After two weeks, colonies were fixed with methanol containing 0.2% crystal violet, and the number of colonies was counted.

Wound healing assay was carried out to test the mobility of HEP2. Cells were trypsinized and seeded equally in 6-well cell culture plates and grew to confluence in 48 h. By using a sterile 100  $\mu$ L pipette tip, artificial homogenous wound in monolayer were created. Cell culture plate was washed with serum-free medium. Cells of migrating into the wound were counted at 0 h and 24 h using an inverted microscope (40 $\times$ ).

#### Bioinformatic analysis and luciferase reporter assay

The potential targets of miR-708-5p were analyzed using TargetScan (<http://www.targetscan.org/>). To generate pGL3-MTSS1-3'UTR plasmid, the human MTSS1 3'-UTR was amplified and cloned into the XbaI site of the pGL3-control vector (Invitrogen), which is in downstream of the luciferase gene. Otherwise, as a control, pGL3-MTSS1-3'UTR-mut plasmids were constructed using cDNA fragments containing mutated nucleotides for miR-708-5p. For the luciferase reporter assay, HEP2 were co-trans-



**Figure 2.** Decrease of miR-708-5p inhibit HEp2 cell growth. The expression of miR-708-5p was detected by qRT-PCR after the transfection of miR-708-5p mimics or miR-708-5p inhibitor. The Cell Counting kit-8 assay demonstrated that miR-708-5p increased cell growth in HEp2 cells. \* $P < 0.05$  vs. the NC-transfected cells. miR, microRNA; NC, negative control.

ected with 100 ng of the luciferase reporter vectors and 20 p mol of control mimic, miR-708 mimic using Lipofectamine 2000 (Invitrogen). The  $\beta$ -actin promoter Renilla luciferase reporter was used for normalization. After 48 h, luciferase activity was analyzed using the Dual-Luciferase Assay System (Promega).

#### Statistical analysis

All of the statistical analyses were carried out with SPSS 16.0 (SPSS, Chicago, IL), by Student's t-test and ANOVA. Data are expressed as the mean  $\pm$  standard deviation (SD). All of results are derived from experiments performed in triplicate. A difference was accepted as significant if  $P < 0.05$ .

## Results

### miR-708 is upregulated in LSCC

The expression of miR-708 in laryngeal carcinoma tissues and corresponding adjacent non-neoplastic tissues was tested using quantitative RT-PCR. The results showed that miR-708-5p expression in laryngeal carcinoma was significantly higher than healthy controls (**Figure 1A**). The results indicated that miR-708-5p may serve important roles in LSCC.

To investigate the roles of miR-708-5p in LSCC cells, miR-708-5p mimic and inhibitor were transfected into HEp2 cells using Lipofectamine

2000. To assess the transfection efficiency, RT-qPCR was performed following transfection. As presented in **Figure 1B**, miR-708-5p was significantly down-regulated in cells transfected with miR-708-5p inhibitor compared with cells transfected with the NC ( $P < 0.05$ ).

### Decrease of miR-708-5p inhibit HEp2 cell growth and proliferation assay

The CCK8 assay was performed to figure out the role of miR-708-5p in laryngeal carcinoma. As presented in **Figure 2**, we transfected HEp2 cells with miR-708 mimic or miR-708 inhibitor. Decrease of miR-708-5p significantly decreased cell growth in HEp2 cells compared with the NC ( $P < 0.05$ ). Colony formation assays indicated that the number of colonies in HEp2 cells transfected with antagomirs group was lower than the control (**Figure 3A**).

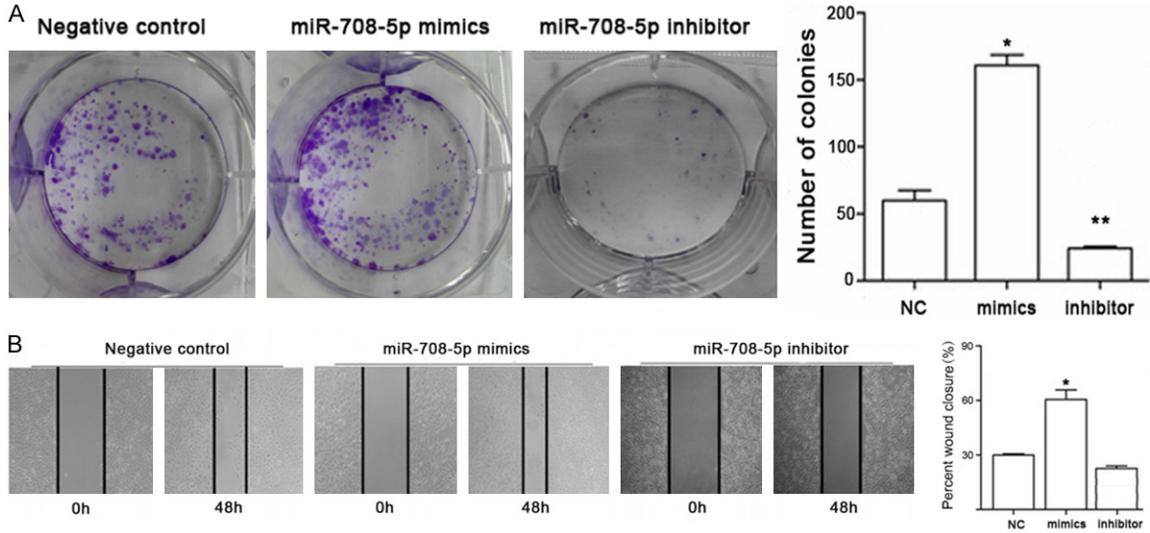
### miR-708-5p promoted LSCC cell migration

The HEp2 migration was measured by cell wound healing assay, which showed that miR-708 inhibitor remarkably restrained, and the miR-708 mimics promoted HEp2 migration in laryngeal carcinoma (**Figure 3B**). These results indicate that miR-708-5p in HEp2 promotes the malignant behavior of HEp2, as migration.

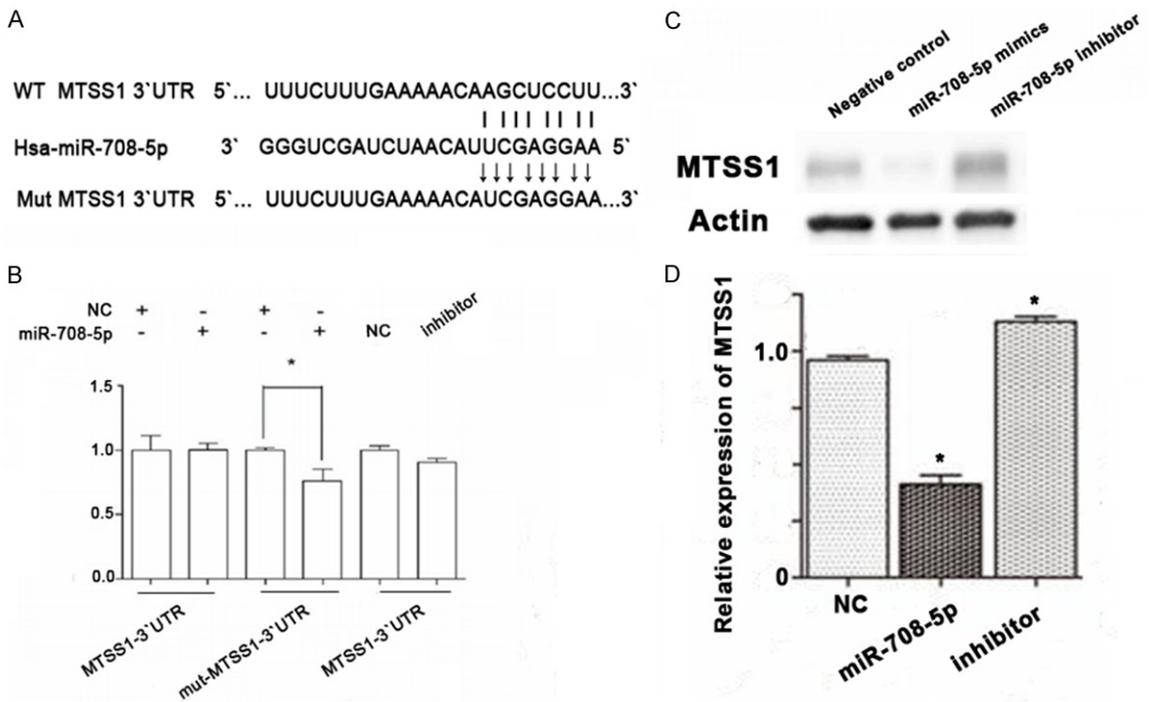
### MTSS1 is a direct target gene of miR-708-5p

By bioinformatic analysis (miRanda and TargetScan), we identified MTSS1 as a potential target gene by miR-708-5p (**Figure 4A**). Subsequently, RT-qPCR and western blot analysis were performed to investigate the regulation of miR-708-5p and MTSS1 expression at the mRNA and protein level in LSCC cells.

As presented in **Figure 4C** and **4D**, MTSS1 was significantly downregulated in HEp2 cells transfected with miR-708-5p compared with cells transfected with the NC ( $P < 0.05$ ). Dual-luciferase reporter assays were also performed to verify the targeting of miR-708-5p. As presented in **Figure 4B**, luciferase activity was significantly downregulated in the miR-708-5p mimics and pGL3-MTSS1-3'UTR-Wt group. While the inhibitory effects miR-708 were not shown in the mutant reporter transfected cells (**Figure 4B**). These results indicated that MTSS1 was a direct gene of miR-708-5p *in vitro*.



**Figure 3.** miR-708-5p promotes the malignant behavior of Hep2. A. Down-regulation of miR708-5p inhibited the cell colony formation ability of HEp2 cells (magnification, ×200). B. Cell wound healing assay. MiR-708-5p significantly promoted cell migration of LSCC.



**Figure 4.** MTSS1 is a direct target gene of miR 708-5p *in vitro*. (A) TargetScan and miRanda assessment demonstrated that MTSS1 contained an miR 708-5p seed match at MTSS1 3'UTR. (B) western blot analysis revealed that MTSS1 was downregulated at the protein levels, respectively, in HEp2 cells following transfection with miR 708-5p. (C and D) miR 708-5p inhibited the pGL3 MTSS1 3'UTR Wt luciferase activity; however, not the pGL3 MTSS1 3'UTR Mut luciferase activity in HEp2 cells. \**P* < 0.05 vs. the respective NC transfected cells. MTSS1, metastasis suppressor-1; miR, microRNA; UTR, untranslated region; Wt, wild type; Mut, mutated; NC, negative control.

**Discussion**

Laryngeal squamous cell carcinoma (LSCC) is the most common form of laryngeal carcinoma

and accounts for one quarter of all head and neck squamous cell carcinomas [9]. Despite the development of therapeutic treatments for LSCC, including surgery, chemotherapy and

radiotherapy, the 5-year overall survival rate of patients with LSCC has not significantly improved over the past 10 years [10]. In the present study, miR-708-5p is proved to be related to the progression of LSCC, which might be a key target in the therapy of LSCC.

In the current study, miR-708-5p is found to be up-regulated in LSCC tissues. In recent years, microRNAs has been studied in many fields, especially in cancers [11]. Many studies have demonstrated that miR serves a significant role in diverse biological processes, including cell proliferation, cell cycle progression, apoptosis, survival, motility, invasion, metastasis, angiogenesis and morphogenesis. Many studies have indicated that miR-708-5p was abnormally expressed in a number of types of human cancer. For example, in hepatocellular carcinoma, miR-708-5p expression was reduced in HCC tissues and cell lines. While in other cancers, miR-708 functions as oncogene. For example, the expression level of miR-708-5p was increased and correlated with poor survival in lung adenocarcinoma and the high expression level of miR-708 in the tumors was most strongly associated with an increased risk of death [12].

Thus, we speculated that miR-708-5p might be a potential indicator for poor prognosis of LSCC. The evidence has been reached via cell growth and proliferation assay. Transfection of LSCC cells with miR-708-5p inhibitor demonstrated to inhibit cell proliferation and invasion by CCK8, colony formation and cell wound healing assay. MicroRNAs function by controlling expression of other genes.

Here, we found that the expression of metastasis suppressor 1 (MTSS1) is regulated by miR-708-5p. As an actin-binding protein, MTSS1 is involved in cytoskeleton function. The protein contains a WASP-homology 2 (WH2) actin-binding motif and an IMD (IRSp53 MIM domain) which regulate cytoskeletal dynamics by restriction of actin polymerization [13]. MTSS1 is expressed in various tissues including spleen, thymus, prostate, uterus, colon or peripheral blood but absent or low expressed in different cancer types including bladder, gastric, colorectal and breast cancer where its reduced expression correlates with poor patient survival [14]. In basal cell carcinomas MTSS1 has also been identified as a Sonic Hedgehog (SHH) respon-

sive gene, potentiating Gli dependent transcription [15].

In conclusion, our research found that miR-708-5p is deregulated in LSCC and promoted the malignant behavior. Furthermore, the cancer promoting effects was realized by the way of restraining MTSS1, which is a cancer suppressor gene.

#### Disclosure of conflict of interest

None.

**Address correspondence to:** Lihua Dong, Intensive Care Unit, The First Hospital of Jilin University, Changchun 130021, Jilin Province, China. Fax: 0431-88782629; E-mail: Lihua\_dong16@163.com

#### References

- [1] Chen Y, Wang W, Li Z, Zhou J, Xiao L, Liu H and Liu B. Expression and significance of MTDH and MMP-9 in laryngeal squamous cell carcinoma. *Lin Chung Er Bi Yan Hou Tou Jing Wai Ke Za Zhi* 2016; 30: 60-63.
- [2] Zou Y, Fang F, Ding YJ, Dai MY, Yi X, Chen C, Tao ZZ and Chen SM. Notch 2 signaling contributes to cell growth, anti-apoptosis and metastasis in laryngeal squamous cell carcinoma. *Mol Med Rep* 2016; 14: 3517-3524.
- [3] Mulholland GB, Zhang H, Nguyen NT, Tkaczek N, Seikaly H, O'Connell D, Biron VL and Harris JR. Optimal detection of hypothyroidism in early stage laryngeal cancer treated with radiotherapy. *J Otolaryngol Head Neck Surg* 2015; 44: 34.
- [4] Ozturk K, Akyildiz S, Gode S, Turhal G, Kirazli T, Aysel A and Uluoz U. Post-surgical and oncologic outcomes of supracricoid partial laryngectomy: a single-institution report of ninety cases. *ORL J Otorhinolaryngol Relat Spec* 2016; 78: 86-93.
- [5] Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009; 136: 215-233.
- [6] Sun X, Song Y, Tai X, Liu B and Ji W. MicroRNA expression and its detection in human supraglottic laryngeal squamous cell carcinoma. *Biomed Rep* 2013; 1: 743-746.
- [7] Cao P, Zhou L, Zhang J, Zheng F, Wang H, Ma D and Tian J. Comprehensive expression profiling of microRNAs in laryngeal squamous cell carcinoma. *Head Neck* 2013; 35: 720-728.
- [8] Li Q, Li S, Wu Y and Gao F. miRNA-708 functions as a tumour suppressor in hepatocellular carcinoma by targeting SMAD3. *Oncol Lett* 2017; 14: 2552-2558.

- [9] El Bagdadi K, Kubesch A, Yu X, Al-Maawi S, Orłowska A, Dias A, Booms P, Dohle E, Sader R, Kirkpatrick CJ, Choukroun J and Ghanaati S. Reduction of relative centrifugal forces increases growth factor release within solid platelet-rich-fibrin (PRF)-based matrices: a proof of concept of LSCC (low speed centrifugation concept). *Eur J Trauma Emerg Surg* 2017; [Epub ahead of print].
- [10] Lacy PD and Piccirillo JF. Development of a new staging system for patients with recurrent laryngeal squamous cell carcinoma. *Cancer* 1998; 83: 910-917.
- [11] Zhao Y and Srivastava D. A developmental view of microRNA function. *Trends Biochem Sci* 2007; 32: 189-197.
- [12] Wu X, Liu T, Fang O, Dong W, Zhang F, Leach L, Hu X and Luo Z. MicroRNA-708-5p acts as a therapeutic agent against metastatic lung cancer. *Oncotarget* 2016; 7: 2417-2432.
- [13] Li DW, Gao S, Shen B and Dong P. Effect of apoptotic and proliferative indices, P-glycoprotein and survivin expression on prognosis in laryngeal squamous cell carcinoma. *Med Oncol* 2011; 28 Suppl 1: S333-340.
- [14] Du P, Ye L, Ruge F, Yang Y and Jiang WG. Metastasis suppressor-1, MTSS1, acts as a putative tumour suppressor in human bladder cancer. *Anticancer Res* 2011; 31: 3205-3212.
- [15] Gonzalez-Quevedo R, Shoffer M, Horng L and Oro AE. Receptor tyrosine phosphatase-dependent cytoskeletal remodeling by the hedgehog-responsive gene MIM/BEG4. *J Cell Biol* 2005; 168: 453-463.