



LncRNA *MALAT1* Promote Cell Proliferation and Invasion by Sponging miR-125b to Modulate *HMG A1* Expression in Laryngocarcinoma

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Abstract

Background: Laryngocarcinoma is the most frequent head and neck malignant tumor. MALAT1 have a role in promoting cell proliferation and metastasis in several tumors. This research aimed to investigate the great roles of MALAT1 in laryngocarcinoma.

Methods: Overall, 54 cases of laryngocarcinoma tissues pathological specimens and paracancerous tissues were collected by surgical resection from the Department of Otolaryngology-Head and Neck Surgery at the Shandong Provincial Hospital affiliated to Shandong University, China from Jan 2012 to Oct 2015. The microRNA and protein levels of genes were evaluated by RT-qPCR and western blot. The proliferative and invasive ability were calculated using CCK8 and transwell assays. Kaplan-Meier method was used to assess the survival of laryngocarcinoma patients.

Results: In laryngocarcinoma tissues and cells, lncRNA MALAT1 expression was significantly increased compared to normal tissues and cells. LncRNA MALAT1 promotes proliferation and migration of laryngocarcinoma cells. LncRNA MALAT1 upregulates *HMG A1* expression by acting as a competitive endogenous RNA (ceRNA) for miR-125b. Rescue experiments showed that microRNA-125b inhibitor reversed the change in cell viability and invasion induced by sh-MALAT1. Down regulation of lncRNA MALAT1 inhibits laryngocarcinoma proliferation and invasion by modulating miR-125b /*HMG A1*.

Conclusion: LncRNA MALAT1 promotes the development of laryngocarcinoma by regulating the expression level of *HMG A1* by acting as a miR-125b ceRNA and may be considered as a new strategy for the development of laryngocarcinoma.

Keywords: MALAT1; Proliferation; Invasion; Laryngocarcinoma

Introduction



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Laryngocarcinoma, the most frequent head and neck malignant tumor, has a high incidence and mortality (1). Laryngocarcinoma originates from the throat skin and accounts for approximately 2.4% of new cancer cases each year (2). In recent years, the incidence of laryngocarcinoma has increased, and laryngocarcinoma has seriously affected human health. Although the treatment of early laryngocarcinoma is satisfactory, the prognosis of patients with advanced laryngocarcinoma is still not satisfactory, and the 5-year overall survival rate is less than 50% (3). Hence, exploring new molecular markers will have important implications for the treatment of laryngocarcinoma. Long non-coding RNAs (lncRNAs) are identified as non-protein coding transcripts more than 200 nucleotides in length, which accounts for at least 70% of human transcripts (4, 5). Accumulating evidence demonstrated that lncRNAs were involved in multiple biological processes, including tumorigenesis, tumor metastasis, cell apoptosis and angiogenesis (6, 7). Long non-coding RNA metastasis associated lung adenocarcinoma transcript (lncRNA MALAT1) produces a precursor transcript from which a long non-coding RNA is derived by RNase P cleavage of a tRNA-like small ncRNA (known as mascRNA) from its 3'-UTR end (8). LncRNA MALAT1 upregulation in multiple cancerous tissues has been associated with the proliferation and metastasis of tumor cells. For instance, MALAT1 promotes cardiomyocyte apoptosis after myocardial infarction (9). MALAT1 enhanced cell proliferation and inhibited cell apoptosis in colorectal cancer (10). LncRNA MALAT1 acted as a potential novel biomarkers for diagnosis of acute myocardial infarction (11). However, the role of lncRNA MALAT1 in laryngocarcinoma is still under investigation.

MicroRNAs (miRNAs), small non-coding RNAs with 22-28 nucleotides in length, bind to the seed region in the 3'-untranslated region (3'-UTR) of target genes, inducing mRNA destabilization or inhibiting protein translation (12). MiRNAs, act as oncogenes or tumor suppressors, have been identified in various cancers and are involved in cancer development and progression (13). MiR-

125b usually acted as tumor suppressor in several cancers, such as oral carcinoma, osteosarcoma and non-small cell lung cancer (14-16). MiR-125b inhibited cell growth and induced cell apoptosis in esophageal squamous cell carcinoma (17). MiR-125b suppressed cell proliferation and invasion in pediatric low grade glioma and breast cancer (18,19). However, miR-125b acted as oncogene to promote cell proliferation in cervical cancer and glioma (20,21). miR-125b suppressed glycolysis and lactate production in laryngeal squamous cell carcinoma (22).

Methods

Patients and tissues

Overall, 54 cases of laryngocarcinoma tissues pathological specimens and paracancerous tissues were collected by surgical resection from the Department of Otolaryngology-Head and Neck Surgery at the Shandong Provincial Hospital affiliated to Shandong University from Jan 2012 to Oct 2015. All the patients did not receive any radiotherapy and/or chemotherapy before the operation. All the tissue samples were immediately frozen in liquid nitrogen and then stored at -80 °C.

Informed consent was obtained from each patient, and the research protocols were approved by the Ethics Committee of Shandong Provincial Hospital affiliated to Shandong University.

Cell culture and treatment

Human laryngocarcinoma cells TU212, M2E and M4E, and nasopharynx epithelium cell NP69 were obtained from American Type Culture Collection (ATCC, USA). All the cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS, Gibco, USA), penicillin (100 U/mL) and streptomycin (100 U/mL). All the cells were cultivated in at 37 °C in a humidified atmosphere of 5% CO₂.

Transfection

The miR-125b mimic, miR-125b inhibitor shRNA-MALAT1, pEX-MALAT1, pcDNA3.1-

HMGA1 and negative control plasmids were designed and synthesized from Gene-Pharma (Shanghai, China). M2E cells were incubated in 6-well plate, used to transfect the vectors. Lipofectamine 2000 Reagent (Invitrogen, USA) was used to the transfection, diluted by Opti-MEM/Reduced serum medium (Thermo Scientific, Shanghai, China). For the stable transfection, the cells were selected by Geneticin (G418; Thermo Scientific, Shanghai, China), while the cells with transient transfection were harvest after transfected 48 h.

Quantitative real-time PCR

miRN easy Mini Kit (Qiagen, Hilden, Germany) be applied to extract total miRNAs from tissues or cells. The first cDNA chain was synthesized by TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Followed miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems) was employed to perform the qPCR. The relative levels of miRNA were derived using $2^{-\Delta\Delta Ct}$ method with U6 small nuclear RNA as normalization.

Total RNA was extracted by using TRIzol Reagent (Invitrogen) and the first cDNA chain was synthesized by using Omniscript Reverse Transcription Kit (Qiagen) from total RNA. Quantitect SYBR Green PCR Kit (Qiagen) was conducted to perform the RT-qPCR in a Quantitect SYBR green PCR system (Qiagen). $A2^{-\Delta\Delta Ct}$ method was used for the mRNA quantification, which normalized by GAPDH.

The following primers were used for analysis:
MALAT1 forward: 5'-AGGTAAAGCTTGAGAAGAT-3', reverse: 5'-GGAAGTAATTCAAGATCAA-3';
miR-125b forward: 5'-CCAGATACTGCGTATGTGTG-3', reverse: 5'-GTCACCTGATCCCATCTAAC-3';
U6 forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3';
GAPDH forward: 5'-CGGATTTGGTTCGTATTGGG-3', reverse: 5'-TGGAAGATGGTGTGATGGGATT-3'.

CCK-8 assay

The cell proliferation ability was measured by using Cell Counting Kit-8 (CCK-8) assay (Dojindo Co.Ltd., Japan). Briefly, M2E cells transfected with special vectors were incubated in 96-well plates and cultured for 24 h, 48 h, 72 h and 96 h at 37 °C with 5% CO₂, followed we added 10 μl CCK-8 solution into each well and incubated 2 hours. Finally, the absorbance at 450 nm was measured to use the microplatereader.

Transwell assay

To perform the cell migration ability, the transwell insert (8 μm membrane, Corning, Cambridge, MA) were placed in 24-well plate and formed up and low two chambers. The M2E cells were suspended by 200 μl RPMI-1640 medium and added in the upper chamber. Meanwhile, 500 μl medium was filled into the lower chamber, which containing 15% FBS. After incubated the cells at 37 °C for 24 h, were moved the non-invasive cells, which still on the upper surface, by cotton swab. For the invasive cells, we fixed and then stained by 4% paraformaldehyde and 10% crystal violet respectively, and followed counted the cells under microscope (Olympus Corporation, Tokyo, Japan).

miRNA targets prediction and dual-luciferase reporter assay

TargetScan (<http://www.targetscan.org>) was used to predict the target gene of miR-125b and we discovered that *HMGA1* was one of potential target gene. The binding sequences were mutated from GUGUCAU to CACAGUA, to validate whether miR-125b binding to *HMGA1* in colorectal cancer cells. Subsequently, the wide type and the mutant 3'-UTR of *HMGA1* were inserted into the dual luciferase reporter vectors, designated as WT or MUT. For the luciferase assay, Lipofectamine 2000 Reagent (Invitrogen, USA) was applied to co-transfected miR-125b mimic and the WT or MUT plasmid into M2E cells. Then, was applied to measure the luciferase activity was measured to apply dual luciferase reporter assay system (Promega, USA).

Statistical analysis

SPSS version 16.0 (IBM Chicago, IL, USA) was used to perform the statistical analysis. All the data are presented as the means \pm SD of at least 3 separate experiments. The difference between two or multiple groups was analyzed using Student's t-test or One-way ANOVA with post hoc Turkey test. $P < 0.05$ was considered to be statistically significant.

Results

LncRNAMALAT1 is overexpressed in laryngocarcinoma tissues and cells

The expression of lncRNA MALAT1 in laryngocarcinoma tissues and cells was detected by qRT-PCR assay. As shown in Fig. 1A, the expression of lncRNA MALAT1 was significantly higher in laryngocarcinoma tissues than that of non-tumor tissues ($P < 0.05$). Similarly, the expression of lncRNA MALAT1 was also significantly up-regulated in TU212 ($P < 0.05$), M2E ($P < 0.01$), M4E ($P < 0.05$) cells compared with normal NP69 cells (Fig.1B). Thus, the M2E cells were used to perform the next experiment.

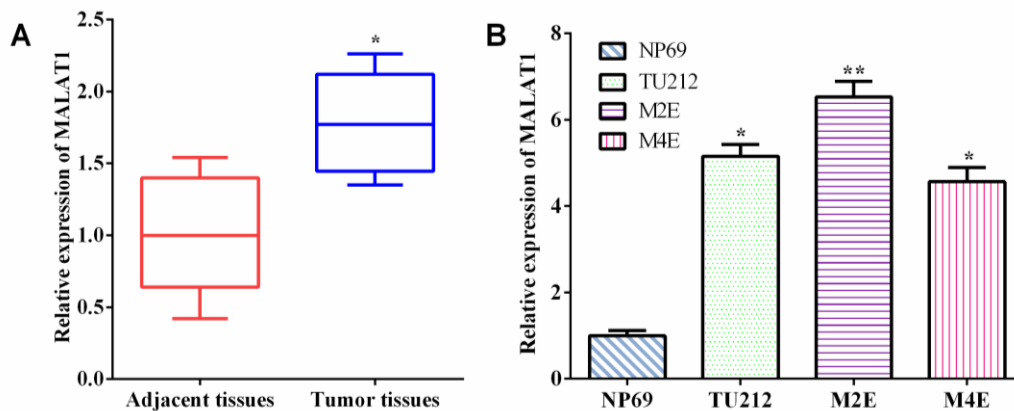


Fig. 1: LncRNA MALAT1 is overexpressed in laryngocarcinoma tissues and cells **(A)** The expression of lncRNA-MALAT1 was significantly higher in laryngocarcinoma tissues than that of non-tumor tissues. **(B)** The expression of lncRNA MALAT1 was also significantly up-regulated in TU212, M2E and M4E cells compared with normal NP69 cells

LncRNAMALAT1 inhibits laryngocarcinoma cell proliferation

To investigate the function of lncRNAMALAT1 in laryngocarcinoma, lncRNA MALAT1 was downregulated or upregulated in M2E cells. The transfection efficiency of transfection of the shRNA ($P < 0.05$) or overexpression plasmid ($P < 0.05$) was calculated by qRT-PCR assay (Fig.

2A,C). Cell proliferative ability was measured by CCK8 assay in M2E cells. As expected, the cell proliferation of M2E cells was significantly impaired by silencing of the lncRNA MALAT1 ($P < 0.05$) (Fig. 2B). On the contrary, overexpression of lncRNA MALAT1 enhanced cell viability in M2E cells ($P < 0.05$) (Fig. 2D).

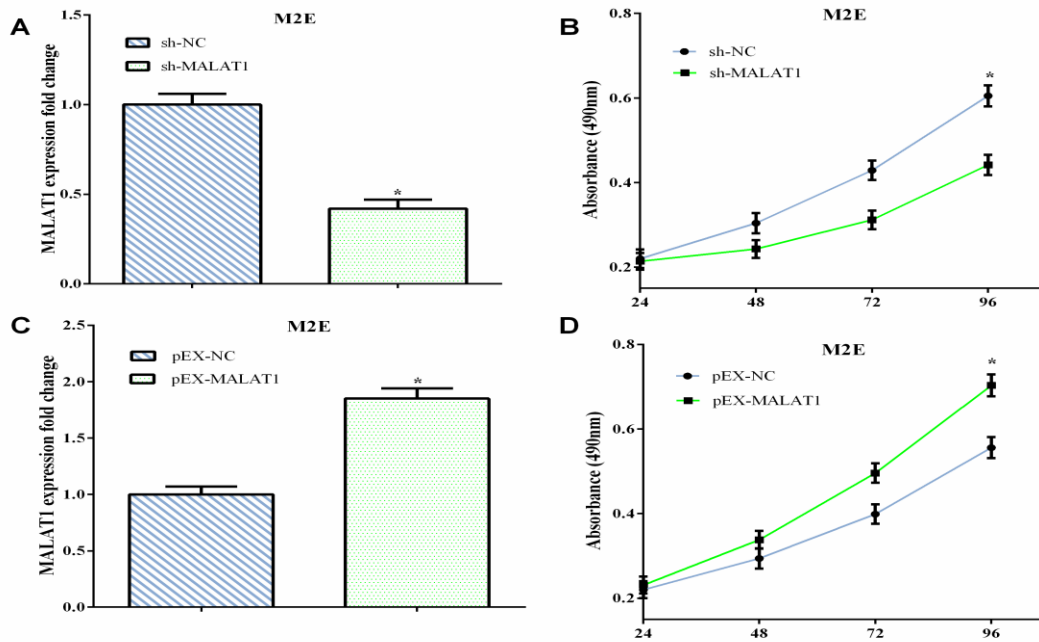


Fig. 2: LncRNA MALAT1 inhibits laryngocarcinoma cell proliferation (A) The transfection efficiency of transfection of the shRNA-MALAT1. (B) The cell proliferation of M2E cells was significantly impaired by silencing of the lncRNA MALAT1. (C) The transfection efficiency of transfection of the overexpression plasmid. (D) Overexpression of lncRNA MALAT1 enhanced cell viability in M2E cells

LncRNA MALAT1 suppressed laryngocarcinoma cell invasion

Transwell assay was applied to calculate cell invasion after downregulating or upregulating lncRNA MALAT1 in M2E cells. Similarly results

with proliferation, cell invasion was reduced by knockdown of lncRNA MALAT1 ($P < 0.05$) (Fig. 3A), whereas it was increased by overexpressing lncRNA MALAT1 in M2E cells ($P < 0.05$) (Fig. 3B).

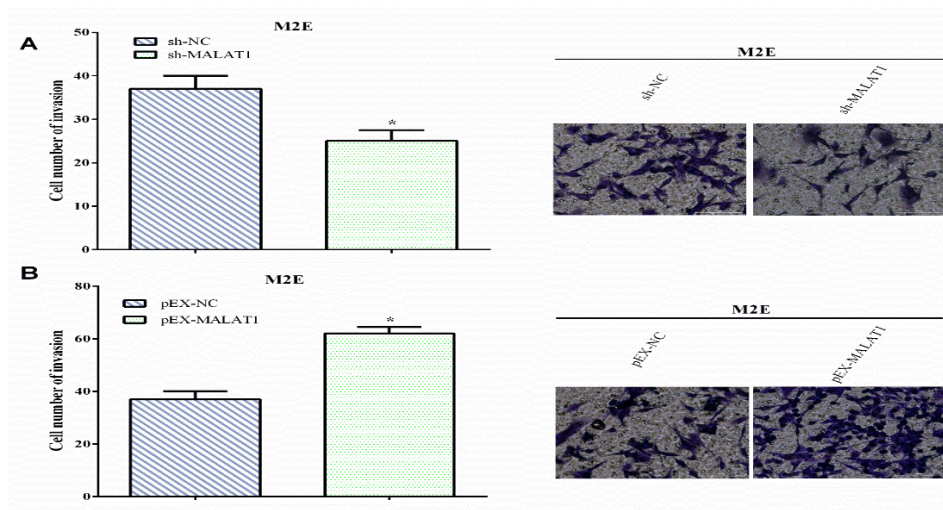


Fig. 3: LncRNA MALAT1 suppressed laryngocarcinoma cell invasion (A) Cell invasion was reduced by knockdown of lncRNA MALAT1. (B) Cell invasion was increased by overexpressing lncRNA MALAT1 in M2E cells

LncRNA MALAT1 acts as a ceRNA by competitively binding to MiR-125b

LncRNA can competitively bind to miRNA through complementary sequences, thereby downregulating its expression, called ceRNA. Starbase online software was used to predict the possible ceRNAs of lncRNA MALAT1. miR-125b maybe interact with lncRNA MALAT1. To investigate the binding relation between lncRNA MALAT1 and miR-125b, the potential binding sequences on MALAT1 mRNA were mutated from UCAGGA to AGUCCU, and followed dual-Luciferase reporter assays was carried out in M2E cells (Fig. 4A). miR-125b mimic reduced

the luciferase activity of the lncRNA MALAT1 compared with NC group ($P < 0.05$), while this reduction disappeared when the target site was mutated ($P > 0.05$) (Fig. 4B). What's more, the expression of miR-125b was measured after altering MALAT1 in M2E cells. Not unfortunately, miR-125b expression was effectively downregulated by overexpressing lncRNA MALAT1 ($P < 0.05$) (Fig. 4C). In contrast, transfection of sh-MALAT1 enhanced the expression of lncRNA MALAT1 in M2E cells ($P < 0.05$) (Fig. 4D). Taken together, all the findings suggested that lncRNAMALAT1 may act as a ceRNA via binding with miR-125b in laryngocarcinoma.

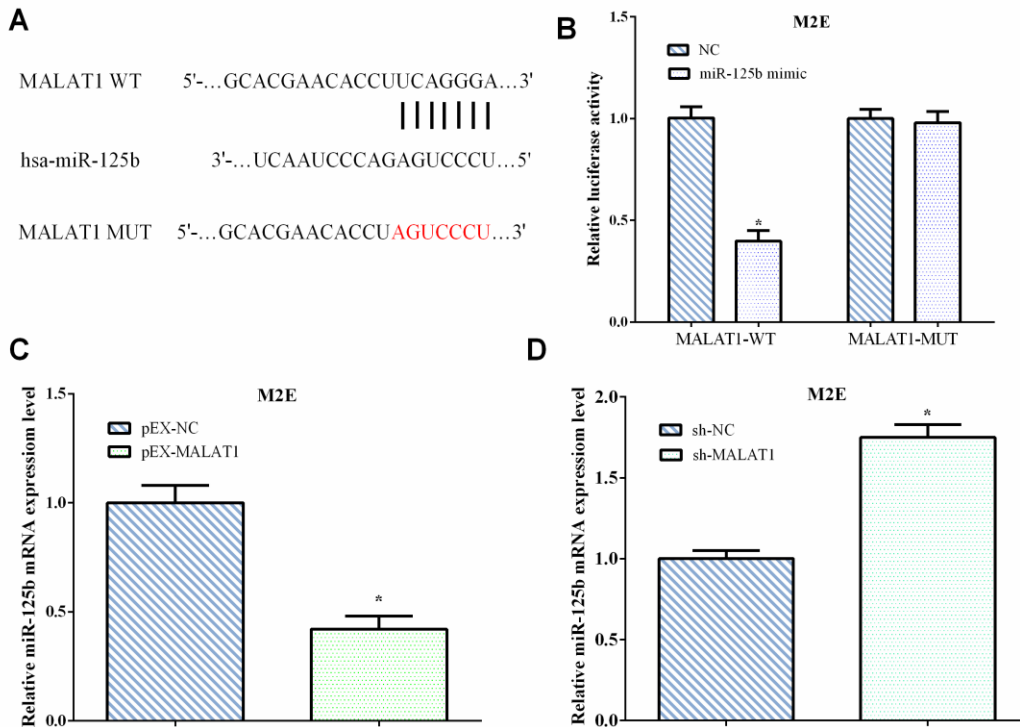


Fig. 4: LncRNA MALAT1 acts as a ceRNA by competitively binding to MiR-125b (A) Starbase online software was used to predict the possible ceRNAs of lncRNA MALAT1. (B) miR-125b mimic reduced the luciferase activity of the lncRNA MALAT1 compared with NC group, while this reduction disappeared when the target site was mutated. (C) miR-125b expression was effectively downregulated by overexpressing lncRNA MALAT1. (D) Transfection of sh-MALAT1 enhanced the expression of lncRNA MALAT1 in M2E cells

MiR-125b impaired cell viability and invasion

To investigate the important roles of miR-125b in laryngocarcinoma, we measured the expression

of miR-125b in laryngocarcinoma tissues and cell lines. MiR-125b was downregulated in laryngocarcinoma tissues compared with the adjacent

tissues ($P<0.05$) (Fig. 5A). Likewise, the expression of miR-125b was evaluated in cell lines, and miR-125b expression was lower in TU212 ($P<0.05$), M2E ($P<0.01$), M4E ($P<0.05$) cells than normal nasopharynx epithelium NP69 cells (Fig. 5B). To explore the effect of miR-125b on cell viability and invasion, the CCK8 and transwell assays were performed after transfection of miR-125b mimic in M2E cells. qRT-PCR

was used to measure the transfection efficiency of transfected miR-125b mimic in M2E cells ($P<0.05$) (Fig. 5C). CCK8 assay revealed that miR-125b mimic decreased cell proliferation compared with the control ($P<0.05$) (Fig. 5D). Transwell assay indicated that cell invasive ability was reduced by overexpression of miR-125b mimic in M2E cells ($P<0.05$) (Fig. 5E).

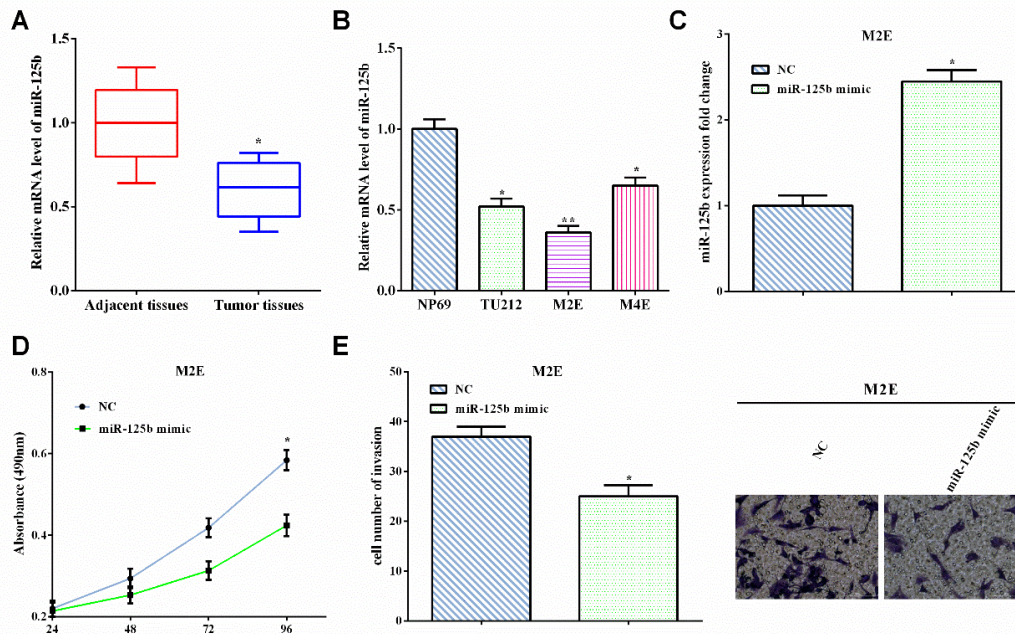


Fig. 5: MiR-125b impaired cell viability and invasion (A) MiR-125b was downregulated in laryngocarcinoma tissues compared with the adjacent tissues. (B) miR-125b expression was lower in TU212, M2E and M4E cells than normal nasopharynx epithelium NP69 cells. (C) The transfection efficiency of transfected miR-125b mimic was measured in M2E cells. (D) CCK8 assay revealed that miR-125b mimic decreased cell proliferation compared with the control. (E) Cell invasive ability was reduced by overexpression of miR-125b mimic in M2E cells

MALAT1 regulated cell proliferation and invasion via miR-125b axis

Since lncRNA MALAT1 functions as a ceRNA of miR-125b, the rescue experiments were carried out to explore whether miR-125b participated in lncRNA MALAT1-mediated cell activity change. To investigate the function of miR-125b in human laryngocarcinoma cell lines, miR-125b inhibitor was transfected in sh-MALAT1 transfected M2E cells, in order to downregulate miR-125b

($P<0.05$) (Fig. 6A). Intriguingly, miR-125b inhibitor promoted cell proliferation and invasion in M2E cells with MALAT1 downregulated ($P<0.05$) (Fig. 6B and C). MiR-125b inhibitor significantly reversed the inhibitory effect of sh-MALAT1 on cell proliferation and invasion in M2E cells. Altogether, lncRNA MALAT1 promotes proliferation, invasion through downregulating of miR-125b in laryngocarcinoma.

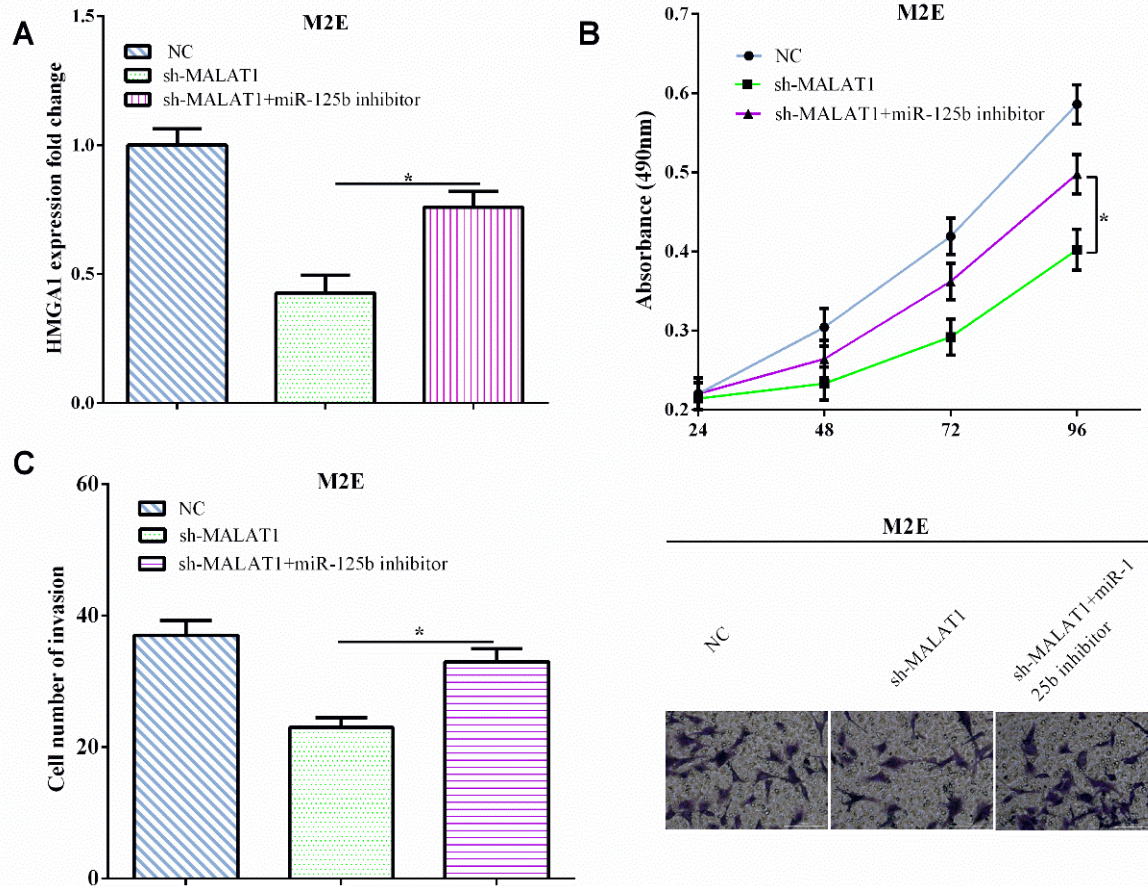


Fig. 6: MALAT1 regulated cell proliferation and invasion via miR-125b axis (A) MiR-125b inhibitor was transfected in sh-MALAT1 transfected M2E cells. (B) MiR-125b inhibitor promoted cell proliferation in M2E cells with MALAT1 downregulated. (C) MiR-125b inhibitor significantly reversed the inhibitory effect of sh-MALAT1 on cell invasion in M2E cells

HMGAI was a target gene of miR-125b

TargetScan software was utilized to predict the potential target genes of miR-125b, and we found *HMGAI* was a candidate (Fig. 7A). To investigate whether miR-125b directly binding to the mRNA 3'-UTR of *HMGAI*, the potential binding sequences was mutated and then evaluated the luciferase activity. As expected, the luciferase activity of the wild type mRNA was reduced by

miR-125b mimic compared with the negative control. However, the luciferase activity has no obvious change after co-transfected with miR-125b mimic and the mutant *HMGAI* mRNA (Fig. 7B). In addition, the expression of *HMGAI* was decreased by miR-125b in M2E cells. Moreover, the expression of *HMGAI* was impaired after knockdown of MALAT1, while it was increased by overexpressing MALAT1.

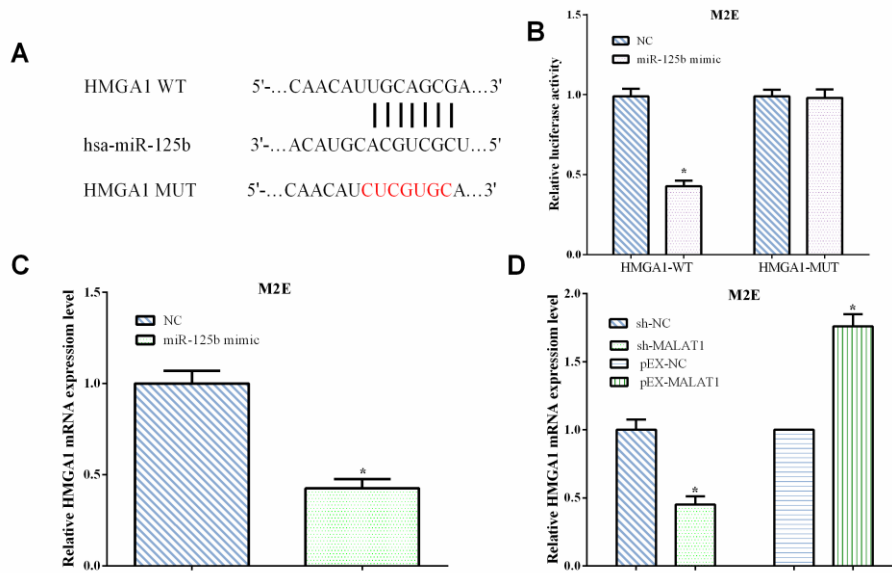


Fig. 7: *HMGAI1* was a target gene of miR-125b (A) TargetScan software was utilized to predict the potential target genes of miR-125b. (B) The luciferase activity of the wild type mRNA was reduced by miR-125b mimic compared with the negative control. (C) The expression of *HMGAI1* was decreased by miR-125b in M2E cells. (D) The expression of *HMGAI1* was impaired after knockdown of MALAT1, while it was increased by overexpressing MALAT1

Discussion

Recently, lncRNA appears to play great role in the development of cancer, and multiple lncRNAs associated with cancers have been identified (23). MALAT1, an evolutionary conserved lncRNA, initially identified as a tumor-related lncRNA (24). Overexpression of MALAT1 promoted cell proliferation in periodontal ligament stem cells (25). Overexpression of MALAT1 improved cell proliferation, migration and invasion in gastric cancer and non-small cell lung cancer (26, 27). Our results were consistent with all the findings, we found that MALAT1 was overexpressed in laryngocarcinoma tissues and cell lines compared with adjacent normal tissues and normal cells. What's more, knockdown of lncRNA-MALAT1 inhibited cell proliferation and invasion, while upregulation of MALAT1 enhanced cell proliferation and invasion.

lncRNA contains a miRNA binding site and acts as a ceRNA-inhibiting sponge miRNA to antagonize its function (28, 29). For instance, Similarly, lncRNA TDRG1 functions as an oncogene

through sponging miR-330 in cervical cancer (30). MALAT1 regulated tumor growth of hepatocellular carcinoma via sponging microRNA-200a (31). In this study, MALAT1 regulated the expression of miR-125b via sponging miR-125b in M2E cells, which were consistent with the findings in Alzheimer's Disease (32). StarBase revealed that MALAT1 has a potential binding site with miR-125b. And luciferase assay was performed to verify that MALAT1 directly binding to miR-125b in M2E cells. Downregulation of miR-125b was associated with poor prognosis in pediatric acute lymphoblastic leukemia (33). Moreover, miR-125b inhibited cell proliferation, migration, and invasion in hepatocellular carcinoma and triple negative breast cancer (34,35). Consistent with all the findings, we found that miR-125b was low expressed in laryngocarcinoma tissues and cell lines, and overexpression of miR-125b suppressed M2E cell proliferation and invasion. MALAT1 sponging miR-125b, miR-125b inhibitor was transfected in cells that interference of MALAT1. Cell proliferation and invasion was reversed in cells that co-transfected with

sh-MALAT1 and miR-125b inhibitor, compared with only interference of MALAT1. Finally, *HMGAI* was verified to be a direct target gene of miR-125b by luciferase assay. In addition, the expression of *HMGAI* was reduced by miR-125b mimic or sh-MALAT1, while it was increased by overexpression of MALAT1.

In laryngocarcinoma tissues and cells, LncRNA MALAT1 expression was significantly increased compared to normal tissues and cells. LncRNA MALAT1 promotes proliferation and migration of laryngocarcinoma cells. Mechanistic studies indicated that lncRNA MALAT1 upregulates *HMGAI* expression by acting as a competitive endogenous RNA (ceRNA) for miR-125b. MiR-125b inhibitor reversed the change in cell viability and invasion induced by sh-MALAT1.

Conclusion

This study demonstrated that downregulation of lncRNA MALAT1 inhibits laryngocarcinoma proliferation and invasion by modulating miR-125b /*HMGAI*.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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