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Long non-coding RNA Dleu2 affects proliferation, migration and invasion ability of laryngeal carcinoma cells through triggering miR-16-1 pathway

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Abstract. – OBJECTIVE: Laryngeal cancer is a common malignant tumor in the head and neck, which affects swallowing, breathing, and pronunciation function. In recent years, many long non-coding RNAs (lncRNAs) have been shown to be involved in the progression of cancer with the development of gene sequencing, transcriptomics, and bioinformatics. LncRNA Dleu2 is a cancer-related lncRNA that down-regulates tumor progression in a variety of cancers. However, its possible effects and related signaling pathway in the development of laryngeal cancer are not clear.

PATIENTS AND METHODS: Real-time PCR was applied to test lncRNA Dleu2 and microRNA-16-1 (miR-16-1) expressions in laryngeal carcinoma tissues. LncRNA Dleu2 and miR-16-1 levels were over-expressed by transfection. Cell proliferation was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Cell migration was evaluated by using wound-healing assay. Cell invasion was determined by using transwell assay.

RESULTS: LncRNA Dleu2 and miR-16-1 levels were significantly declined in the laryngeal carcinoma tissue compared to para-carcinoma tissue ($p < 0.05$). LncRNA Dleu2 and miR-16-1 up-regulation significantly reduced cell proliferation, migration, and invasion compared to the control group ($p < 0.05$). Ago-miR16-1 transfection significantly enhanced the luciferase activity of wild-type Dleu2 compared to control group ($p < 0.05$), suggesting their interaction with each other.

CONCLUSIONS: LncRNA Dleu2 influences the proliferation, migration, and invasion of laryngeal cancer cells through miR-16-1. Therefore, lncRNA Dleu2 and miR-16-1 may serve as potential biomarkers and targets for laryngeal cancer diagnosis and treatment.

Key Words:
Laryngeal carcinoma, lncRNA, Proliferation, Migration, Invasion.

Introduction

Laryngeal cancer is a common malignant tumor in the head and neck, which affects swallowing, breathing, and pronunciation function[1,2]. Therefore, revealing the molecular mechanism of laryngeal cancer and improving the diagnosis and treatment of laryngeal cancer is of great significance.

In the recent years, with the development of gene sequencing, transcriptomics, and bioinformatics, more and more cancer-related non-coding RNAs have been found, including microRNAs (miRNAs) and LncRNAs, providing a strong basis for the diagnostic markers and therapeutic targets of laryngeal cancer screening[3]. LncRNA refers to non-coding RNAs that are more than 200 nt in length, most of which are transcribed and spliced by RNA polymerase 2. They are mainly located in the nucleus or cytoplasm and can regulate the transcription process of genes from a variety of lateral sides and influence miRNA expressions to affect cell behavior. It was showed that lncRNA Dleu2 is a cancer-related lncRNA that decreases in gastric cancer, leukemia, and other cancers. However, its potential effects and related signaling pathway in the development of laryngeal cancer are not clear[4]. Therefore, investigating the effect of lncRNA Dleu2 on cell proliferation, migration, and invasion of laryngeal carcinoma and

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screening the potential molecular mechanism and molecular target become hot spots in the process of laryngeal carcinoma prevention and treatment.

We found that lncRNA Dleu2 and miR-16-1 may have potential interactions by reviewing the literature and the bioinformatics database, such as Targetscan[5]. Therefore, we aim at exploring the expressions of lncRNA Dleu2 and miR-16-1 in laryngeal carcinoma and over-express them to explore the impact on cell proliferation, migration, and invasion. This may provide a theoretical foundation for laryngeal cancer-related diagnosis and treatment.

Patients and Methods

Main Reagents

Laryngeal carcinoma cell line was provided by the Chinese Academy of Medical Sciences Institute of Cell Bank (Beijing, China). Laryngeal cancer tissues and the corresponding adjacent tissues (non-cancerous tissues at less than 3 cm from laryngeal cancer) were taken from laryngeal carcinoma patients received operation in our hospital. All the patients had signed informed consent. This investigation was approved by the Ethics Committee of Third Xiangya Hospital, Central South University, Changsha, China. Reverse Transcription Kit was purchased from TaKaRa (Dalian, China). Real-time PCR kit was purchased from TransGen Biotech (Beijing, China). Dulbecco's modified eagle medium (DMEM) medium, trypsin, and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). Plasmid extraction kit was purchased from Promega (Madison, WI, USA). The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) reagent was derived from Tongren Co., Ltd. (Tokyo, Japan). Matrigel was got from BD Biosciences (Franklin Lakes, NJ, USA). Lipofectamine 2000 and TRIzol were provided by Invitrogen/Life Technologies (Carlsbad, CA, USA). Dleu2 gene was synthesized by Taihe Biotechnology Co. Ltd. (Beijing, China). PCR primers were synthesized by AuGCT (Beijing, China). Other common reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Main Instruments

The clean bench was bought from BoXun Industrial Co., Ltd. (Shanghai, China). UVP Multispectral Imaging System was obtained from UVP cooperation (Sacramento, CA, USA). PS-9 semi-dry transfer electrophoresis instrument was purchased from Jim-x Scientific (Dalian, China). BD FACSCalibur flow cytometry was obtainde from BD Biosciences (San Diego, CA, USA). Carbon dioxide (CO_2) incubator and Thermo-354 micro-plate were purchased from Thermo Fisher Scientific (Waltham, MA, USA). CX23 fluorescence microscope was purchased from Olympus Corporation (Tokyo, Japan).

Laryngeal Carcinoma Cell Cultivation

Human laryngeal carcinoma cell lines TU212 and A549 were purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were maintained at Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The cells were cultured at 37°C and 5% CO_2.

Total RNA Extraction

The cells were collected and added with 1 ml TRIzol reagent for 5 min. Next, the sample was added with 200 μl chloroform and set at room temperature for 15 min. After centrifuged at 4°C and 10000 × g for 15 min, the supernatant was treated by 0.5 ml isopropyl and set at room temperature for 10 min. Next, the sample was centrifuged at 4°C and 10000 × g for 10 min, and the RNA sediment was collected. At last, the RNA was washed by 75% alcohol and solved in 15-60 μl diethyl pyrocarbonate (DEPC) water.

Real-Time PCR

Total RNA was reverse transcribed to cDNA to test Dleu2 expression by Real-time PCR. Dleu2 reverse transcription primer: 5'-TCTCATACAGGTTACAGTTC-3'. Dleu2 primer: forward, 5'-TCTGGAGAACAGCCTCACTTC-3', reverse, 5'-TGCTGAGCTAAGTAGAGGTCTC-3'; GAPDH primer: forward, 5'-ACCCAGAAGACTGTGGATGG-3', reverse, 5'-TTCTAGACGGCAGGTCAGG-3'. The Real-time PCR was performed at 94°C for 4 min, followed by 45 cycles of 94°C for 10 s, 63°C for 10 s, and 72°C for 12 s.

Recombinant Plasmid Construction and Cell Transfection

DNA plasmid or agomir-miR-16-1 was synthesized by Sigma-Aldrich (St. Louis, MO, USA). The cells were passaged on the day before transfection with the confluent degree at 30-50% in the 24-well plate. A total of 1.25 μl siRNA liquid (20 μM) was

dissolved in 100 μl Opti-MEM medium as solution A. Lipofectamine 2000 or Lipofectamine™ RNAiMAX was dissolved in the Opti-MEM medium as solution B. After 5 min, the solutions A and B were mixed and set for 20 min. Next, the mixture was added to the plate and cultured for 4 h. Next, the medium was changed back to Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were observed under a fluorescence microscope to evaluate the transfection efficiency.

MTT Assay

MTT assay was performed according to the reference[6]. TU212 cells in logarithmic phase were digested by 0.25% trypsin and seeded in 96-well plate at a volume of 100 μl per well. The cells were transfected by lncRNA Dleu2 or miR-16-1 plasmid with eight replicates. After 24 h incubation, 10 μl MTT were added to the plate and incubated for 4 h. The plate was observed under the microscope. Next, the crystal was solved by 150 μl dimethyl sulphoxide (DMSO). At last, the plate was tested at 490 nm wavelength to measure the absorbance (A) and calculate the cell survival rate.

Cell viability (%) = (experimental group optical density (OD)-blank group OD)/(control group OD-blank group OD).

Wound Healing Assay

Wound healing assay was performed[7]; the cells were seeded in 6-well plate and transfected to over-express lncRNA Dleu2 or miR-16-1. When the cell fusion reached 90%, 200 μl tip were used to scratch the bottom of the plate. The plate was taken pictures under the microscope. After 24 h, the plate was photographed at the same location to assess cell migration.

Transwell Assay

Transwell chamber and all the equipment used were pre-cooled at 4°C overnight. ECM matrigel was unfrozen at 4°C. The transwell chamber was put into the 24-well plate and 50 μl ECM matrigel were added to the chamber. After setting at 37°C for 4 h, the cells were seeded into the upper chamber at 10000/ml. DMEM containing 10% FBS was added to the lower chamber. After some time, the membrane was stained by crystal violet and observed under the microscope.

Dual Luciferase Reporter Gene Assay

MiR-161 or control, luciferase labeled plasmid (pIS0-Dleu2-3'UTR-mut or pIS0-Dleu2-3'UTR) or control plasmid, was co-transfected to A549 cells. Luciferase activity was detected using the dual luciferase reporter gene kit according to the manual.

Statistical Analysis

All data analyses were performed on SPSS 19.0 software (IBM, Armonk, NY, USA). Measurement data were presented as mean ± standard deviation and received normality test and homogeneity test of variance. The data were presented as mean ± standard deviation. The Student's *t*-test was used to compare the differences between two groups. Tukey's post hoc test was used to validate ANOVA for comparing measurement data between groups. $p < 0.05$ depicted statistical significance.

Results

LncRNA Dleu2 Expression in Laryngeal Carcinoma Tissue and Corresponding Adjacent Tissue

As shown in Figure 1, lncRNA Dleu2 expression in laryngeal carcinoma tissue was significantly down-regulated compared with that in adjacent tissue ($p < 0.05$).

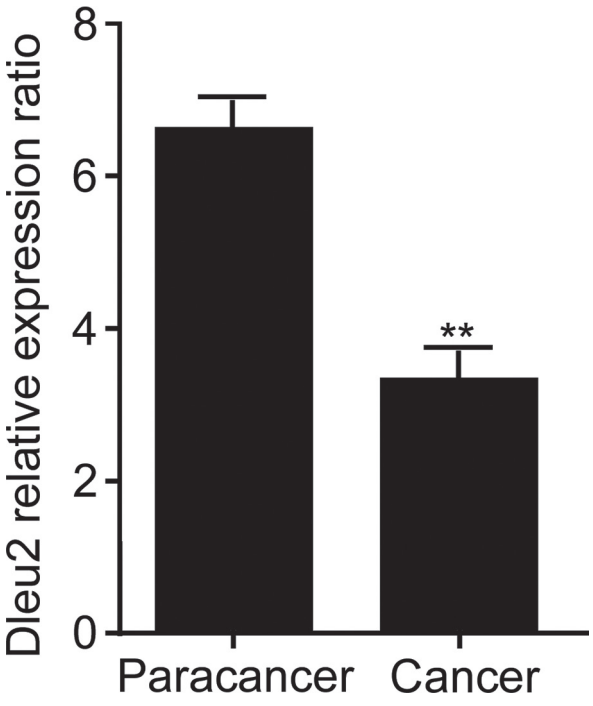

Figure 1. LncRNA Dleu2 expression in laryngeal carcinoma tissue and corresponding adjacent tissue. $^{**}p < 0.05$, compared with paracancer tissue.

MiR-16-1 Expression In Laryngeal Carcinoma Tissue and Corresponding Adjacent Tissue

As shown in Figure 2, Real-time PCR revealed that miR-16-1 expression in laryngeal carcinoma tissue was reduced compared with that in adjacent tissue ($p < 0.05$).

Effects of lncRNA Dleu2 and miR-16-1 on Cell Proliferation

To investigate the effects of lncRNA Dleu2 and miR-16-1 on laryngeal carcinoma cell proliferation, we over-expressed lncRNA Dleu2 and miR-16-1 in laryngeal carcinoma cells, respectively. Cell proliferation was markedly restrained after lncRNA Dleu2 and miR-16-1 over-expression, suggesting that lncRNA Dleu2 and miR-16-1 were related to cell proliferation (Figure 3).

Effects of lncRNA Dleu2 and miR-16-1 on Cell Migration

As shown in Figure 4, cell migration was significantly inhibited after lncRNA Dleu2 and miR-16-1 over-expression compared with control group ($p < 0.05$).

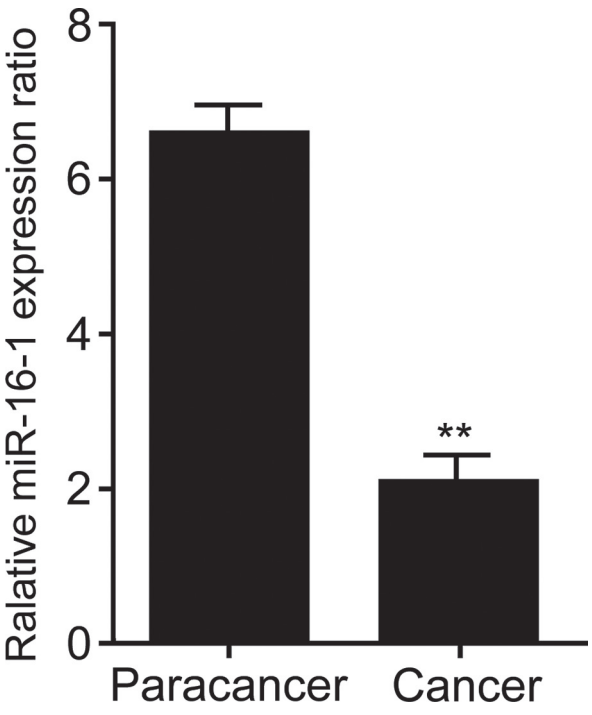

Figure 2. MiR-16-1 expression in laryngeal carcinoma tissue and corresponding adjacent tissue. $^{**}p < 0.05$, compared with paracancer tissue.

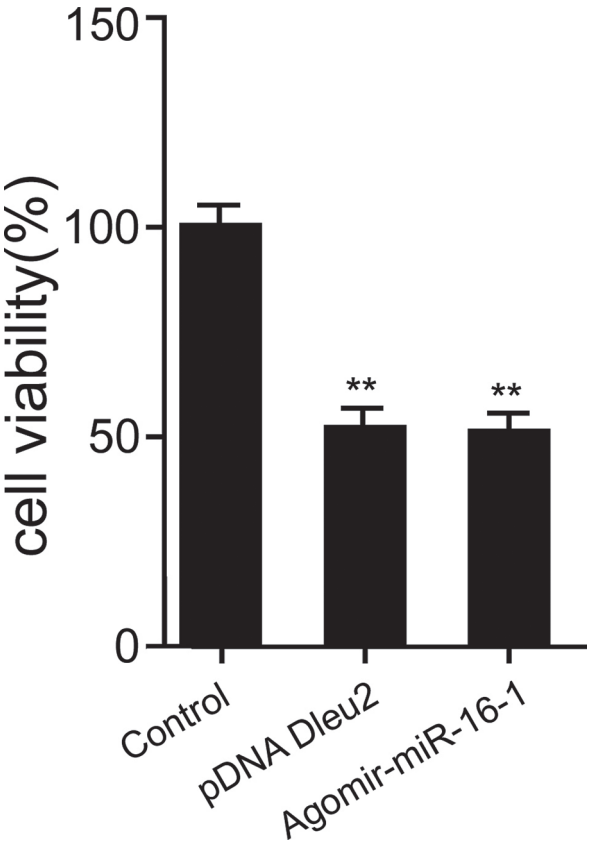

Figure 3. The impact of lncRNA Dleu2 and miR-16-1 on cell proliferation. $^{**}p < 0.05$, compared with control.

Effects of lncRNA Dleu2 and miR-16-1 on Cell Invasion

Transwell assay was used to evaluate the effects of lncRNA Dleu2 and miR-16-1 on cell invasion. As shown in Figure 5, the cell number penetrated the membrane after that lncRNA Dleu2 and miR-16-1 over-expression for 24 h was significantly decreased compared with control ($p < 0.05$).

lncRNA Dleu2 and miR-16-1 Interaction

It was demonstrated that lncRNA Dleu2 and miR-16-1 over-expression can suppress laryngeal carcinoma cell proliferation, migration, and invasion. Combining literature and bioinformatics database, we found that lncRNA Dleu2 may regulate miR-16-1 directly[5,8]. Therefore, we applied dual luciferase reporter gene assay. As shown in Figure 6A, wild-type and mutant Dleu2 luciferase vector were constructed, respectively. The activity of luciferase was detected after Ago-miR-16-1 transfection. As shown in Figure 6B, luciferase activity elevated in wild-type Dleu2 transfected

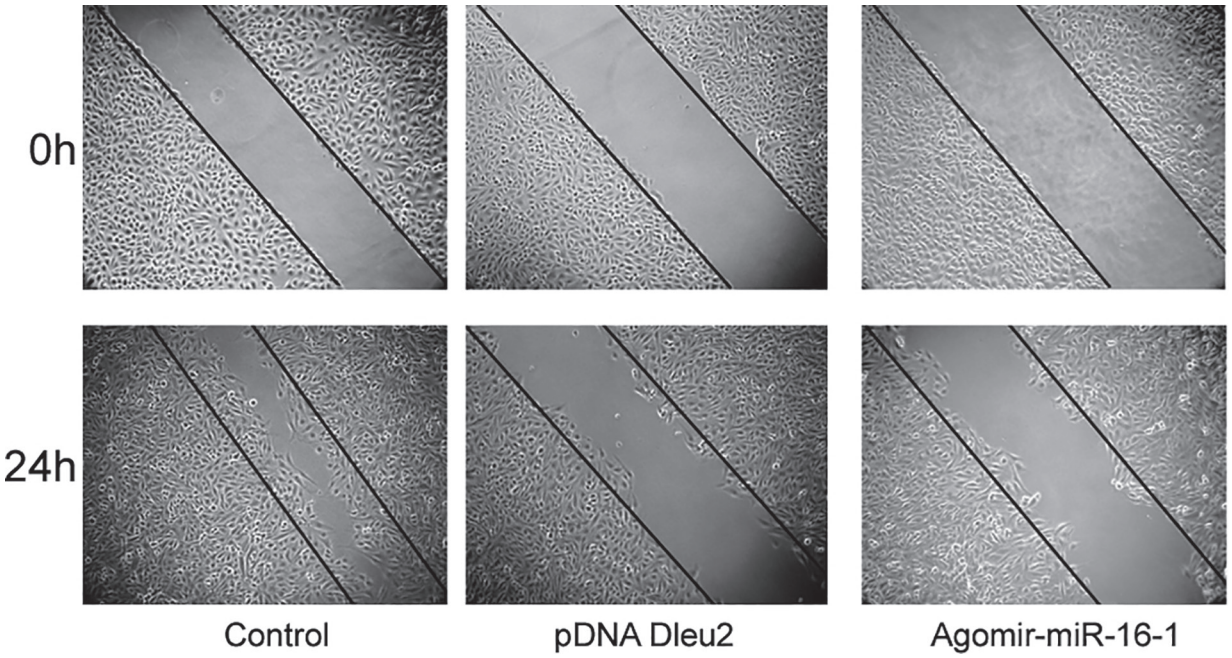

Figure 4. The influence of lncRNA Dleu2 and miR-16-1 on cell migration.

by Ago-miR-16-1, whereas it exhibited no statistical change in mutant Dleu2, indicating the interaction between lncRNA Dleu2 and miR-16-1.

Discussion

Laryngeal carcinoma is a common malignant tumor in the head and neck. It was found that some non-coding RNAs are abnormally expressed in laryngeal carcinoma cells, which are predominantly downregulated in miRNAs and up-regulated in lncRNAs[9].

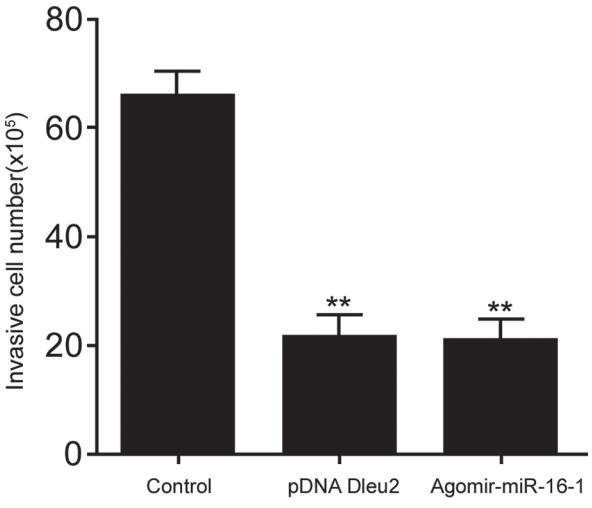

Figure 5. The effect of lncRNA Dleu2 and miR-16-1 on cell invasion. $^{**}p < 0.05$, compared with control.

LncRNA is a class of RNA molecules with transcript longer than 200 nt that do not encode proteins. LncRNAs are regulated by development and characterized as tissue and cell specificity[10]. They contain many types of transcripts that are structurally similar to mRNA. They also can transcribe into the antisense transcripts of encode genes. Under physiological and pathological conditions, lncRNAs perform important regulatory functions and are closely related to the development of the disease[11].

Transcriptional and post-transcriptional regulation of key genes are important processes for normal cells to perform function. Its destruction or alternation may promote the development of malignant tumors[12]. The deletion of chromosome 13q14 telomere region to RB1 is a common genetic aberration in many tumor types, which is also the most common area of chromosomal loss in chronic lymphocytic leukemia. This phenomenon not only occurs in T cell lymphoma and multiple myeloma, but also in head and neck cancer, prostate cancer, and other solid tumors. The deletion of 13q14 is most finely mapped to a 10 kb minimal deletion region (MDR) in chronic lymphocytic leukemia, which contains the promoter and the first exon of the DLEU2 gene[13]. Interestingly,

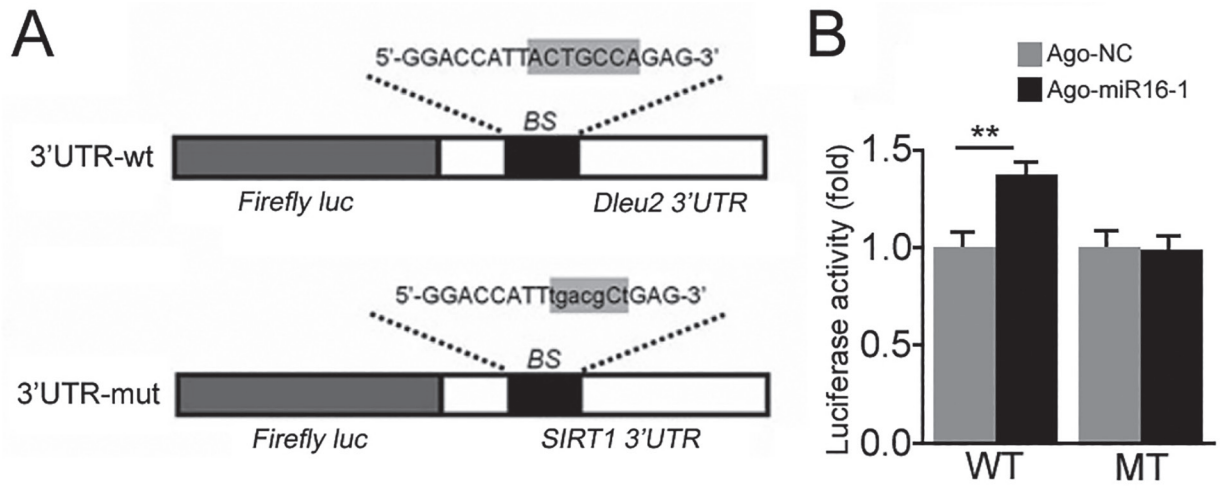

Figure 6. The interaction between lncRNA Dleu2 and miR-16-1. $^{**}p < 0.05$, compared with control.

DLEU2 is evolutionarily conserved in nucleotides rather than encoded protein, indicating that it may function at the RNA level[14].

Transcription miR-16-1 is highly expressed in a variety of human tissues[15]. Furthermore, miR-16-1 is identified to be down-regulated or deleted in chronic lymphocytic leukemia, prostate[16], breast cancer[17], gastric cancer[18], and pituitary tumors[19]. Nevertheless, there is still lack of research on its transcriptional regulation or biological function. However, it was reported that the expression of DLEU2 was correlated with miR-16-1[20-23]. MiR-16-1 is located around 13q14 MDR in cell chronic lymphocytic leukemia[24]. It is found that they have anti-proliferative and anti-apoptotic functions, making it possible to play a regulatory role in malignant tumors[25]. Up to now, there is no systematic study of whether miR-16-1 and Dleu2 have a regulatory effect in laryngeal carcinoma and their effects on cell proliferation, migration, and invasion. Therefore, in this report, we first investigated the expression of lncRNA Dleu2 and miR-16-1 in laryngeal carcinoma, and then over-expressed lncRNA Dleu2 and miR-16-1 by transfection technique to observe their influence on cell proliferation, migration, and invasion. At last, we assessed their interaction to evaluate their potential therapeutic effect in laryngeal carcinoma.

In summary, we showed that lncRNA Dleu2 is functionally coupled with miR-16-1 to inhibit the proliferation, migration, and invasion of laryngeal cancer cells. Up-regulation of lncRNA Dleu2 may be one of the targets of inhibiting laryngeal cancer growth and metastasis. However, its specific role still needs further verification in animal experiments. In addition, it has been suggested that lncRNA Dleu2 and miR-16-1 may have an effect on the cell cycle[26,27]. Therefore, their regulation of cell cycle requires further exploration.

Conclusions

We showed that lncRNA Dleu2 influences the proliferation, migration, and invasion of laryngeal cancer cells through miR-16-1. Therefore, lncRNA Dleu2 and miR-16-1 may serve as potential biomarkers and targets for laryngeal cancer diagnosis and treatment.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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