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Original Article

Identification of laryngeal carcinoma related gene 1 as a target gene of microRNA-21-5p in laryngeal carcinoma

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Abstract: The laryngeal carcinoma related gene 1 (*LCRG1*) has been implicated as a tumor suppressor in laryngeal cancer. However, the microRNAs (small non-coding RNAs of approximately 22 nucleotides in length) that regulate *LCRG1* expression remain elusive. In this study, quantitative PCR analysis first showed that the average level of miR-21-5p was elevated in primary laryngeal carcinoma tissues compared to paired adjacent non-tumor tissues. Thus, we explored the potential regulation of miR-21-5p on *LCRG1*. PITA algorithm predicted that two sites within *LCRG1* mRNA 3'UTR, which reside at +743 position and +938 position, respectively, might be targeted by miR-21-5p. Dual luciferase reporter assay confirmed that miR-21-5p mimic specifically decreased luciferase activity of the reporter harboring 3'UTR+743 target site. With respect to the 3'UTR+938 site, the influence of miR-21-5p on luciferase activity was not sequence specific. Further, we observed that miR-21-5p mimic transfection decreased the LCRG1 protein in human laryngeal carcinoma Hep2 cells as indicated by Western blotting analysis, and promoted cell growth, migration and invasion as evidenced by MTT assay and transwell migration assay and matrigel invasion assay. Of note, the effects of miR-21-5p inhibitor treatment are opposite to those of the miR-21-5p mimic. Taken together, this study identifies that the tumor suppressor *LCRG1* is targeted by the oncogenic miR-21-5p. This finding may help us to better understand the dysregulation of cancer-associated genes associated with microRNAs and eventually improve the diagnosis and target therapy for laryngeal carcinoma.

Keywords: Laryngeal carcinoma related gene 1, tumor suppressor, miR-21

Introduction

Laryngeal carcinoma (LC) is a common type of head and neck cancer [1]. In terms of the GLOBOCAN data of 2012, the number of LC patients reached to 156,877 worldwide, of which 20,014 (12.8%) were reported in China. Moreover, an annual addition of 5,590 patients with LC has been estimated for the next five years in China, with most patients predicted to have poor prognosis and limited response to surgical treatment, radiotherapy, and chemotherapy [2]. Therefore, there is an urgent need to understand the pathobiology of LC, which may help to establish methods for effective target therapy.

Recent advances in molecular biology implicate an intricate gene regulation network in cancer, involving the traditional encoding genes, as well as the non-coding transcripts of genome [3, 4]. With respect to LC, apart from the key oncogenes (e.g. *RAS*, *BCL-2, c-Myc*, *C-erbB2*, *cyclin D1*, and *EGFR*) and tumor suppressor genes (e.g. *p21*, *p27*, *p53*, and *Rb)* [5-7] which have been demonstrated to play important roles in tumor initiation and progression, the pathological significance of microRNA is also being uncovered [8, 9].

MicroRNA is a class of non-coding RNAs, with approximately 22 nucleotides in its mature form. It is estimated that more than one thirds encoding genes are under the regulation of microRNAs, which usually bind to 3' un-translated regions (UTR) of target mRNAs via imprecise pairing, to cause mRNA degradation or translation suppression [10]. Substantial studies sup-

Table 1. Primers used for luciferase activity reporter construction

Reporter	Direction	Primer sequences (5'-3')
pcDNA3.1-Luc-3'UTR+743	Sense	CGGAATTCAAAGACTGGCCTGGTG
	Antisense	GCTCTAGAGCTATACAAGCATGCATGTC
pcDNA3.1-Luc-3'UTR+938	Sense	CGGAATTCGCAATACGAGGACT
	Antisense	GCTCTAGAGCAGGTCAGAACTTC
pcDNA3.1-Luc-3'UTR+743 mutant	Antisense	GTGTCCGACTGTATGTAGACACAAC
	Sense	GTGTCTACATACAGTCGGACACTTCTATG
pcDNA3.1-Luc-3'UTR+938 mutant	Antisense	CAATGTCGAATCCTAGTAACAAGACAG
	Sense	GGATTCGACATTGTGTTAAAACCTG

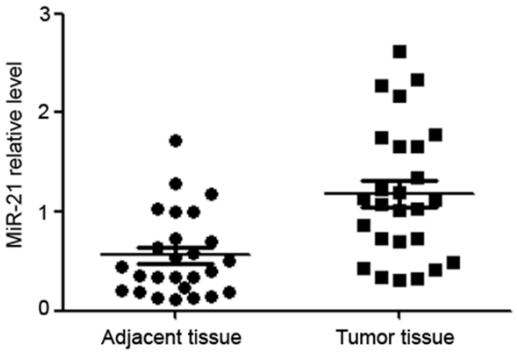

Figure 1. The relative expression levels of miR-21-5p in laryngeal carcinoma tissues and adjacent non-cancerous tissues were determined by quantitative RT-PCR. Tissue samples from 26 patients with laryngeal carcinoma who received radical surgery were analyzed. Paired student t test was conducted for the comparison between two groups (P=0.0002).

port that microRNAs play significant roles in tumor cell proliferation, differentiation, and apoptosis, directly or indirectly influencing cancer development [11]. MicroRNAs may function as cancer-promoting factor or tumor suppressor. For instance, it has been shown that miR-16 targets zyxin protein in human LC cells to promote cell motility [12], whereas miR-34 has been reported to suppress growth and invasion of LC cells through targeting c-Met [13].

The tumor suppressor laryngeal carcinoma related gene 1 (*LCRG1*), alternatively called gametogenetin-binding protein 2 (*GGNBP2*) elsewhere, was originally identified by means of mRNA differential display and found significantly down-regulated in primary LC tissues and LC cell lines [14]. However, up to date, the mechanism by which *LCRG1* is dysregulated in LC has not been revealed. The present study aimed to explore the microRNAs that may potentially target *LCRG1* mRNA and the resulting effects on cell proliferation, migration and invasion.

Materials and methods

Specimen

Primary LC tissues were from 26 patients who received radical surgery at the First People's Hospital of Chenzhou City affiliated to University of South China between January 2008 and August 2012. All patients had no history of chemotherapy prior to the surgery. All patients received written informed consent for the use of tissue samples and this study was conducted with approval from Medical Ethics Committee of University of South China.

Cell lines

Human laryngeal squamous cell carcinoma cell line Hep2 was generously provided by Professor Chun-Mei He at State Key Laboratory of Chemo/Biosensing and Chemometrics, Hunan University, and maintained in RPMI1640 medium containing 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Beijing, China). HEK293 T cells were a kind gift from Shanghai Oncology Institute and grown in DMEM medium supplemented with 10% FBS.

Prediction of microRNA recognition site

LCRG1 cDNA sequences were retrieved from GenBank (No. AF268387). The 3'UTR sequences were uploaded to PITA (http://genie.weizmann.ac.il/pubs/mir07) for prediction of microRNAs that may target *LCRG1* mRNA.

Construction of luciferase-LCRG1 3'UTR gene reporter

To construct luciferase-LCRG1 3'UTR gene reporter, a 300-bp region including a miR-21-5p target site and the upstream and downstream sequences within LCRG1 3'UTR was amplified

Table 2. PITA predicted miR-21-5p target sites within LCRG1 3'UTR

MicroRNA	Position	Seed	dGduplex	dGopen	ddG
hsa-miR-21	938	8:01:01	-12.8	-10.01	-2.78
hsa-miR-21	743	8:01:01	-11.9	-15.97	4.07

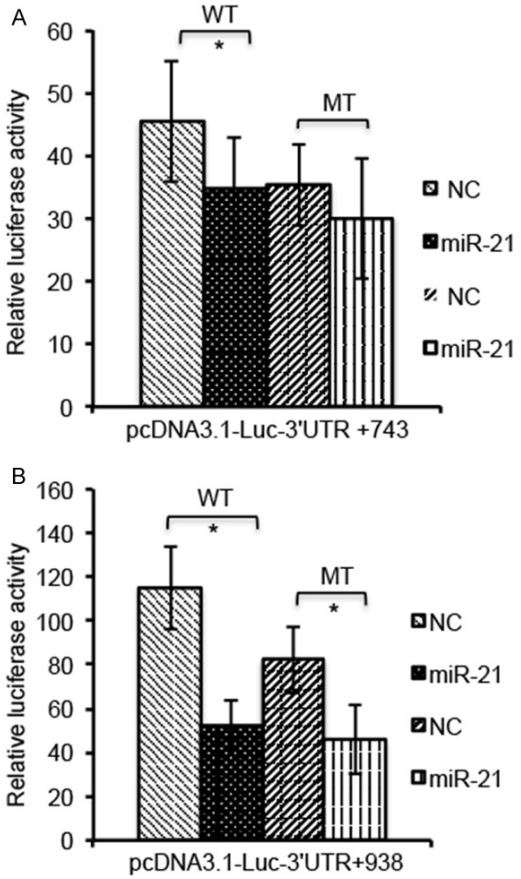

Figure 2. MiR-21-5p down-regulated the activity of luciferase reporter harboring target site of *LCRG1* 3'UTR. Dual luciferase assay was carried out with the luciferase activity reporter harboring the miR-21-5p target site at *LCRG1* 3'UTR+743 position (A) or +938 position (B).

using Hep2 cell genomic DNA as template and then inserted into pcDNA3.1-Luc backbone by *EcoRI/XbaI* restriction enzyme sites. Mutations in LCRG1 3'UTR that were made on purpose to abolish miR-21-5p binding were introduced by PCR using specific primers. The primers used for amplification are listed in **Table 1**.

Dual luciferase reporter assay

HEK293T cells were seeded in 96-well plate at 7000 cells per well. After 24 hours, cells in the well were transfected with 50 ng pcDNA3.1-Luc-LCRG1 3'UTR, 10 pmol miR-21-5p mimic or negative control (NC) microRNA (provided by Ribobio, Guangzhou, China), 5 ng Renilla vector using lipofectamin 2000 (Invitrogen, Beijing, China). The luciferase activity was measured using dual luciferase reporter assay kit (Promega, USA) following the manufacturer's instructions.

Real-time quantitative PCR

Total RNA extraction of primary LC tissue samples was conducted using Trizol reagent (Invitrogen) according to protocols as recommended by the supplier. The cDNA synthesis was performed with PrimeScript reverse transcription reagents kit (TaKaRa, Dalian, China) and stem-loop primers. Real-time PCR was run in a 10 μl volume containing TaKaRa SYBR Premix Ex Taq (2×) 5 μl, forward and reverse primer (10 μM) 0.4 μl for each, ROX Dye (50×) 0.2 μl, cDNA template 1 μl at 95°C, 30 s followed by 95°C, 30 s; 60°C, 30 s for 40 cycles on ABI 7500 PCR system. The amplification of snRNA U6 served as endogenous control. The relative expression level was calculated using ΔΔCT method [15].

Western blotting analysis

Hep2 Cells were harvested and washed twice with cold phosphate buffer saline (PBS) prior to the lysis in 50 mM Hepes pH 7.4, 135 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 0.1% SDS supplemented with proteinase inhibitor cocktails (Roche). Protein concentration of the lysate was determined using BCA assay kit provided by Pierce (Thermo Scientific). 20 μg proteins for each sample were loaded onto 10% SDS-PAGE for separation and then electro-transferred onto nitrocellulose membrane (Millipore). The membranes were blocked in PBS buffer with 5% nonfat milk and 0.02% Teween-20 at room temperature for one hour, followed by primary antibody (Cat#: sc-164839, Santa Cruz Biotechnology) incubation overnight at 4°C. HRP-conjugated secondary antibody (Jackson Immunoresearch laboratory, USA) incubation proceeded at room temperature for one hour. The blots were visualized by adding chemiluminescence substrate (Beyotime Biotechnology, Beijing, China) followed by exposure to X-ray films.

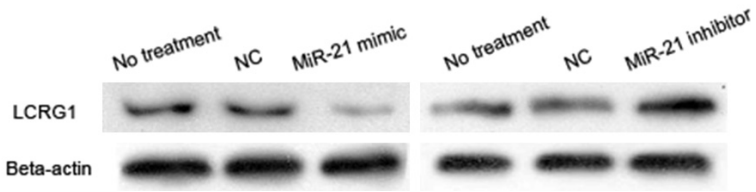

Figure 3. MiR-21-5p regulated LCRG1 protein level in Hep2 cells. MiR-21-5p mimic and inhibitor transfection followed by Western blotting analysis showed that LCRG1 protein was decreased and increased, respectively.

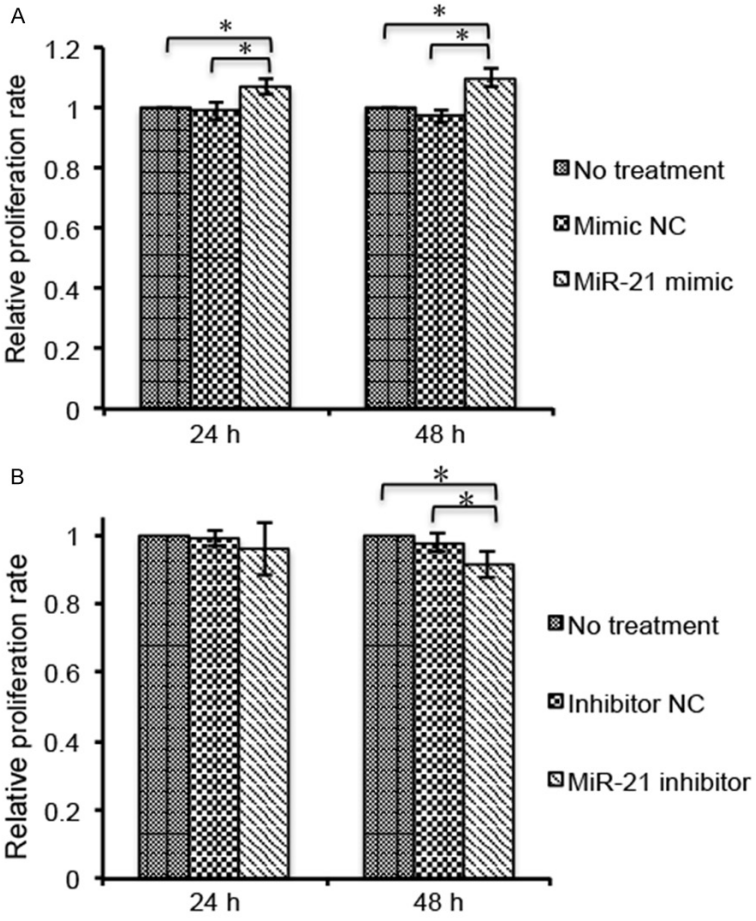

Figure 4. MiR-21-5p mimic and inhibitor transfection promoted and inhibited Hep2 cell growth, respectively. Hep2 cells were transfected with miR-21-5p mimic (A) or inhibitor (B). MTT assay was performed at 24 and 48 hours upon transfection, and the relative proliferation rate was determined with the no-treatment control serving as reference.

MTT assay

Hep2 Cells were seeded in 96-well plate at 5,000 cells per well. Next day, cells were transfected with miR-21-5p mimic/inhibitor or the negative control microRNA. For MTT assay, 20 μl 5 mg/ml MTT (Sigma-Aldrich, USA) were added for each well and the plate was kept for another 4 hours at 37°C with 5% CO_2. Upon removal of the medium, 100 μl dimethyl sulphoxide were added into each well. The absorbance of the solution at 490 nm was measured on a plate reader. Each experimental condition was measured in triplicates at the indicated time.

Cell migration and invasion assay

100 μl of cell suspension (10^6 cells/ml) were added into the upper chamber of a transwell insert placed in 24-well plate. After incubation for 12 hours in a cell incubator, the transwell insert was taken out and washed with PBS for three times. The Hep2 cells on the upper surface were swiped using cotton swabs, and then the transwell membrane was exposed to 4% polyformaldehyde for 18 minutes at room temperature. After fixation, the membrane was air-dried and then stained using 0.1% crystal violet for 18 min. Images of six fields were captured under a light microscope. For invasion assay, matrigel (BD biosciences) was paved at the lower surface of the chamber, and the following procedures are similar to those in migration assay.

Flow cytometry

To detect cell apoptosis, cells were collected and washed with PBS, followed by incubation with Annexin V-FITC and propidium iodine (PI) for 20 min on ice. For cell cycle analysis, cells were washed twice using PBS and then fixed in 75% ethanol overnight. Next day, the fixed cells were stained with PI for 30 min prior to analysis on a flow cytometer.

Statistical analysis

The experimental data were statistically processed using Graphpad prim 5 software and presented as mean ± standard deviation. For numerical data, unpaired student t test was employed for comparison on mean value between two groups. P<0.05 was considered statistically significant.

Results

MiR-21-5p was up regulated in LC and possibly targets LCRG1

As previous study showed that *LCRG1* was down regulated in LC, we sought for the microRNAs that are up regulated to suppress *LCRG1*. Since miR-21-5p has been reported to increase in many cancers, we first performed quantitative RT-PCR to analyze its expression level in primary LC tissues. Results show that the average level of miR-21-5p in primary LC tissues is significantly higher than that in adjacent noncancerous tissues (**Figure 1**). Next, PITA algorithm predicted 649 microRNAs that may potentially target *LCRG1* mRNA, and miR-21-5p is a top candidate on the list, as the seed sequence of miR-21-5p is highly complementary to two sites within *LCRG1* 3'UTR. Of the two sites, one is located at +743 position and the other at +938 position (**Table 2**).

MiR-21-5p suppressed luciferase activity by targeting LCRG1 3'UTR+743 site

To verify the binding of miR-21-5p to either LCRG1 3'UTR+743 site or the +938 site, luciferase activity gene reporter was constructed by inserting a 300-bp *LCRG1* 3'UTR fragment (containing the binding site and it upstream and downstream sequences) into the pcDNA3.1-Luc backbone. Dual luciferase assay results showed that the luciferase activity in HEK293T cells transfected with pcDNA3.1-Luc-LCRG1 3'UTR+743 reporter and miR-21-5p mimic significantly decreased compared to that in HEK293T cells transfected with the reporter and negative control microRNA (P<0.01) (**Figure 2**). As to the reporter with mutations that abolished the complementary binding between *LCRG1*-3'UTR+743 site and miR-21-5p, the miR-21-5p mimic had no significant inhibition on luciferase activity compared to the control microRNA (P=0.059) (**Figure 2**). Regarding to the *LCRG1*-3'UTR+938 binding site, miR-21-5p mimic dramatically suppressed the luciferase activity of the wild-type *LCRG1*-3'UTR+938 reporter. However, miR-21-5p mimic also showed inhibition to the luciferase activity of the mutated 3'UTR+938 reporter, which apparently is nonspecific.

Increase of miR-21-5p caused downregulation of LCRG1 expression

Western blotting analysis show that the level of LCRG1 protein was obviously decreased in Hep2 cells transfected with miR-21-5p mimic compared to Hep2 cells transfected with NC microRNA or non-treated, and oppositely that the LCRG1 level was appreciably increased in Hep2 cells treated with miR-21-5p inhibitor relative to the Hep2 cells treated with NC microRNA or non-treated (**Figure 3**).

MiR-21-5p promoted growth, migration and invasion of Hep2 cells

To determine the impact of miR-21-5p on cell growth, Hep2 cells transfected with miR-21-5p mimic or inhibitor were subjected to MTT assay. Results show that the growth rate of Hep2 cells in the presence of miR-21-5p mimic was increased compared to that of Hep2 cells treated with NC microRNA or non-treated, whereas the growth of Hep2 cells in the presence of miR-21-5p inhibitor was suppressed at 48 hours upon treatment (**Figure 4**). To evaluate the effect of miR-21-5p on cell migration, transwell assay without matrigel was performed. Results show that the migration of Hep2 cells was enhanced in the presence of miR-21-5p mimic and inhibited by the miR-21-5p inhibitor (**Figure 5**). Further, matrigel invasion assay was performed to evaluate the invasion ability of Hep2 cells transfected with miR-21-5p mimic or inhibitor. Crystal violet staining showed that the invasive cells were significantly increased under miR-21-5p mimic treatment compared to NC microRNA treatment and no treatment (**Figure 6**).

Knockdown of miR-21-5p promoted cell cycle arrest and cell apoptosis

To observe the influence of miR-21-5p on cell cycle progression and cell survival, Hep2 cells were transfected with miR-21-5p inhibitor and subjected to flow cytometric analysis. Results showed that down regulation of miR-21-5p in Hep2 cells led to an increase of both G1 and S

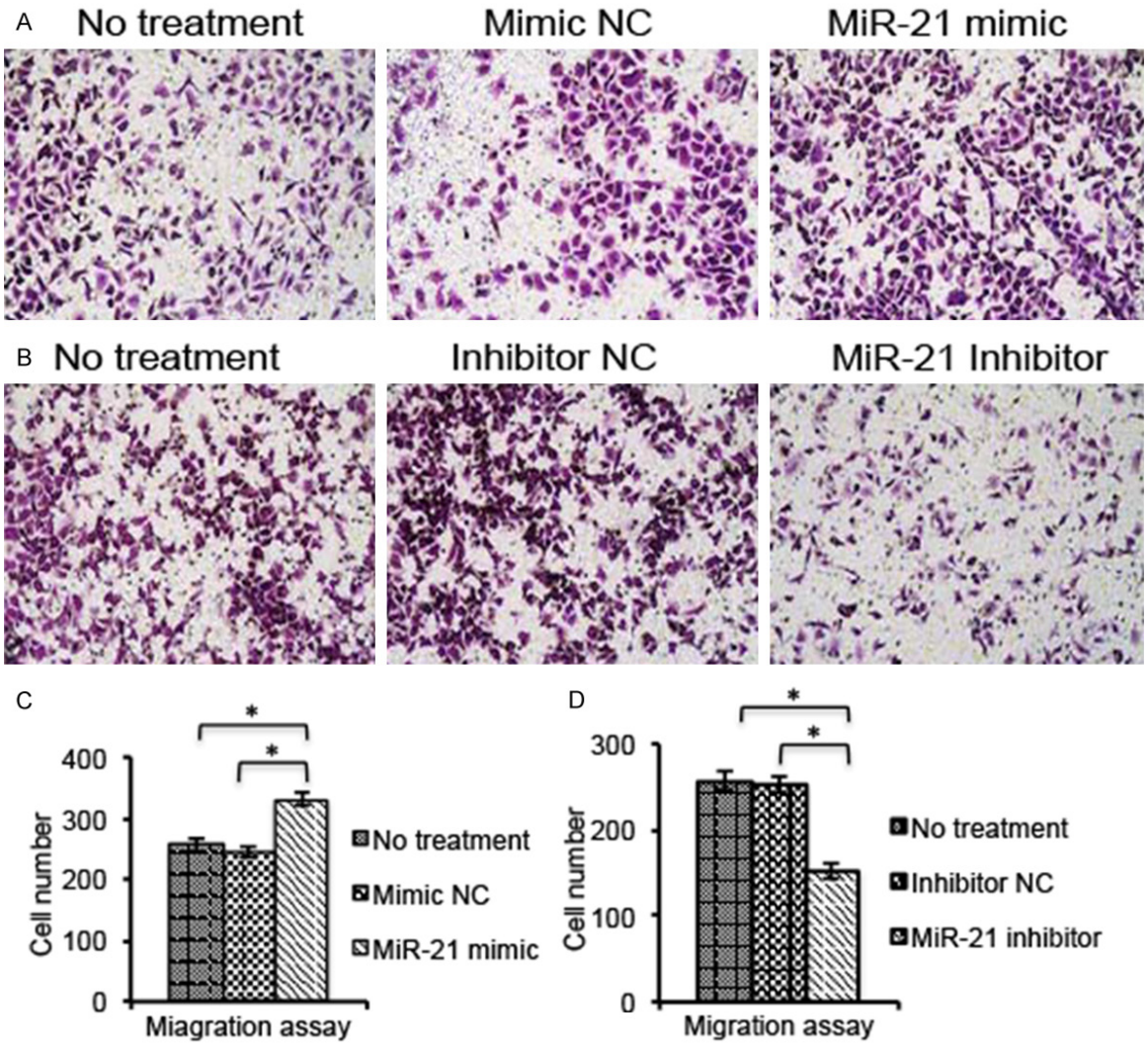

Figure 5. Transwell assay shows that miR-21-5p mimic (A) and inhibitor (B) treatment enhanced and suppressed cell migration, respectively. The migrated cells on transwell membranes were stained by crystal violet. Representative images and statistical data (C and D) were shown (*, P<0.05).

stage cells and meantime a dramatic decrease in G2 stage cells compared to the negative control and non-treated control (**Figure 7**). By dual labeling using FITC-Annexin V and PI, it was observed that miR-21-5p inhibitor transfection induced significant cell apoptosis compared to the controls (**Figure 7**).

Discussion

Although *LCRG1* was originally identified by means of differential mRNA display and suggested as a tumor suppressor over a decade ago [14], little is known about the molecular basis of its deregulation in LC. The finding in this study that *LCRG1* is targeted by miR-21-5p highlights a post-translational regulation on *LCRG1* in laryngeal cancer. In addition, our results implicate that the increase of miR-21 and down-regulation of LCRG1 may promote LC cell growth and motility, thereby contributing to cancer development and progression. It would be interesting to further explore the clincopathological significance of *LCRG1* in the context of laryngeal cancer. For example, how is the level of *LCRG1* correlated with LC diagnosis and prognosis in clinic?

The oncogenic role of miR-21 has been well known [16]. It targets a number of tumor suppressive genes in various cancers, for instance, *PTEN* in lung cancer [17], *FASLG* in glioblasto-

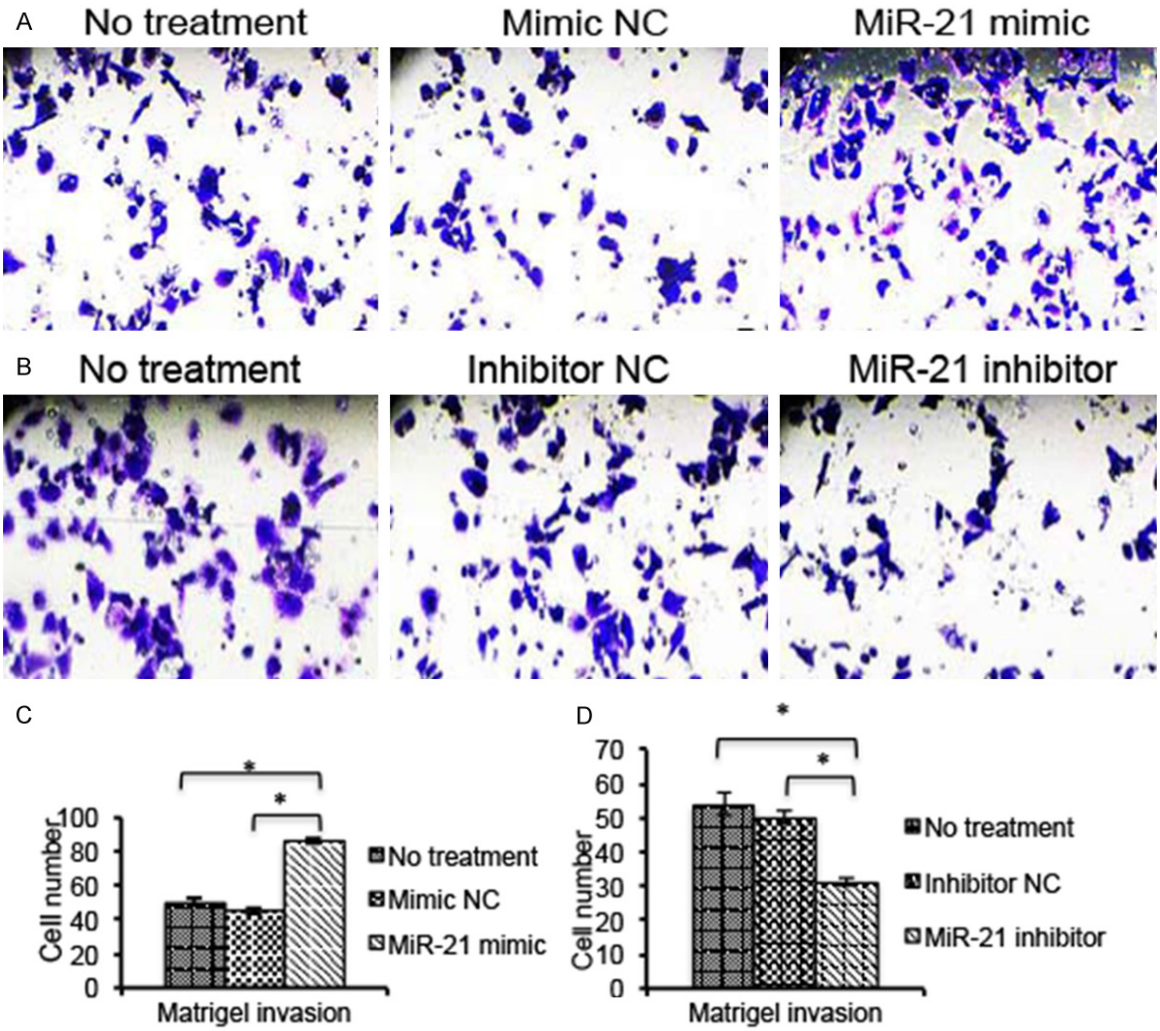

Figure 6. Matrigel invasion assay shows that miR-21-5p mimic (A) and inhibitor (B) treatment promoted and inhibited cell invasion, respectively. Transwell matrigel invasion assay was conducted. The cells that invaded into matrigel were stained by crystal violet. Representative images and statistical data (C and D) were shown (*, $P<0.05$).

ma [18], *BTG2* in human laryngeal carcinoma [19], and et al. As a microRNA is able to target many encoding genes and simultaneously these genes may be regulated by many other microRNAs [20], the complexity of regulatory network associated with miR-21 in LC increases with the identification of *LCRG1* as a miR-21 target gene. To fully uncover the target genes of miR-21 in laryngeal cancer will help us to better understand the pathobiology of this malignancy, as well as to develop the potential use of miR-21 as a biomarker and therapy target for LC [16].

We realize that our study may simply reflect one possible mechanism by which *LCRG1* is regulated in LC, and other epigenetic and transcriptional mechanisms may collaboratively act to suppress the tumor suppressor *LCRG1*. Thus, further investigation into the LCRG1 gene regulation is anticipated. Moreover, our current knowledge about the molecular basis of the tumor suppressive function of *LCRG1* remains largely unknown. To identify the signaling pathways that involve LCRG1 is of significance to develop LCRG1 as a potential molecular target for cancer therapy.

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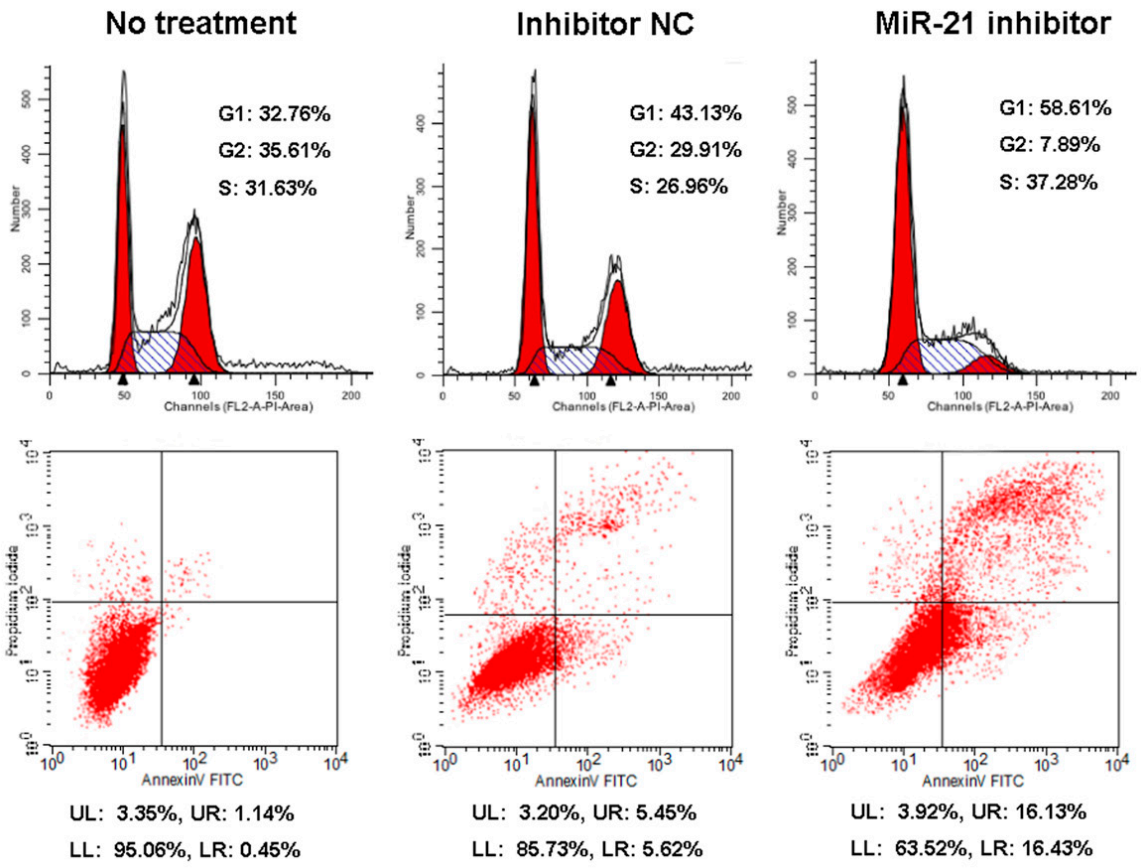

Figure 7. Knockdown of miR-21-5p induced cell cycle arrest and apoptosis. Cells were fixed and stained with PI for cell cycle analysis on a flow cytometer. The percentages of G1, S, and G2 stage cells were indicated. For apoptosis analysis, cells were dual-labeled by PI and Annexin V-FITC prior to loading onto a flow cytometer. Upper left (UL) quadrant represent PI positive and Annexin negative cells; Upper right (UR), both PI and Annexin positive; Lower left (LL), both PI and Annexin V negative; Lower right (LR), PI negative and Annexin positive.

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Disclosure of conflict of interest

None.

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