

Effect of miR-363 on the proliferation, invasion and apoptosis of laryngeal cancer by targeting Mcl-1

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Abstract. – OBJECTIVE: To investigate the potential effect of miR-363 on the development of laryngeal cancer and to reveal the relevant mechanism.

PATIENTS AND METHODS: The expression level of miR-363 was detected in laryngeal cancer tissues and cells (TU-177), respectively. Luciferase assay was performed to evaluate the interaction between miR-363 and myeloid cell leukemia-1 (Mcl-1). The effect of the miR-363/Mcl-1 axis on TU-177 cells was determined by subsequent experiments including cell proliferation, invasion, apoptosis and the expression level of Mcl-1.

RESULTS: In the present study, we found that miR-363 was both repressed in laryngeal cancer tissues and cells (TU-177). To find the relevant target of miR-363, we searched three publicly available algorithms, including TargetScan, miRDB, and microRNA. Results showed that Mcl-1 was a direct target of miR-363, and the Luciferase assay confirmed our suggestion. Subsequent experiments indicated that the increased expression of Mcl-1 resulted from the down-regulation of miR-363 could lead to the cell proliferation and invasion and accelerate cell apoptosis in laryngeal cancer cells.

CONCLUSION: Our research revealed the suppressed expression of miR-363 in laryngeal cancer by targeting Mcl-1. Meanwhile, we found that the up-regulation of miR-363 could serve as a potential therapeutic strategy for the treatment of laryngeal cancer.

Key words: Laryngeal cancer (LCA), miR-363, Myeloid cell leukemia-1 (Mcl-1), proliferation, invasion, apoptosis

Introduction

Laryngeal cancer (LCA) is one of the common malignant tumors of the head and neck, whose incidence rate is second only to nasopharyngeal carcinoma. LCA frequently occurred in middle-aged

men. In recent years, its incidence rate has shown a younger trend. With the economic development and the acceleration of industrial pollution, the incidence of LCA in urban area is higher than that in rural area^{1,2}. The pathogenesis of LCA is still not yet clear. Researcher have demonstrated that the development of LCA is a result of multiple factors, including smoking, drinking, environmental pollution, chronic inflammation, viral infection, vitamin deficiency, endocrine disorders, previous lesions, and genetic factors. Although the medical level is constantly improved with the progress of science and technology now, the early diagnosis of early LCA is still difficult. Most patients with LCA have been in the advanced stage when treated. Radical resection combined with radiotherapy and chemotherapy is the main treatment for middle-advanced LCA; however, the postoperative recurrence rate is high and the curative effect is not satisfactory, leading to a low 5-year survival rate²⁻⁵. Therefore, LCA is a kind of malignant tumor seriously threatening people's health and life. The early diagnosis of LCA is the key to improving the treatment effect and the life quality of LCA patients.

MiRNA is a kind of non-coding small RNA that is mostly composed of 20-25 nucleotides. miRNAs are involved in a number of important biological processes in life, including cell development, proliferation, apoptosis, death, fat metabolism, and cell differentiation⁶⁻⁸. Normal cell growth, proliferation, development, differentiation, and death are highly-ordered processes, and the disorder of these processes can lead to tumorigenesis. Studies have found that miRNA also plays a key role in the occurrence and development of tumors⁹. Scholars^{10,11} have confirmed that miRNA-25 and miRNA-221 are abnormally expressed in LCA, which are expected to be used for early diagnosis. Other studies have also found that miRNA is closely related to tumor metastasis.

sis, recurrence, staging, and prognosis¹². Multiple researches have shown that down-regulated miRNAs in LCa may inhibit the occurrence and development of LCa via regulating some tumor suppressor genes, which may play a role as tumor suppressor genes. Meanwhile, miRNAs that are up-regulated may promote the occurrence and development of LCa through regulating some oncogenes, which may play a role as oncogenes. Hence, those up-regulated and down-regulated miRNAs in LCa constitute a complex gene control network to regulate the proliferation, apoptosis, differentiation, and migration of tumor cells.

As a kind of endogenous non-coding and single-stranded RNA, miR-363 can down-regulate the expression of target genes by binding to the 3'-untranslated region (UTR) of the target gene mRNAs. Many studies^{13,14} have shown that the expression disorder of miR-363 will lead to tumorigenesis, and tumor cells may often increase the expression of oncogenes and inactivate tumor suppressor genes through reconstructing the expression of miR-363 in cells. It is reported that miR-363 is down-regulated in a variety of tumors, suggesting that it is a tumor suppressor gene¹⁵. However, whether miR-363 is involved in the progression of LCa remains unclear. In this study, we found that miR-363 targeted to Mcl-1, and the transfection of miR-363 mimics could inhibit LCa cell proliferation and invasion and promote its apoptosis.

Patients and Methods

Laryngeal Cancer Cases and Tissues

This study included 48 laryngeal cancer (LCa) patients, and 20 of them underwent a surgical resection procedure in the Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University. The tissues were then pathologically diagnosed and confirmed LCa. Preoperative chemotherapy or radiotherapy treatment were forbidden. The liquid nitrogen was used to freeze the LCa tissues and the corresponding adjacent normal tissues were kept at -70°C for storage. The adjacent normal tissues were confirmed by biological biopsy to insure that they did not include cancer cells. After all, the Declaration of Helsinki should be mentioned and respected. This work was approved by the Ethics Committee of the Affiliated Huai'an No. 1 People's Hospital of Nanjing Medical University. Signed written informed consents were obtained from all the participants before the study.

The human laryngeal cell line (TU-177) and normal bronchial epithelial cell line (16HBE) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen, Carlsbad, CA, USA) complemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin and 100 IU/mL penicillin (Invitrogen, Carlsbad, CA, USA), and were incubated in a 5% CO₂ culture incubator.

Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was procured by RNeasy Lysis Reagent (ThermoFisher Scientific, Inc. Waltham, MA, USA) in accordance with the manufacturer's protocol. SYBR green qPCR assay (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to detect the expression level of miRNAs in myeloid cell leukemia 1 (Mcl-1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. TaqMan miRNA assay (Applied Biosystems, Waltham, MA, USA) was used to determine the expression level of miR-363 normalized to miRNA U6.

Luciferase Reporter Assay

In TargetScan, miRDB, and microRNA websites, we found that Mcl-1 was the target gene of miR-363. TU-177 cells were co-transfected with pMIR-30UTR-Mcl-1/pMIR-30UTR-Mut Mcl-1, miR-363 mimic/negative control (NC), and the pMIR-Renilla plasmid (Promega, Madison, WI, USA), followed by being seeded into a 12-well plate. The cells were then lysed after transfection. The luciferase activity was tested by a multi-function microplate reader (Promega Corporation, Madison, WI, USA), and the results were normalized to the Renilla luciferase activity.

Cell Transfection

miR-363 mimics, control, and inhibitor were synthesized and transfected to the LC cell line to analyze the biological function of miR-363. Then, three groups were established to study the potential relevance between miR-363 and TU-177 cells: the NC group (negative control), the miR-363 mimics group (TU-177 cells transfected with miR-363 mimics), and the mimics+Mcl-1 group (TU-177 cells transfected with miR-363 mimics and siMcl-1). All the reagents were purchased from RiboBio (Guangzhou, China), and were transfected by using the lipofectamine

RNAiMAX (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions.

Western Blot Analysis

The protein concentration was measured by the bicinchoninic acid (BCA) Reagent Kit (Merck, Billerica, MA, USA). Totally 20 μg protein of TU-177 cells were separated on polyacrylamide gels, and were then transferred to nitrocellulose membranes. Subsequently, the membranes were blocked with blocking buffer (TBS with 0.05% Tween 20, pH 7.6 with 5% skimmed milk). Next, the membranes were washed and incubated with anti-Mcl-1 and anti- β -actin (1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA) antibody at 4°C overnight. Then, the membranes were sealed with 5% skim milk powder at room temperature for 2 h. After washing for 3 times, the membranes were incubated with enhanced chemiluminescence (ECL) (Millipore, Billerica, MA, USA) for luminescence generation. The proteins were visualized and detected, and the grey level of each protein was normalized to β -actin. All the results were analyzed by the Image-J Software.

Cell Proliferation Assay

TU-177 cells were harvested and inoculated into 96-well plates at a density of 2×10^3 cells for 24 hours. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (5 mg/mL, MultiSciences, Nanjing, China) was added to each well after 4-hour incubation. Then, 100 μL dimethyl sulfoxide (DMSO) (Aldrich, St. Louis, MO, USA) was added to each well for solubilizing the formazan. After half an hour, OD (optical density) value at 490 nm was measured by a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell Invasion Assay

Cell invasion assay was performed by transwell plates (Corning, Corning, NY, USA) with 8- μm pore size membranes with Matrigel. Briefly, 2×10^5 cells were seeded into the upper chamber with serum-free medium. Meanwhile, the lower chambers were added with culture medium containing 10% fetal bovine serum as a chemoattractant. After 24-days incubation, cells on the top of the membranes were wiped by a brush. Subsequently, the membranes were stained with 0.2% crystal violet, followed by drench with 95% ethanol. The number of invading cells was observed under an inverted microscope.

TUNEL Assay

The apoptosis of TU-177 cells was detected by TUNEL assay according to the manufacturer's instructions (Roche, Basel, Switzerland). Horseradish peroxidase-mediated diaminobenzidine reaction was used to visualize the TUNEL-positive cells, followed by counterstaining. Fields were randomly selected and photographed at a magnification of 200 \times . The apoptosis index was used to measure the extent of apoptosis.

Statistical Analysis

Data were analyzed by the SPSS 16.0 software (La Jolla, CA, USA). Statistical analysis was performed by Student's *t*-test or *F*-test. All *p*-values were two-sided and $P < 0.05$ were considered statistically significant.

Results

The Expression Level of miR-363 Was Decreased in LCa Tissues and Cells

MiR-363 has been found lowly expressed in laryngeal cancer (LCa) in recent studies¹⁶, but its mechanism remains unclear. To investigate the role of miR-363 in LCa, qRT-PCR was performed to detect the expression level of miR-363 in LCa tissues and adjacent normal tissues. Result showed that the expression of miR-363 was significantly lower in LCa tissues than that of adjacent normal tissues (Figure 1A), which was consistent with the paper description. Further, we detected miR-363 expression in TU-177 cells and 16HBE cell, and got the same results (Figure 1B). Together, we thought miR-363 might have a regulatory effect on LCa progression.

Mcl-1 Was a Direct Target of miR-363 in LCa Cells

To elucidate the putative and possible targets of miR-363, we searched three publicly available algorithms, including TargetScan, miRDB, and microRNA. Finally, we found that Mcl-1 was a supposed target of miR-363 (Figure 2A). Recent studies¹⁷⁻¹⁹ have demonstrated that Mcl-1 has the function of promoting tumor progression. Thus, Mcl-1 has caught our attention. To confirm whether miR-363 has a regulatory effect on Mcl-1, we established luciferase reporter vectors containing the wild or mutant-type miR-363 seed sequences of the Mcl-1 3'UTR. The increased expression of miR-363 with mimics resulted in

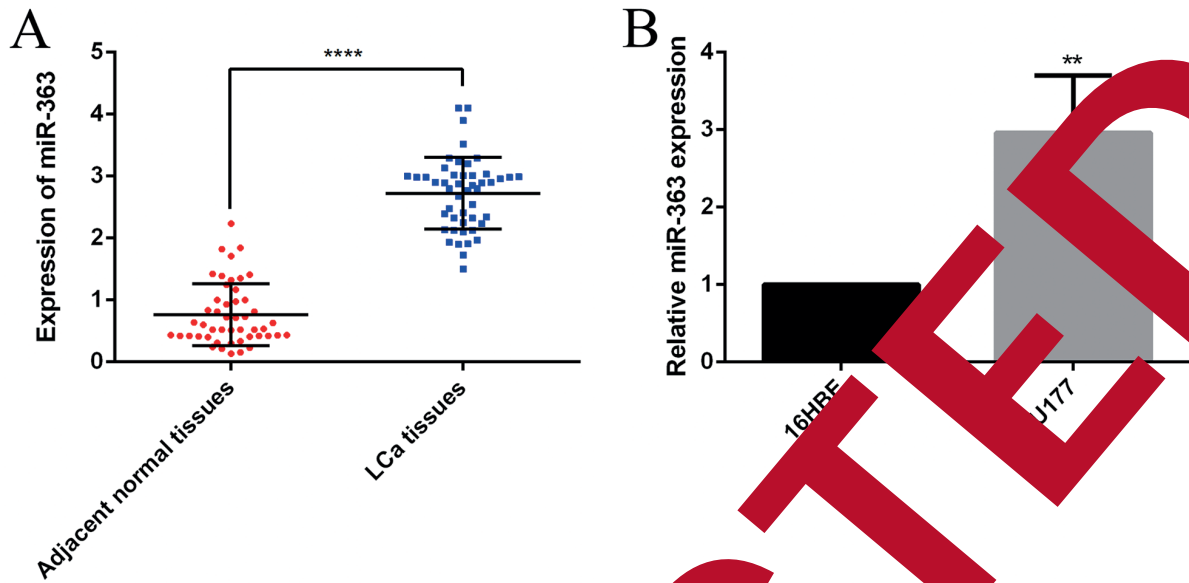


Figure 1. The expression of miR-363 in LCa tissues and cells. **A**, Difference in the expression of miR-363 between LCa tissues and corresponding adjacent normal tissues (**** $p < 0.0001$ compared with adjacent normal gastric tissue). **B**, The expression of miR-363 in the LCa cell line (TU-177) and normal bronchial epithelial cell line (16HBE) (** $p < 0.01$ compared with 16HBE).

the decrease of the luciferase activity of the wild-type Mcl-1 3'UTR reporter gene. However, no effect was found on the mutant-type, suggesting that the expression of Mcl-1 could be regulated by miRNA-363 (Figure 2B).

MiR-363 Decreased the Expression of Mcl-1

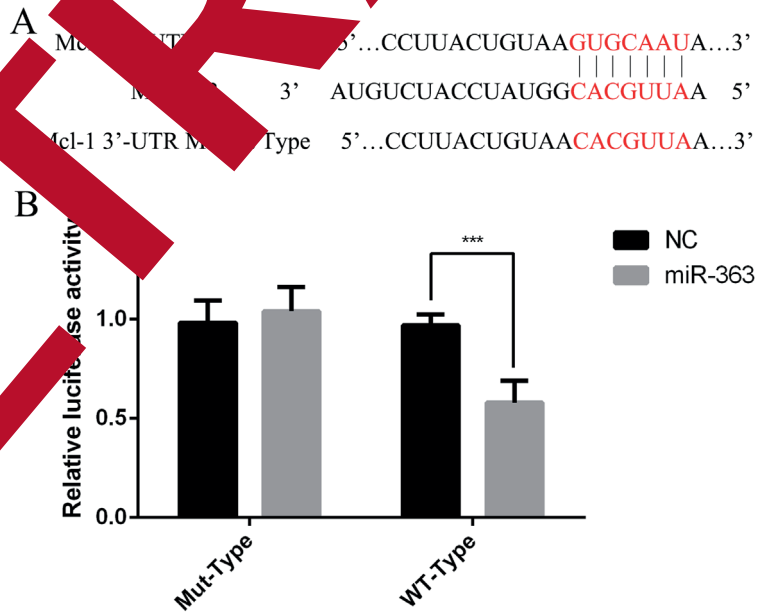


Figure 2. Mcl-1 was a direct and functional target of miR-363. TU-177 cells were transfected with miR-363 mimics and inhibitor. **A**, Diagram of putative miR-363 binding sites of Mcl-1. **B**, Relative activities of luciferase reporters (***) $p < 0.001$.

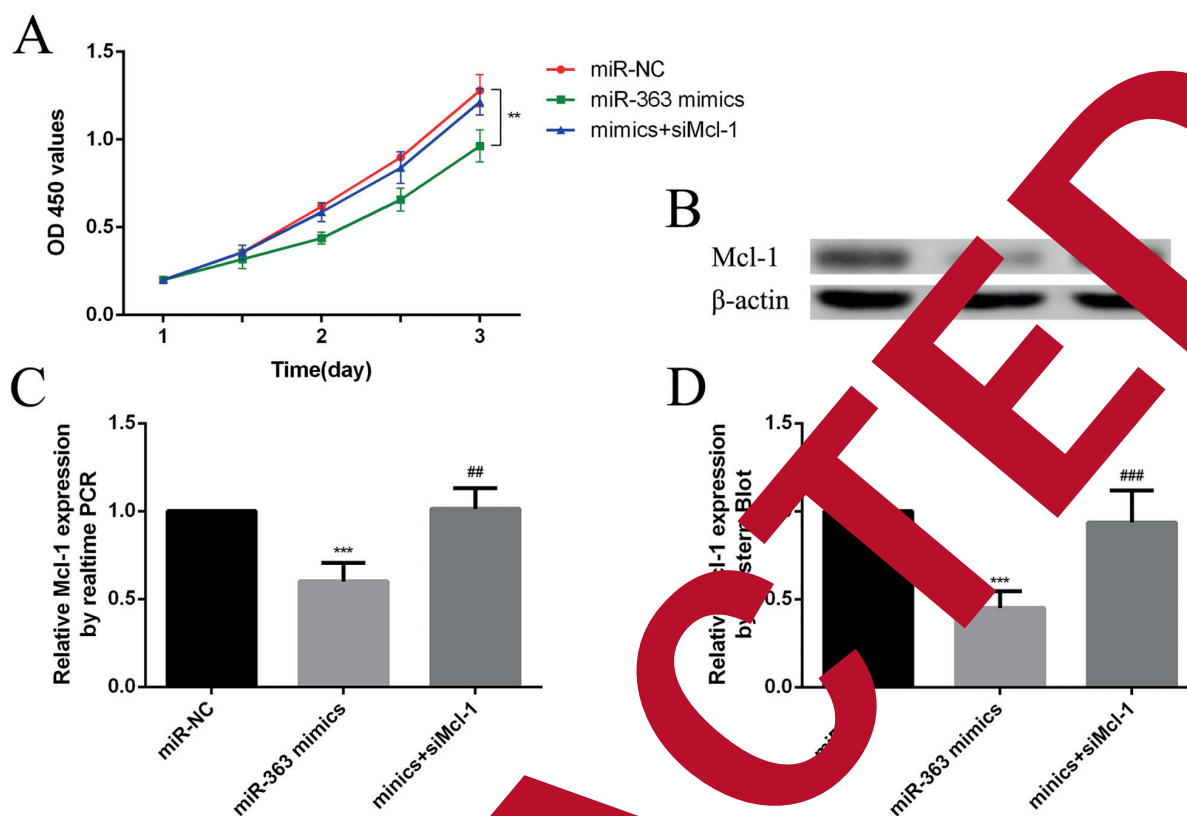


Figure 3. MiR-363 decreased the proliferation of LCa cells and the expression level of Mcl-1. **A**, MiR-363 inhibited the proliferation of LCa cells (** $p < 0.01$). **B**, The protein expression level of Mcl-1 by Western blot. **C**, The expression level of Mcl-1 by real-time PCR analysis. **D**, The expression level of Mcl-1 by Western blot (** $p < 0.001$ vs. the NC group; ## $p < 0.01$, ### $p < 0.001$ vs. the mimics group).

TU-177 cells to study the correlation between miR-363 and Mcl-1. Results of RT-PCR and Western blot showed that the expression of Mcl-1 was suppressed by the regulation of miR-363 in TU-177 cells (Figure 3B, 3D). These data confirmed that Mcl-1 could be negatively regulated by miR-363.

MiR-363 Suppressed the Proliferation of LCa Cells

To examine the function of miR-363 on the proliferation of LCa cells, MTT assay was performed to detect the cell proliferation rate. MTT results indicated that the cell proliferation rates of TU-177 cells were decreased by the up-regulation of miR-363 using mimics transfection. After the Mcl-1 siRNA was increased, the proliferation of TU-177 cells was also increased (Figure 3A).

MiR-363 Inhibited the Invasion of LCa Cells

Invasion was found to be a key factor in cancer cell proliferation. In the transwell investigations,

we found that the invasion ability of TU-177 cells was elevated in the miR-NC group. The invasion ability of TU-177 cells was limited by the intervention of miR-363. Meanwhile, the addition of siMcl-1 could subside the effect of miR-363 (Figure 4A, 4C).

MiR-363 Enhanced the Apoptosis of LCa Cells

TUNEL assay revealed that the apoptosis level was decreased by the intervention of miR-363. However, after increasing Mcl-1 siRNA, the apoptosis index of TU-177 cells was also appeared, suggesting that miR-363 could facilitate apoptosis through targeting Mcl-1 (Figure 4B, 4D).

Discussion

LCa is one of the most common malignant tumors of head and neck, ranking secondly only to nasopharyngeal carcinoma. The incidence rate of LCa shows an increasing trend year by year. Cur-

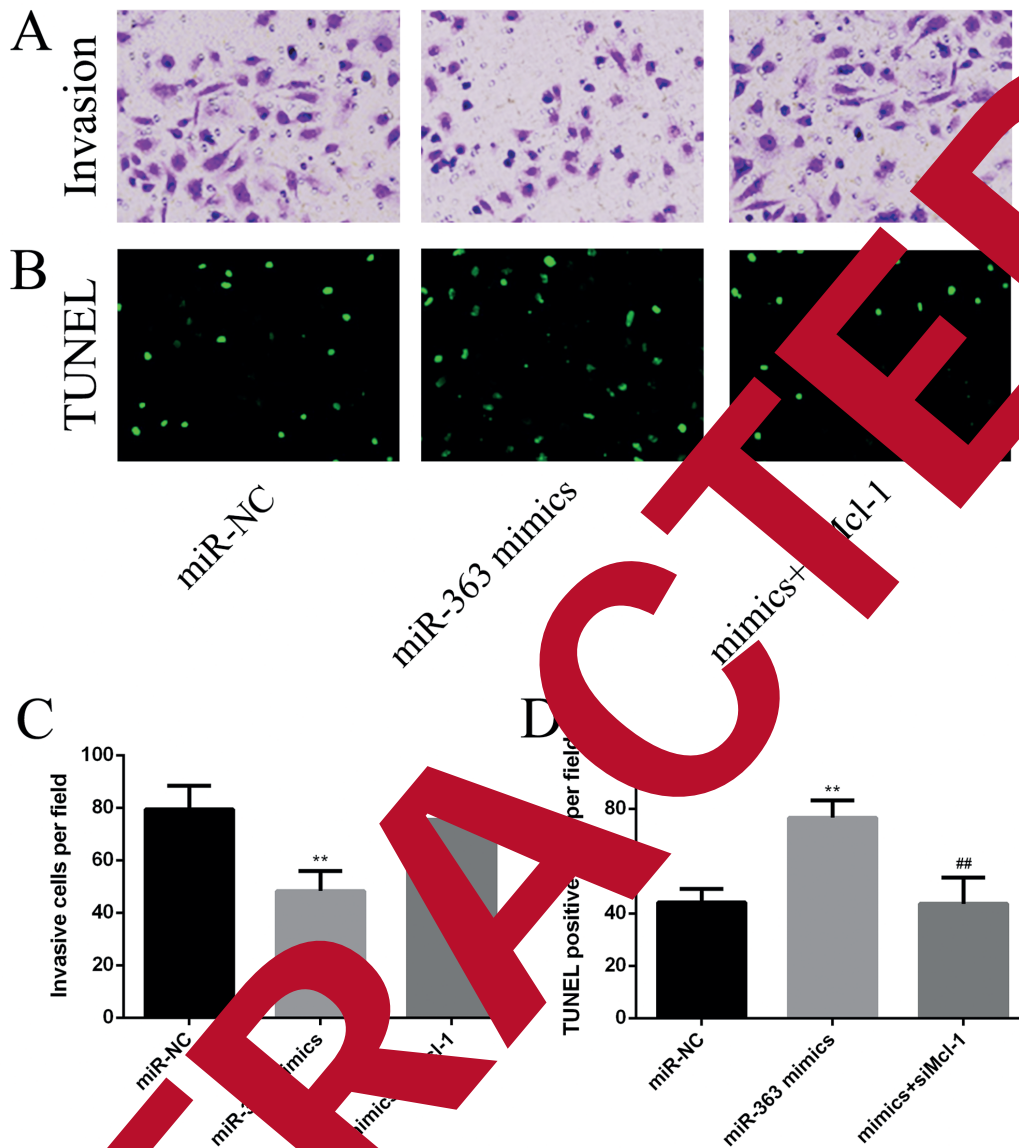


Figure 4. MiR-363 inhibits the invasion and apoptosis of LCa cells. Mcl-1 overexpression attenuated the suppressive effect of miR-363 on TUC177 cells. **A**, The invasion ability by transwell assay. **B**, The apoptosis test by TUNEL assay. **C**, and **D**, were statistical analysis of **A**, and **B**, respectively. Data were presented as means \pm standard deviations. (Magnification, $\times 200$, ** $p < 0.01$ vs. the NC group, # $p < 0.05$, ## $p < 0.01$ vs. the mimics group).

rently, the main treatments for LCa are surgery and radiotherapy, but the long-term efficacy is unsatisfactory, especially for patients in the advanced stage^{20,21}. This is mainly because the molecular mechanism of the pathogenesis of LCa is still unclear. The early diagnosis and treatment are unsatisfactory. Therefore, deeply studying the pathogenesis of LCa, searching new targets for early diagnosis and treatment, and finding new therapies at the genetic level have become problems urgently to be solved.

Mcl-1 gene is a member of the B-cell lymphoma (Bcl)-2 gene family²², which is 35% homologous to Bcl-2. It is also a member of the anti-apoptotic genes. Unlike other Bcl-2 family genes, Mcl-1 has a very short half-life (30-40 min) and high transcriptional, translational, and post-translational processing levels. The discovery of this gene is a new breakthrough in the study of tumor occurrence and development. In recent years, Mcl-1 has become a research hotspot in China and foreign countries due to its own advantages.

Several studies have shown that Mcl-1 is overexpressed in almost all malignant tumors, such as colon cancer²³, liver cancer²⁴, ovarian cancer²⁵, leukemia²⁶, pancreatic cancer²⁷, and gastric cancer²⁸, indicating that Mcl-1 is closely related to the occurrence and development of malignant tumors. Some studies^{29,30} have shown that reducing the expression of Mcl-1 can promote tumor cell apoptosis and inhibit tumor cell proliferation, indicating that Mcl-1 is a potential therapeutic target for malignant tumors.

In recent years, researches on miRNAs have suggested that miRNAs play an extremely important regulatory role in the occurrence and development of tumor progression. Numerous studies have also confirmed that miRNAs are involved in almost all the important physiological processes, such as the formation and development of LCa.

Here in our work, we analyzed the expression level of miR-363 in LCa tissues and cells. Data showed that the expression level of miR-363 was significantly up-regulated. Subsequently, we searched three publicly available algorithms and found that Mcl-1 was a regulatory target of miR-363. As expected, the expression of miR-363 was negatively correlated with the expression of Mcl-1 in the luciferase assay, Real-time PCR and Western blot analysis. These results all revealed the regulatory role of miR-363 on Mcl-1.

Cancerization is essentially that normal cells get free from the inherent regulatory mechanism, eventually forming an uncontrolled and proliferative group. The process of forming abnormal proliferation ability is a key step in cancerization. In our study, luciferase assay indicated that after up-regulating the expression of miR-363 in LCa cells, the proliferation ability of LCa cells was significantly suppressed, proving the function of miR-363 on inhibiting the proliferation of LCa cells.

Due to the recurrence and metastasis of patients in the advanced stage, the 5-year survival rate of patients remains high. The fundamental reason of malignant tumors different from benign tumors are invasive growth and metastasis. Potential tumor cells need some motion capacity when falling off from the tumor and invading blood flow and surrounding tissues. Highly-aggressive tumors usually have a stronger invasion capacity. We detected the invasion ability of LCa cells by the transwell experiment and found that miR-363 could significantly suppress the invasion ability of LCa cells.

Cell apoptosis is a process that nucleated cells initiate the intracellular death mechanism under the action of apoptosis-stimulating signals, eventually leading to programmed cell death. Cell apoptosis and death through a series of signal transduction pathways. Cell apoptosis plays an important role in maintaining the normal morphology and function of tissues and organs. Avoiding apoptosis is one of the necessary characteristics of cell malignant transformation, and tumor cells may obtain this characteristic via activating anti-apoptotic signals or inhibiting pro-apoptotic signals. In our study, after the intervention with miR-363, the apoptosis index of LCa cells was significantly increased. These findings suggested that miR-363 had an effect on promoting the apoptosis of LCa cells.

Since the discovery of miRNA, it has become a research hotspot in life sciences. It has wide regulatory functions in life activities, and possesses profound and complex effects on gene expression, growth development, and behaviors. Our study suggested that there was a specific correlation between the occurrence and development of LCa and the up-regulation of miR-363. Therefore, miR-363 is very likely to become a new biomarker for the diagnosis of LCa, or to serve as a target of intervention in new drug research and development, eventually providing a new mean for the treatment of LCa.

Conclusions

In the present study, we showed that Mcl-1 was a direct and functional target of miR-363. Meanwhile, the decreased expression of Mcl-1 resulting from the up-regulation of miR-363 could decelerate cell proliferation and invasion, and accelerate the cell apoptosis in laryngeal cancer cells. Our study revealed that the restoration of miR-363 could be a potential therapeutic strategy for the treatment of LCa.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

of Bcl-2, Bax, Bcl-X, and Mcl-1 expression in pancreatic cancers. *Oncology* 1999; 56: 73-82.

- 28) LIKUI W, QUN L, WANGQING Z, HAIFENG S, FANGQIU L, XIAOJUN L. Prognostic role of myeloid cell leukemia-1 protein (Mcl-1) expression in human gastric cancer. *J Surg Oncol* 2009; 100: 396-400.
- 29) NIJHAWAN D, FANG M, TRAER E, ZHONG Q, GAO W, DU F, WANG X. Elimination of Mcl-1 is required for the

initiation of apoptosis following ultraviolet irradiation. *Genes Dev* 2003; 17: 1475-1486.

- 30) SUBRAMANIAM D, NATARAJAN G, RAMALINGAM CHANDRAN I, MAY R, QUEIMADO L, HOUGH S. Translation inhibition during cell cycle arrest and apoptosis: Mcl-1 is a novel target for RNA binding protein CUGBP2. *Am J Physiol Gastrointest Liver Physiol* 2008; 294: G1025-

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