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MiR-140-5p inhibits larynx carcinoma invasion and angiogenesis by targeting VEGF-A

J.-R. ZHANG[1], R.-H. ZHU[2], X.-P. HAN[2]

[1]Department of Physical Examination Center, Jinan Central Hospital Affiliated to Shandong University, Jinan, Shandong, China
[2]Department of Otolaryngology, Jinan Central Hospital Affiliated to Shandong University, Jinan, Shandong, China

Abstract. – OBJECTIVE: Larynx carcinoma is a common head-neck malignant tumor. Recent investigations showed the involvement of microRNA (miR) in regulation of multiple tumors. miR-140-5p showed decreased expression in various cancers, but without knowledge regarding its expression in larynx carcinoma and effects on cell invasion and angiogenesis.

MATERIALS AND METHODS: Real-time quantitative PCR was firstly employed to measure miR-140-5p expression in larynx carcinoma and controlled tumor adjacent tissues. In larynx cancer cell line, agomir or antagomir of miR-140-5p was applied to up-regulate or down-regulate miR-140-5p, respectively. Western blot was used to evaluate vascular endothelial growth factor A (VEGF-A) expression, and cell proliferation was modified by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H- tetrazolium bromide (MTT) approach. Transwell approach was used to measure cell invasion, and angiogenesis assay was used to detect the effect on angiogenesis. Luciferase report assay (LRA) measured targeting binding between miR-140-5p and VEGF-A.

RESULTS: Comparing to tumor adjacent tissues, larynx carcinoma cells showed significantly decreased miR-140-5p expression. Agomir up-regulated miR-140-5p expression and weakened proliferation and invasion potency, and inhibited angiogenesis. Antagomir down-regulated miR-140-5p and presented the opposite results. Finally, LRA confirmed direct binding between miR-140-5p and VEGF-A.

CONCLUSIONS: MiR-140-5p can target VEGF-A in larynx carcinoma cell line to inhibit cell invasion and angiogenesis. MiR-140-5p thus may work as the direct molecular target of larynx carcinoma.

Key Words:
Larynx carcinoma, miRNA, Proliferation, Invasion, Angiogenesis.

Introduction

Laryngeal cancer is one commonly occurred malignant tumor in head and neck segment. Patient's swallowing, respiration and vocalization functions may be compromised, and patients were thus suffered for life quality[1,2]. Therefore, the investigation of molecular mechanism governing laryngeal cancer is of critical importance for early diagnosis and treatment of laryngeal cancer. In recent years, with the development of gene sequencing, transcriptome and bioinformatics, increasing number of non-coding RNA molecules have been found, and many microRNA (miR) have been demonstrated to be involved in cancer pathogenesis and progression. These non-coding RNA molecules provide strong evidence for screening of laryngeal cancer related diagnosis and treatment markers as well as targets[3]. miR is a group of small molecule non-coding single stranded RNA with 18-24 bp length, and can completely or incompletely bind with 3' un-translated region (3'UTR) of target mRNA. Under the direction of RNA exonuclease, mRNA can be selectively degraded, thus inhibiting or activating downstream genes[4]. MiR mainly exerts regulation on downstream genes, and in the meantime, regulation on mRNA degradation or activation can also affect cellular function or oncogene activation via suppressing certain protein expression[5]. Angiogenesis provides necessary condition for providing oxygen and nutrients of tumor tissues. Various studies have found angiogenesis related factors. Vascular endothelial growth factor (VEGF)-A is one member of VEGF family, and was initially cloned and separated by Smith et al[6] from anti-sense DNA of human prostate cancer cell line PC-3 by receptor affinity chromatography, along with tyrosine kinase activity of VEGF receptor. VEGF-A has been widely studied and plays important roles in tumor growth, migration and invasion[7]. Under normal physiological conditions, VEGF-A and its receptor have relatively low expression level, but is largely up-regulated in those tissues with sufficient blood supply and active metabolism. Previous investigations[8-10] showed the close relationship between

Corresponding Author: Xiaopan Han, MD; e-mail: j627843774@sina.com

VEGF-A expression and tumor malignancy. In serum samples of multiple solid tumor and metastatic tumors, people found significantly increased VEGF-A expression, which is thus believed as a biomarker for tumor metastasis. With reference to literatures and bioinformatics database, we found potentially interaction between miR-140-5p and VEGF-A[11,12]. Therefore, this work aimed to investigate the expression of miR-140-5p and VEGF-A in laryngeal carcinoma tissues. We thus utilized cell transfection approach to knockdown or to overexpress miR-140-5p, and measured the condition of cell proliferation, migration, invasion and angiogenesis, in order to provide evidence for early diagnosis of laryngeal cancer and biomarkers/targeted for early diagnosis.

Materials and Methods

Major Materials and Reagents

Polyclonal rabbit anti-VEGF-A antibody was purchased from Sanying Biotech. Inc. (Wuhan, China). Reverse transcription kit was purchased from Toyobo Co. Ltd. (Osaka, Japan). Real-time quantitative PCR kit was purchased from Quanshijin Biotech. (Beijing, China). Dulbecco's Modified Eagle Medium (DMEM) medium, trypsin and fetal bovine serum (FBS) were purchased from Gibco BRL. Co. Ltd. (Grand Island, NY, USA). Plasmid extraction kit was purchased from Promega (Madison, WI, USA). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H- tetrazolium bromide (MTT) assay reagent was purchased from Toyobo Co. Ltd. (Osaka, Japan). Matrigel matrix gel was purchased from BD Biosciences (San Jose, CA, USA). Lipofectamine 2000 and TRIzol were purchased from Invitrogen/Life Technologies (Carlsbad, CA, USA). MiR-140-5p inhibitor or mimic and respective negative control sequences were purchased from Gimma Gene (Shanghai, China). PCR primer was designed by Aoke Dingsheng Biotech. Co. Ltd. (Beijing, China). This study was approved by the Ethical Committee of Jinan Central Hospital affiliated to Shandong University (Ji'nan, China).

Major Equipment

Ultrapure workstation was provided by Boxun (Shanghai, China). Gel imaging system was obtained from UVP Multispectral Imaging System (UVP, Sacramento, CA, USA). PS-9 semi-dry transferring electrophoresis was purchased from Jingmai Trade Co. Ltd. (Shenzhen, China). CO_2 chamber and Thermo-354 micro-plate reader were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Inverted fluorescent microscope was purchased from Olympus (Mode: IX81, Tokyo, Japan).

Tissue Collection

Clinical tissues were collected from Shandong Cancer Hospital affiliated to Shandong University. After signing informed consents, both laryngeal cancer and adjacent tissues were collected during the surgery and were kept at -80°C.

Cell Line and Culture

Human laryngeal cancer cell line (TU212) and human aorta endothelial cell line (HUVECs) were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% sterile fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, in a 37°C chamber with 5% CO_2.

MTT Assay for Cell Proliferation

Cells were transfected with miR-150-5p antagomir or agomir. Cells at log-growth phase were collected to adjust cell concentration to 5-10 $\times 10^4$/ml and were seeded into 96-well plate (100 μl/well). Each group was tested in 10 replicated wells. After 24 h incubation and attached growth, cells were continuously cultured for 24 h, and 10 μl MTT solution were added into each well. After 4 h, the supernatant was carefully removed and tripled solution was added (100 μl/well). The plate was vortexed to completely dissolve crystals, followed by 12-15 h incubation. Optical density (OD) values at 450 nm were measured, and proliferation efficiency of each group was calculated using normal group as 100%. Cell viability rate (%) = (OD of experimental group-OD of blank group)/(OD of control group-OD of empty group).

Recombinant Plasmid Construction and Cell Transfection

MiR-140-5p antagomir and agomir were synthesized by Gimma (Shanghai, China). One day before transfection, cells were passed in 24-well plate reaching 30-50% confluence. 1.25 μl siRNA stock solution (20 μM) was diluted in 100 μl option-minimum essential medium (Opti-MEM) medium as solution A, and 1 μl Lipofectamine 2000 or Lipofectamine RNAiMAX was diluted in Optim-MEM as solution B. After 5 min, solution A and B were mixed, incubated still for 20 min, and were added into cell culture plate. After

4 h incubation, cell growth medium was switched and transfection efficiency was observed under a fluorescent microscope. One day before transfection, cells were seeded into 6-well plate. 10 pmol miR-140-5p antagomir or agomir and 5 µl POL-Deliver 3000 were diluted into 100 µl for 5 min incubation after mixture, which was added into 6-well plate for gentle mixture and 48 h incubation. Protein and miR expression was measured along with cell proliferation rate.

Protein Extraction From Laryngeal Cancer Tissues and Cells

Tissues or cells from each group were rinsed in phosphate-buffered saline (PBS), and were dried by filter paper. For 1 ml lysate, 10 µl 100 mM phenylmethanesulfonyl fluoride (PMSF) were added for vortex, and each 100 mg tissue was mixed with 1000 µl lysis buffer. With homogenization, the lysate was processed on ice for 5-10 min, and was centrifuged at 12000 r/m for 5 min. The supernatant was collected to prepare the total protein solution.

Western Blot

Total protein solution was quantified by bicinchoninic acid (BCA) approach, and was diluted to equal concentration using saline. The loading buffer was added and the mixture was boiled for 5 min to denature. Samples were loaded onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for electrophoresis separation of target protein against adjacent proteins. The target protein was then transferred to polyvinylidene difluoride (PVDF) membrane under 300 mA for 1 h. The membrane was incubated at 4°C overnight using polyclonal rabbit anti-VEGF-A antibody, and was rinsed in Tris-buffered saline-Tween-20 solution (TTBS) for three times. Secondary antibody (1:1000) was added for 37°C 2 h incubation. Enhanced chemiluminescence (ECL) developing substrate was employed to develop the protein band.

Total RNA Extraction

For all tissues or cells, each 100 mg sample was mixed with 1 ml TRIzol for 5 min iced incubation and pipetting mixture. Lysate from each well was collected into 1.5 ml tube, which was added with 200 µl chloroform for 15 s vigorous mixture and 3 min room temperature incubation. The mixture was centrifuged at 4°C with 12000 ×g for 15 min. After centrifugation, the upper aqueous phase was carefully removed to new tubes with 500 µl isopropanol, and was incubated at room temperature for 10 min, followed by 12000 ×g centrifugation for 10 min. The upper aqueous phase was discarded and 1 ml ethanol was added for three times of washing. The supernatant was carefully removed and 20 µl diethy pyrocarbonate (DEPC) water was added to dissolve mRNA.

Real-Time Quantitative PCR

MiR-140-5p primer was designed and synthesized by Sigma-Aldrich (St. Louis, MO, USA) under sequences: forward 5'-TTGAA TTCTA ACACC TTCGT GGCTA CAGAG-3'; reverse 5'-TTAGA TCTCA TTTAT CGAGG GAAGG ATTG-3'; U6 was used as the internal reference: forward 5'-CTCGC TTCGG CAGCA CA-3'; reverse 5'-AACGC TTCAC GAATT TGCGT-3'. PCR was performed in a 50 µl system, following manual instruction of test kit. Reaction conditions were: 50°C for 30 min, followed by 95°C 5 min, and 40 cycles each containing 95°C 30 s, 55°C 30 s, 72°C 50 s, ended with 72°C for 5 min. After reaction, Real-time PCR amplification curve and melting curve were confirmed. Relative expression was calculated by comparing Ct values of target gene and internal reference gene, using $2^{-\Delta\Delta Ct}$ approach.

Transwell Assay for Cell Migration

Transwell chamber and other equipment were pre-cold in 4°C fridge one night before assay. ECM gel was thawed in 4°C fridge, and transwell chamber was placed into 24-well plate. 50 µl diluted extracellular matrix (ECM) gel were added into each well of transwell chamber, which was incubated at 37°C for 4 h. Residual liquid was removed for air-dry. Cholangiocarcinoma cells were harvested for 12 h and mixed with trypsin to prepare cell suspension. Cell concentration was adjusted and added into the upper chamber, whilst lower chamber was filled with culture medium containing 10% FBS. The experimental was divided into normal control group, miR-140-5p agomir group, miR-122 antagomir group, with triplicates in each group. After adding liquid, the chamber was removed at specific time point. After 0.1% crystal violet staining, cells were counted in an inverted microscope from five fields in each corner and the center.

Micro-Vessel Formation Assay

Cryopreserved extracellular matrix gel was thawed at 4°C for complete solving. 50 µl matrix gel was added into 96-well plate for gentle mixture. The plate was incubated at 37°C with

5% CO_2 until condensation of gel. Cells at log-growth phase were seeded into culture dish for 24 h incubation after transfection. The supernatant was collected and cell debris was removed by centrifugation. DMEM medium was used to re-suspend HUVECs cell to generate cell suspension. Cell density was adjusted to inoculate into 96-well plate with matrix gel. Cell supernatant was added for further incubation. After 18 h, 96-well plate was collected for observing tube formation under a microscope. Five randomly selected fields were chosen for imaging and statistics.

Dual Luciferase Reporter Gene

Liposome reagent was used to transfect miR-140-5p knockdown, over-expression or controlled NC, or luciferase labeled plasmid (pIS0-VEGF-A-3'UTR-mut or pIS0-VEGF-A-3'UTR) and controlled plasmid were co-transfected into laryngeal cancer cells. Dual luciferase reporter gene assay kit was used to measure luciferase activity following the manual instruction.

Statistical Analysis

SPSS 10.0 software (SPSS Inc., Chicago, IL, USA) was used to process all data, which were presented as mean ± standard deviation (SD). The Student's *t*-test was used to compare the differences between two groups. Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. $p<0.05$ was considered statistically different.

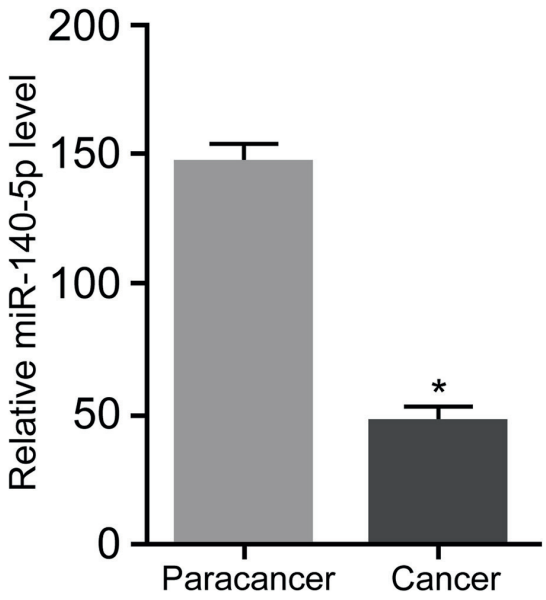

Figure 1. MiR-140-5p expression in laryngeal cancer tissues and cancer adjacent tissues. $^*p<0.05$ comparing to tumor adjacent tissues.

Results

Assay for miR-140-5p Level in Laryngeal Carcinoma Clinical Samples

We measured miR-140-5p expression in laryngeal cancer and adjacent tissues. As shown in Figure 1, comparing cancer adjacent tissues, laryngeal cancer tissues showed significantly suppressed miR-140-5p expression ($p<0.05$).

VEGF-A Expression in Laryngeal Cancer Tissues and Adjacent Tissues

As shown in Figure 2, we used Western blot to measure VEGF-A expression. Comparing to adjacent tissues, laryngeal cancer tissues showed remarkably elevated VEGF-A expression ($p<0.05$).

Transfection of Laryngeal Cancer Cell Lines With miR-140-5p Agomir or Antagomir

To investigate the effect of miR-140-5p on behavior of laryngeal carcinoma cells, we utilized miR-140-5p agomir and antagomir to transfect laryngeal carcinoma cells. As shown in Figure 3, after giving miR-140-5p agomir, the expression level of miR-140-5p was significantly elevated, with significant difference with control group. The transfection of miR-140-5p antagomir, miR-140-5p presented prominent down-regulation, with significant difference with control group.

MTT Assay for Proliferation Potency of Laryngeal Cancer Cells

Figure 4 showed the assay for cell proliferation potency. After potentiating miR-140-5p level using agomir, cell proliferation was remarkably potentiated. In contrast, miR-140-5p antagomir transfection inhibited miR-140-5p expression and inhibited cell proliferation, with significant difference against that of control group.

Transwell Assay for Cell Invasion

Cell invasion potency within matrix gel was shown in Figure 5. After up-regulating miR-140-5p level, cell invasion potency was remarkably inhibited. In contrast, inhibition of miR-140-5p level could potentiate cell invasion potency.

VEGFA expression level

VEGF-A is one important factor affecting tumor angiogenesis. MiR-140-5p agomir or an-

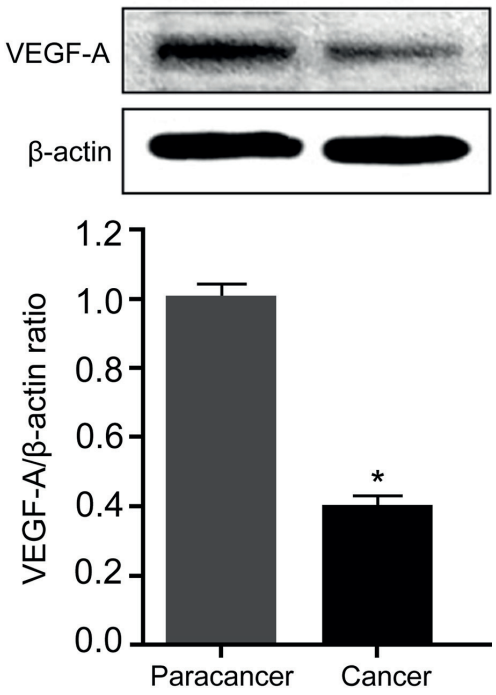

Figure 2. VEGF-A expression in laryngeal cancer and adjacent tissues. $^{*}p<0.05$ comparing to tumor adjacent tissues.

tagomir effect on VEGF-A expression level was shown in Figure 6. In general, miR-140-5p presented a negative correlation with VEGF-A expression level. After up-regulation of miR-140-5p, VEGFA expression was remarkably suppressed; when miR-140-5p level was suppressed, VEGF-A expression was prominently elevated.

Evaluation of Angiogenesis Potency By Micro-Vessel Formation Assay

We also used matrix gel tube formation assay to investigate the effect of miR-140-5p on angiogenesis potency. As shown in Figure 7, inhibition of miR-140-5p expression significantly enhanced angiogenesis: comparing with control group, tube formation number was significantly increased with densely distributed cells in miR-140-5p inhibited cells. Over expression of miR-140-5p weakened tube formation potency of cells, as presented by sparsely distributed cell colonies without typical vascular morphology.

Dual Luciferase Reporter Gene For Targeted Effect of miR-140-5p On VEGF-A

Abovementioned results showed that miR-140-5p could negatively regulate VEGF-A. From previous literatures and bioinformatics database,

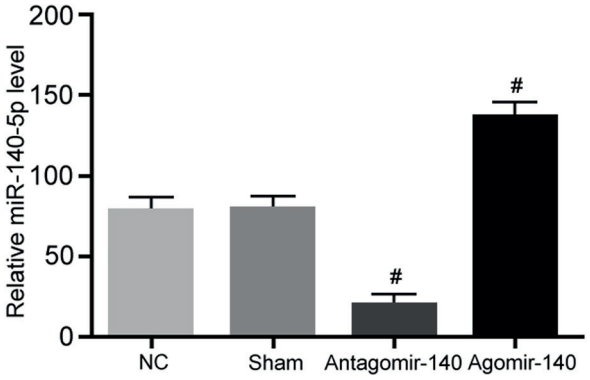

Figure 3. Effect of agomir and antagomir on miR-140-5p expression. $^{\#}p<0.05$ comparing to Sham group.

we found that miR-140-5p might directly regulate VEGF-A. We thus designed these experiments and utilized dual luciferase marker gene approach for substantiation. As shown in Figure 8, we constructed wild type and mutant form of VEGFA luciferase plasmid. After transfecting Ago-miR-140, luciferase activity was measured. With transfection using Ago-miR-140 by wild type VEGFA, luciferase activity was significantly elevated, whilst no significant change was found in mutant form of VEGF-A. Therefore, it is proved that an interaction existed between VEGF-A and miR-140-5p.

Discussion

Laryngeal carcinoma is one commonly occurred head-neck malignant tumor. By recent years study, we have found some non-coding RNA with abnormal expression in laryngeal carcinoma. Among those miRNA plays important roles. In this study, we investigated the effect of

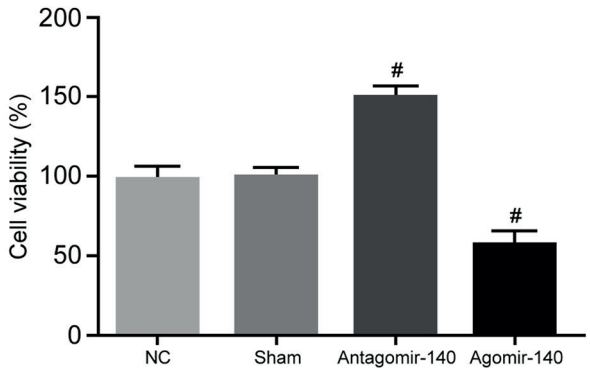

Figure 4. Effects of agomir and antagomir on cell proliferation potency. $^{\#}p<0.05$ comparing to sham.

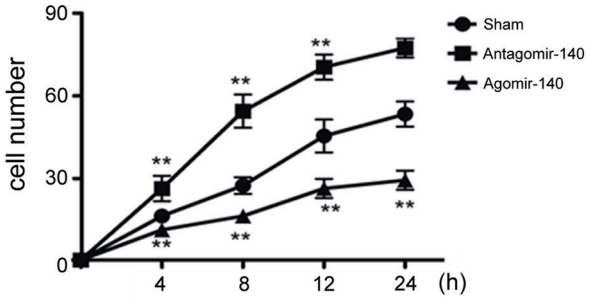

Figure 5. Effects of agomir and antagomir on cell invasion potency. $^{**}p<0.01$ comparing to Sham group.

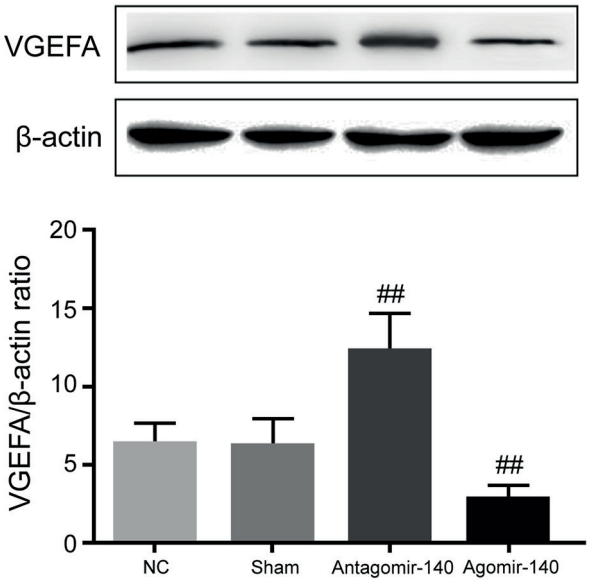

Figure 6. Effects of agomir and antagomir on VEGF-A expression level. $^{\#\#}p<0.01$ comparing to Sham group.

miR-140-5p on laryngeal carcinoma cell proliferation, migration, invasion and angiogenesis. We also for the first-time utilized luciferase reporter gene to substantiate targeted role of miR-140-5p on VEGF-A. miRNA is a group of newly discovered non-coding RNA with 20-24 nucleotide sequence, and can bind with 3' un-translated region of mRNA protein coding sequence, to regulate mRNA expression at post-transcriptional level[13]. The regulation of miR on protein synthesis at post-transcription level is of critical importance. Abnormal regulation of miR is correlated with onset and progress of multiple human cancer, including thyroid carcinoma[14], liver cancer[15], and cholangiocarcinoma[16]. Across malignant tumors at different sites, miR molecules exerted critical roles. Transcript of miR-140-5p is widely expressed in multiple human tissues[17]. A lot of studies have been performed regarding miR-140-5p. Some studies showed that miR-140-5p can facilitate cardiac oxidative stress via targeting Nrf2, thus aggravating Adriamycin-induced cardiac toxicity[18]. In tumor related work, miR-140-5p has been confirmed to be down-regulated or deleted in chronic lymphocyte leukemia, as well as prostate adenoma[19], breast cancer[20], gastric carcinoma[21] and pituitary tumor[22]. Nevertheless, few researches have been performed regarding its function on transcription modulation or biological function. How about the expression pattern of miR-140-5p in laryngeal cancer and what role is it, still are unclear. In this paper we for the first time demonstrated down-regulation of miR-140-5p in laryngeal cancer, and inhibitory role of miR-140-5p on cancer cell proliferation or invasion. Cell migration and proliferation are critical steps for tumor growth and metastasis as well as angiogenesis. VEGF-A is a potent angiogenesis promoting factor, and can be synthesized and secreted by tumor cell, endothelial cell and supporting cell. It is also the most potent endothelial mitogen and can affect cardiovascular endothelial cells both in vivo and in vitro to induced division and proliferation of endothelial cells, for inducing angiogenesis. Previous reports showed large amounts of VEGFA secretion both inside and outside of tumor tissues. Moreover, its expression level was

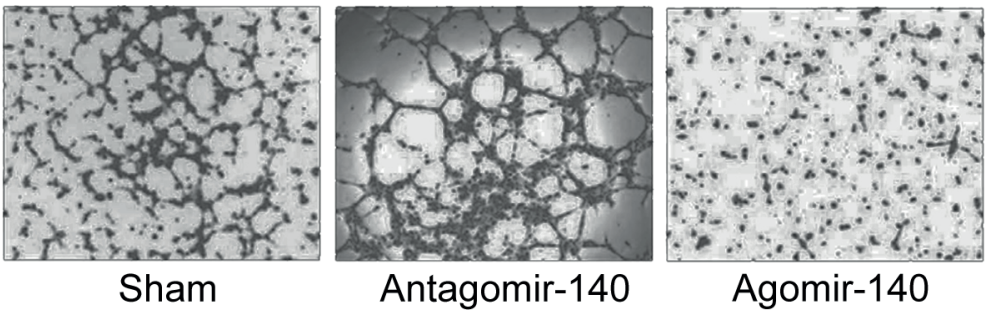

Figure 7. Effects of agomir and antagomir on angiogenesis.

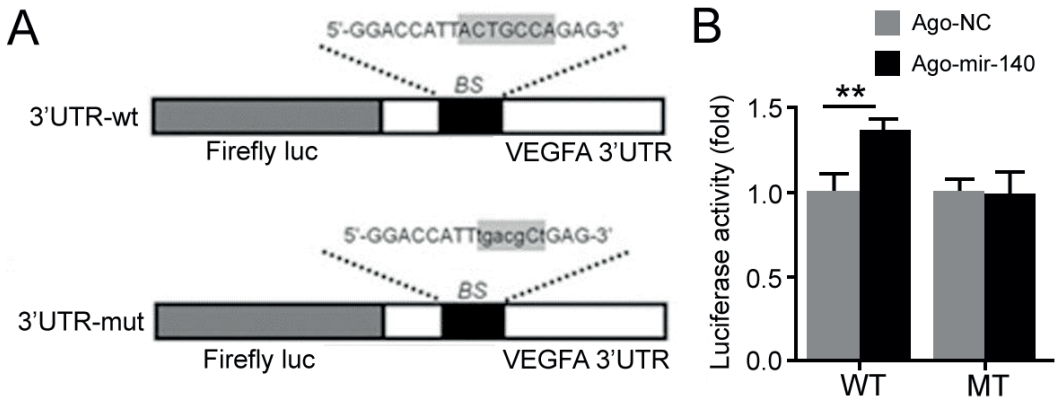

Figure 8. Interaction between miR-140-5p and VEGF-A. $^{**}p<0.01$ comparing to control group.

significantly higher than normal cells[23,24]. Recent studies[25-28] have postulated correlation between VEGF-A and miR-140-5p expression. On pulmonary hypertensive patients, VEGF-A was predicted to be the target gene of miR-140-5p[11]. MiR-140-5p mediated VEGF-A also participated in angiogenesis of post-ischemia stroke and progression of colorectal carcinoma[29, 30]. To date, no systemic study has been performed regarding whether miR-140-5p could regulate VEGF-A, or its effect on cell proliferation, migration, invasion activity or angiogenesis in laryngeal cancer. Therefore, we firstly investigated expressional profile of VEGF-A and miR-140-5p in laryngeal cancer tissues, and utilized transfection approach to knock-down or to over-express miR-140-5p to investigate the change of cell proliferation, migration, invasion and angiogenesis behaviors. Although this study systematically investigated the effect of miR-140-5p on tumor cell behavior, whether similar role was found at animal level, or whether miR-140-5p up-regulation could inhibit tumor growth requires further proof by animal study.

Conclusions

We observed that miR-140-5p can target VEGF-A in laryngeal cell line to suppress cell invasion and angiogenesis. MiR-140-5p might work as the potential molecular target of laryngeal cancer treatment.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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