Original Article

MiR-448 inhibits laryngeal cancer metastasis by repressing AEG-1 expression

Susheng Miao1*, Xionghui Mao1*, Rong Pei1, Kaibin Song1, Yuanjing Lv1, Huanyv Jiang1, Baojun Li1, Xianguang Yang1, Cheng Xiu1, Hongxue Meng2, Ji Sun1

Departments of 1Head and Neck Surgery, 2Pathology, Harbin Medical University Cancer Hospital, Harbin 150081, China. *Equal contributors and co-first authors.

Received April 10, 2017; Accepted December 2, 2017; Epub March 15, 2018; Published March 30, 2018

Abstract: Incidences of laryngeal carcinoma have been reported to increase recently. MicroRNAs (miRNAs) are globally dysregulated in human carcinomas. However, the mechanism and signaling of miRNA regulation in human carcinomas, especially in laryngeal cancer, remains unclear. In this study, we discovered the lower expression levels of miR-448 in human laryngeal carcinoma tissues, and proved miR-448 inhibited metastatic traits in laryngeal cancer cells, including cell migration, invasion, proliferation, apoptosis and even epithelial-mesenchymal transition (EMT), as well as angiogenesis. These effects were achieved via the down regulation of the miR-448 target gene, AEG-1, which was proved to play a key role in cancer metastasis. These findings provide new insights into the physiological effects of and potential therapeutic uses for miRNAs in laryngeal cancer.

Keywords: Laryngeal cancer, metastasis, miR-448, AEG-1

Introduction

Laryngeal carcinoma is one of the most common head and neck cancers in the world [1]. The main treatment strategy for laryngeal carcinoma is still surgery or total laryngectomy. Therefore, there is an urgent need for understanding the underlying mechanisms of laryngeal carcinoma pathogenesis and development of therapeutic approaches against advanced laryngeal carcinoma cases.

As a new class of signaling modulators, microRNAs (miRNAs) negatively regulate gene expression by usually binding to the 3'UTR of target mRNA, in a post-transcriptional manner [2]. A small number of miRNAs have been demonstrated to actively participate in the regulation of tumor development [3-7]. Recent reports have deciphered the roles of miRNAs in tumor metastatic progress [8]. Accumulating data have pointed out the central regulatory role of miRNAs in the initiation and progression of most cancers [7]. Therefore, there are new hopes for improving therapeutic approaches of cancers based on the utilization of miRNAs.

More and more evidence have suggested that miR-448 is a tumor suppressor regulator [9-12]. However, the clear function and target gene of miR-448 are not yet reported in laryngeal carcinoma. In this study, we explored the roles and mechanism of miR-448 in the development and aggression of laryngeal carcinoma by analyzing the biological characteristics and regulation manner of miR-448 in laryngeal carcinoma.

According to our results, miR-448 was down-regulated in human laryngeal carcinoma tissues. Overexpressed miR-448 in laryngeal carcinoma Hep2 cells resulted in the inhibition of cell migration, invasion and proliferation, together with an increase in cell apoptosis and induction of G1 phase arrest. Further functional detection showed that overexpression of miR-448 inhibited EMT and angiogenesis. Finally, we identified AEG-1 as the direct target of miR-448 in laryngeal carcinoma, and proved that miR-448 functions in laryngeal carcinoma by repressing AEG-1 expression.

Materials and methods

Cell culture and transfection

Hep2 cell was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA)
and cultured in Dulbecco’s Modified Eagle’s Medium containing 10% fetal bovine serum, 100 U ml⁻¹ penicillin and 0.1 mg ml⁻¹ streptomycin under humidified conditions of 95% air and 5% CO₂ at 37°C. The transfection protocol was followed with the Lipofectamine™ RNAiMAX (Invitrogen) transfection reagent instructions.

**RNA extraction and quantitative real-time PCR (qRT-PCR)**

For clinical samples and cultured cell lines, total RNA was purified using the Trizol kit (Tiangen Biotech, Beijing) according to the manufacturer’s protocols. Primers for reverse transcription and PCR were from the hsa-miR-448 detection kit (Ribo Biotech, Guangzhou). The expression levels of miR-448 were quantified by qRT-PCR with the SYBR Premix Ex Taq Kit (Takara) using a DNA Engine Opticon2 system (Bio-Rad). The following PCR protocol was used: denaturation at 95°C for 3 min, amplification for 40 cycles at 95°C for 12 s and 62°C for 40 s. The melting curve was plotted from 62°C to 95°C, read every 0.2°C with a 2 s' hold). U6 small nuclear RNA was used as an internal control. For the analysis of AEG-1 expression, GAPDH was used as the internal control, and the primers were listed in Table S1. The results were represented as fold changes, which were calculated by the 2⁻ΔΔCt method.

**Transwell migration and invasion assay**

For migration assays, Hep2 cells were seeded into the upper chamber of a transwell insert (pore size, 8 µm, Costar) in 100 µl of serum-free medium per well. Medium at a volume of 600 µl, containing 10% serum, was placed in the lower chamber to act as a chemoattractant. Nonmigratory cells were removed from the upper chamber by scraping with a cotton bud. The cells remaining on the lower surface of the insert were fixed with 4% formaldehyde (Sigma) and stained by DAPI (Roche). For invasion assays, the cells were seeded into a Matrigel (Bio-Rad)-coated chamber and were cultured at 37°C for 48 h. The thickness of Matrigel was about 1 mm. The next steps were as same as the migration assay.

**Cell proliferation assay**

Hep2 cells were seeded into a 96-well plate at a density of 3×10³ cells per well. After 1 day, the cells were transfected with the indicated miRNAs or siRNAs. Two days later, the cells were incubated with 10% CCK8 reagent (DoJinDo Laboratories, Japan) for 1 h at 37°C. Then the absorbance was detected by the automatic spectrometer (Multimode Reader; Enspire) at 450 nm.

**Cell apoptosis assay**

Apoptosis was determined by propidium iodide (PI) and Annexin V-FITC staining according to the manufacturer’s instructions (Roche). Briefly, 1×10⁶ Hep2 cells were washed in phosphate-buffered saline (PBS) and resuspended in 100 µl of incubation buffer which was added with 2 µl AnnexinV-FITC labeling reagent and 2 µl PI solution. After incubation for 10 min, the cells were analyzed using a flow cytometer.

**Cell cycle assay**

1×10⁶ Hep2 cells were washed in PBS and resuspended in 500 µl of 70% alcohol for 2 h at 4°C. After washing in PBS, the cells were resuspended in 200 µl PI staining solution and incubated for 20 min in dark. Then the cells were analyzed using a flow cytometer.

**HUVEC tube formation assay**

Human umbilical vein endothelial cells (HUVEC) were seeded into a 96-well Matrigel-coated plate at a density of 3×10⁴ cells per well and cultured for 8 h at 37°C. The cells were then analyzed by a microscope, and the branch and length were recorded.

**Immunofluorescence**

Hep2 cells were seeded onto sterile cover slides and allowed to attach overnight. The cells were then fixed with 4% formaldehyde, permeabilized with 0.1% Triton X-100 and blocked in 2% bovine serum albumin for 1 h at room temperature. The expression of E-cadherin and Vimentin was examined using targeted antibodies (anti-E-cadherin: ab76055; Abcam) (anti-Vimentin: ab8978; Abcam) and visualized using anti-rabbit IgG (H+L), F(ab)2 fragment (Alexa Fluor 488 Conjugate, Cell Signaling Technology). The antibodies were diluted for 1:200. Cell nuclei were stained with DAPI (Roche). Fluorescence was examined using a microscope (EclIPSE Ti-U; Nikon).
Luciferase assay

The 3'-untranslated region (UTR) or the mutant 3'-UTR of AEG-1 was cloned into the luciferase reporter vector to construct the plasmids pGL3-AEG-1 and pGL3-mut-AEG-1. For the luciferase assay, $4 \times 10^4$ Hep2 cells were co-transfected with 200 ng of miR-448 mimics and 200 ng of the indicated pGL3 firefly luciferase construct, and 20 ng of a pGL3 renilla luciferase construct as the normalization control. The medium was changed 6 h post-transfection and luciferase activity was measured 48 h after transfection using the dual luciferase reporter assay system (Promega).

Western blot

Proteins in cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation). After about 1 h of blocking with 5% skimmed milk, the membranes were incubated with the primary antibody against AEG-1 (ab45338; Abcam) at the dilution of 1:1000. Then the membranes were washed and incubated with the HRP-conjugated secondary antibody for 1-2 h. Enhanced chemiluminescence (ECL) reagent was used for the band visualization. β-actin (Santa Cruz, CA, USA) was used as the loading control.

Human laryngeal samples and immunohistochemistry (IHC)

All human laryngeal samples were obtained from the Tumor Hospital Affiliated Harbin Medical University. Before surgery at the center, all patients provided written informed consent to allow any excess tissue to be used for research studies. IHC was accomplished at the Tumor Hospital Affiliated Harbin Medical University. For IHC, the formalin-fixed, paraffin-embedded sections (4 μm thick) of tissues, were treated with 1% H$_2$O$_2$, subjected to antigen retrieval in trypsin for 30 min at 37°C, and then followed by immersion in EDTA buffer (pH 9.0, Maixin, China) for 20 min at 120°C in an autoclave. Sections were then blocked with the Protein Blocking Agent (Streptavidin-Biotin Universal Detection System, Beckman Coulter, Marseille, France) and incubated with the primary antibody rabbit anti-human AEG-1 (1:100, Proteintech, USA) at 4°C overnight. This was followed by the incubation with secondary antibody from the Streptavidin-Biotin Universal Detection System. Sections were visualized using DAB staining. The specific isotype control antibody and PBS (omitting primary antibodies) were used as the negative control. The number of cells stained positively by IHC were scored as 0 (absent), 1+ (< 25% of cells), 2+ (25-50% of cells), 3+ (50-75% of cells), or 4+ (> 75% of cells). The scoring was done in a blinded fashion.

Statistical analysis

All of the results are expressed as the means ± SD derived from at least three independent experiments. When comparing two groups, Student’s unpaired t-test (two-tailed) was used. For all of the tests, a P value < 0.05 was considered significant. *indicates P < 0.05; **indicates P < 0.01.

Results

Decreased expression of miR-448 in human laryngeal cancer tissues

To explore whether miR-448 might have function in human laryngeal cancer tissues, qRT-PCR was first applied to determine the expression levels in carcinoma tissues and adjacent
MiR-448 inhibits laryngeal cancer metastasis

paracarcinoma tissues (normalized to U6 small RNA expression). The results showed that the expression levels of miR-448 in laryngeal carcinoma tissues were significantly decreased than in the paired normal tissues (29 pairs, P < 0.0001) (Figure 1A). Pair-wise comparison indicated that more than 80% (24/29) of tumors showed more than 2-fold of reduction in miR-448 expression compared to their matching controls (Figure 1B).

Figure 2. MiR-448 modulates metastatic traits in Hep2 cells. (A) Over-expressed miR-448 results in reduced migratory and invasive capability. N=5. Scale bar, 50 μm. (B) Over-expressed miR-448 results in reduced cell viability. N=3. (C) Over-expressed miR-448 results in cell cycle arrest. N=3. (D) Quantification and statistical comparison of the proportion in different phases between NC and miR-448 groups in (C). Data were represented as mean ± SD from at least three independent experiments (*P < 0.05).

MiR-448 inhibits metastatic traits in laryngeal cancer cells

To explore the possible role of miR-448 in laryngeal carcinoma cells, we took use of Hep2 cell lines to conduct functional experiments. When miR-448 mimics and NC (Negative control) were transfected into Hep2 cells individually, by transwell migration and invasion assay, the migratory capabilities of the cells with high
MiR-448 inhibits laryngeal cancer metastasis

expression of miR-448 had a 40% reduction compared to those of cells transfected with NC, and the invasion capabilities had a more significant decrease, reduced to 20% (Figure 2A). We additionally detected the cell proliferation activity by CCK8 assay and found that overexpression of miR-448 inhibited cell proliferation to a certain extent (Figure 2B). Quantitative analysis of cell cycle by FACS revealed that miR-448 increased the proportion of cells in G1 phase with a concomitant decrease in G2/M phase compared to the control (Figure 2C, 2D). Furthermore, miR-448 overexpression in Hep2 cells promoted both apoptosis and necrosis compared to the control (Figure S1). These findings showed that

miR-448 played a negative role in regulating metastatic traits in laryngeal cancer cells.

**MiR-448 inhibits EMT in laryngeal cancer cells**

EMT was viewed as an essential early step in cancer metastasis, so we investigated whether miR-448 might affect EMT. Immunofluorescence results displayed that E-cadherin was increased while Vimentin was decreased when miR-448 was overexpressed in Hep2 cells (Figure 3). This finding revealed that miR-448 functions as an EMT repressor to suppress metastasis.

**MiR-448 inhibits tumor angiogenesis in HUVEC cells**

To discover the molecular mechanism of miR-448, we took use of miRNA.org algorithm to predict the potential target genes [13]. Here, the binding of miR-488 to the 3'UTR of AEG-1 was predicted (Figure 5A). In order to verify whether AEG-1 was the target gene of miR-448, the 3'-UTR of AEG-1 was cloned into a luciferase reporter construct. The luciferase activity decreased by 65% when miR-448 was overexpressed compared with NC group. When the binding site was mutated, the luciferase activity was rescued (Figure 5B, 5C). Furthermore, we detected the endogenous mRNA and pro-
MiR-448 inhibits laryngeal cancer metastasis

![Image](image_url)

**Figure 4.** MiR-448 inhibits tubule elongation and branching formation in HUVEC cells. N=3. Scale bar, 200 μm. Data were expressed as the mean ± SD of three independent experiments (*P < 0.05).

**Figure 5.** MiR-448 targets AEG-1 gene in Hep2 cells. A. Prediction of the binding between AEG-1 and miR-448 via miRNA.org algorithm. B. Mutant binding site of AEG-1. C. Luciferase activity in Hep2 cells co-transfected with miR-448 or control with the indicated 3’-UTR-driven reporter plasmid or mutant 3’-UTR-driven reporter plasmid. N=3. D. Real-time PCR results for endogenous AEG-1 expression after transfection with miR-448 or control. N=3. E. Western Blot results for endogenous AEG-1 expression after transfection with miR-448 or control. GAPDH was used as the loading control. Data were represented as mean ± SD from the experiments performed in triplicate (*P < 0.05, **P < 0.01).

Silencing of AEG-1 inhibits metastatic traits in laryngeal cancer cells

To investigate the functions of AEG-1 in laryngeal cancer metastasis, we firstly synthesized three siRNAs against AEG-1 (siRNA sequences listed in Table S2) and detected the silencing effect both at mRNA and protein levels with qRT-PCR and Western blot assays, respectively (Figure S2). Three siRNAs were mixed as a pool and transfected into Hep2 cells. By transwell migration and invasion assays, the migratory and invasive capabilities of the cells were reduced at least by 50% when AEG-1 was silenced (Figure 6A). Furthermore, we detected the cell proliferation activity by CCK8 assay and found that silencing of AEG-1 inhibited cell proliferation to a certain extent (Figure 6B). FACs
MiR-448 inhibits laryngeal cancer metastasis

analysis result showed that silencing of AEG-1 arrested cell cycle at G0/G1 phase (Figure 6C), and induced cell apoptosis and necrosis compared to the control group (Figure S3). Hence, repression of AEG-1 mediates metastasis relevant traits, including cell migration, invasion, proliferation and apoptosis. These findings imply that miR-448 functions in metastasis through silencing the target gene AEG-1.

Figure 6. AEG-1 modulates metastatic traits in Hep2 cells. (A) Silenced AEG-1 results in reduced migratory and invasive capability. N=5. Scale bar, 50 μm. (B) Silenced AEG-1 results in reduced cell viability. N=3. (C) Silenced AEG-1 results in cell cycle arrest. N=3. (D) Quantification and statistical comparison of the proportion in different phases between NC and siAEG-1 groups in (C). Data were expressed as the mean ± SD of at least three independent experiments (*P < 0.05).

Silencing of AEG-1 inhibits EMT in laryngeal cancer cells

To investigate whether AEG-1 was involved in the process of EMT, we determined EMT of the Hep2 cells after AEG-1 silencing by transfection with siRNAs. The results showed that AEG-1 silencing significantly decreased the expression of Vimentin and increased the expression
MiR-448 inhibits laryngeal cancer metastasis

Figure 7. Silenced AEG-1 inhibits EMT in Hep2 cells. A. Silenced AEG-1 results in increased expression of E-cadherin and decreased Vimentin (green). E-cadherin and Vimentin were used as EMT markers. DAPI staining was used to detect nuclei (blue). Scale bar, 50 μm. B. Statistical histogram. N=3. Data were represented as mean ± SD from the experiments performed in triplicate (*P < 0.05, **P < 0.01).

AEG-1 promotes laryngeal cancer metastasis in clinical stage

Patient laryngeal cancer tissues obtained from operations were used to identify the expression pattern of AEG-1. Those patients’ laryngeal tumors have transferred to other places, that is, metastasis has happened. Immunohistochemistry detection was conducted on the sections of carcinoma or paracarcinoma tissues. In total, we tested 20 pairs of samples from 20 different laryngeal cancer patients. In all 20 cases, AEG-1 was found to be expressed at high levels in tumor tissues, compared with the paracarcinoma tissues (Figure 9). Analysis results revealed that AEG-1-positive cells were much more in tumor tissues than in paracarcinoma tissues (P < 0.01). These results prove the more progressive laryngeal cancer tissues express relative higher levels of AEG-1, which accords with our functional detection findings.

Discussion

of E-cadherin compared to the control group (Figure 7). The results demonstrated that silencing of AEG-1 inhibited EMT process in Hep2 cells.

Silencing of AEG-1 inhibits tumor angiogenesis in HUVEC cells

SiRNAs against AEG-1 or NC were transfected into HUVEC cells cultured in EBM medium. Twenty-four hours after transfection, HUVEC cells were seeded onto Matrigel-coated plates in the absence of angiogenic stimuli. The result demonstrated that silencing of AEG-1 significantly inhibited tubule elongation and branching formation in contrast to NC group (Figure 8).

Accumulated evidences have proved that miRNAs participate in cancerogenesis and cancer metastasis. The essential task is to identify physiologically relevant and therapeutically promising miRNAs. Actually, miR-448 as a potential cancer suppressor has been reported in recent decades. Li and coworkers has discovered that miR-448 suppresses proliferation by regulating IGF1R in colorectal cancer [9]. MiR-448 has also been found to suppress gastric cancer proliferation and invasion by regulating ADAM10 [11], and negatively regulate ovarian cancer cell growth and metas-
MiR-448 inhibits laryngeal cancer metastasis

that miR-448 negatively regulated the expression of downstream target gene AEG-1, mainly through repression of translation. Human AEG-1 is a protein with 582 amino acids and a molecular mass of 64 kDa. It occurs downstream of Ha-Ras and c-Myc, and its overexpression leads to the activation of downstream PI3k/Akt and NF-κB, and the Wnt pathway [14]. AEG-1 was identified to involve in metastasis process, and the expression levels of AEG-1 are high in most tumors, including colorectal cancer, ovarian cancer and breast cancer [15-17]. Li and coworkers has found AEG-1 promotes EMT and metastasis through the downstream AKR1C2 and NF1 in liver cancer [18]. Here, silencing of AEG-1 inhibited cell migration, invasion and proliferation, induced cell apoptosis and G1 phase arrest in Hep2 cells, and also inhibited EMT and angiogenesis in vitro. Finally, immunohistochemistry detection results proved that AEG-1 was highly expressed in the clinical samples, suggesting that AEG-1 could be a promoting factor in laryngeal cancer metastasis.

In sum, the results of this study have important significance for the understanding of the roles of miR-448 in human laryngeal carcinoma progression, especially metastasis. We believe that these findings provide new insights into the physiological and therapeutic importance of miRNAs in human laryngeal carcinoma.

Acknowledgements

This research is funded by the China Postdoctoral Science Foundation (2015M581470; 2015M581472), National Nature Science Foundation of China (81600539), Special Financial Grant from the China Postdoctoral Science Foundation (2016T90310), Heilongjiang Postdoctoral Foundation (LBH-Z15173; LBH-TZ06-
MiR-448 inhibits laryngeal cancer metastasis

16), Natural Science Foundation of Heilongjiang (H2016048; LC2016038), Technology Foundation for Selected Overseas Chinese Scholars, Heilongjiang Human Resources and Social Security Bureau (Hongxue Meng), Harbin Medical University Cancer Hospital Foundation (JJ2007-06; JJ2008-08), Harbin Special Fund Project for Science and Technology Innovation (2016RAQJ203, Hongxue Meng) and Haiyan Foundation of Harbin Medical University Cancer Hospital (TJMS2016-05), Youth Elite Training Foundation of Harbin Medical University Cancer Hospital (JY2016-06, Hongxue Meng). We truly thank Cheng Xiang, Lei Wang and Zhennan Yuan from Department of Head and Neck Surgery, the Tumor Hospital Affiliated Harbin Medical University for manuscript revision and suggestions.

Disclosure of conflict of interest

None.

Address correspondence to: Hongxue Meng, Department of Pathology, Harbin Medical University Cancer Hospital, 150 Harping Road, Harbin 150081, China. Tel: +86 13796602126; E-mail: menghongxue15@163.com; Ji Sun, Department of Head and Neck Surgery, Harbin Medical University Cancer Hospital, 150 Harping Road, Harbin 150081, China. Tel: +86 451 85718159; Fax: +86 451 85718156; E-mail: drsunji@126.com

References


MiR-448 inhibits laryngeal cancer metastasis

Table S1. Primers for real-time PCR assays

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEG-1</td>
<td>AEG1-F</td>
<td>CCTGGCCTTGCTGAAGAATC</td>
</tr>
<tr>
<td></td>
<td>AEG1-R</td>
<td>GGCTGCTTTGCTGTTACACT</td>
</tr>
<tr>
<td>GADPH</td>
<td>GADPH-F</td>
<td>CATGAGAAGTGACAGACGCT</td>
</tr>
<tr>
<td></td>
<td>GADPH-R</td>
<td>AGTCCCTCCACGATAACAAAGT</td>
</tr>
</tbody>
</table>

Figure S1. Annexin V-FITC/PI staining assay shows that overexpression of miR-448 in Hep2 cells promotes apoptosis and necrosis.

Table S2. siRNA sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Anti-sense</th>
</tr>
</thead>
<tbody>
<tr>
<td>AEG-1 (A)</td>
<td>CCGAAGUACUCGUCAAAAAtt</td>
<td>UUUUGACGAGUACUUCGGct</td>
</tr>
<tr>
<td>AEG-1 (B)</td>
<td>GAAUCUCCCAAACAAUAAtt</td>
<td>UUAUUUGUUGGGAGAUUCCc</td>
</tr>
<tr>
<td>AEG-1 (C)</td>
<td>GGAUGUAGCCGUAUCAAtt</td>
<td>UUGAUACGCGUAACAUUCca</td>
</tr>
<tr>
<td>Scrambled</td>
<td>UUCUCGAACGUGUCACGUtt</td>
<td>ACGUGACACGUGUCGAGAAtt</td>
</tr>
</tbody>
</table>
MiR-448 inhibits laryngeal cancer metastasis

Figure S2. Validation of the silencing effect by the siRNAs against AEG-1. A. qRT-PCR results indicate that AEG-1 mRNA level can be efficiently silenced by the corresponding siRNAs. N=3. Data were represented as mean ± SD from three independent experiments (***P < 0.001). B. Western bolt results indicate that AEG-1 protein level can be efficiently silenced by the corresponding siRNAs.

Figure S3. Annexin V-FITC/PI staining assay shows that the silencing of AEG-1 in Hep2 cells promotes apoptosis and necrosis.