Original Article

MiR-19a is correlated with prognosis and apoptosis of laryngeal squamous cell carcinoma by regulating TIMP-2 expression

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Abstract: MicroRNAs (miRNAs) are small, noncoding RNA molecules that act as posttranscriptional regulators of gene expression and function as important regulators in cancer-related processes. MiR-19a is overexpressed in various cancers and has been causally related to cellular proliferation and growth. To determine whether miR-19a plays a role in laryngeal squamous cell carcinoma (LSCC), we used quantitative real time PCR to detect miR-19a expression in LSCC tissues. We found that miR-19a is overexpressed in LSCC and correlated with neck nodal metastasis, poor differentiation and advanced stage. Statistical analysis suggests that higher level of miR-19a was associated with reduced overall survival. In vitro functional study showed that inhibition of miR-19a by antisense oligonucleotides (ASO) led to apoptosis and reduction of cell proliferation in LSCC cells. Furthermore, growth of LSCC xenograft tumors was significantly suppressed by repeated injection of ASO-miR-19a lentivirus. The TUNEL stain and transmission electron microscopy also detected increased apoptotic cells in ASO-miR-19a treated LSCC xenografts. In addition, both real-time PCR and western blot showed ASO-miR-19a can upregulate TIMP-2 expression and this suggests miR-19a is related with TIMP-2 pathway in LSCC cells. Taken together, these data suggest that miR-19a plays an oncogenic role in the progression of LSCC, and may serve as a biomarker or therapeutic target for patients with LSCC.

Keywords: miR-19a, LSCC, TIMP-2, apoptosis

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer in the world and laryngeal squamous cancer is an aggressive head and neck malignancy. Conventional management approaches to advanced laryngeal cancer have included radical surgery, radiation therapy and chemotheraphy, but the prognosis for advanced laryngeal cancer remains generally poor. Furthermore, total laryngectomy in advanced patients leads to the loss of natural speech function and lung infection. Therefore, a better understanding of the molecular mechanisms underlying LSCC progression is important to improve diagnosis and therapy of laryngeal cancer. The non-coding RNA (ncRNA) is commonly employed for RNA that does not encode a protein. Recently, increasing evidences have demonstrated that the ncRNAs are functional [1, 2]. MicroRNAs (miRNAs) are a class of small, ncRNAs that are endogenously expressed in animal and plant cells. They target messenger RNAs and regulate the expression of protein-coding genes by suppressing translation or degrading miR-bound mRNA. MiRNAs play important roles in the regulation of gene expression for developmental timing, cell proliferation and apoptosis. Recent evidence has showed that abnormal expression of miRNAs is associated with the progression of various cancers [3]. MiRNA-19a has been identified to be over-expressed in many types of solid tumors, but the biological roles of miR-19a in LSCC are still poorly understood [4, 5]. Therefore, in this study we first examined the expression of miR-19a in LSCC and found upregulation of miR-19a in LSCC cancer tissue. Next we investigated the
miR-19a in LSCC

effects of miR-19a on cell proliferation and apoptosis. Moreover, we found that miR-19a modulates TIMP-2 (Tissue inhibitors of metalloproteinases 2) expression, and thereby is involved in TIMP-2 network. Our findings demonstrate that miR-19a plays an oncogenic role of LSCC.

Material and methods

Patients and samples

Included in the study were 83 patients with laryngeal cancer who underwent partial or total laryngectomy at the Department of Otorhinolaryngology of the Second Affiliated Hospital of Harbin Medical University between October 2005 and January 2007. The patients had not received any therapy before admission. After surgery, the matched specimens of LSCC and the corresponding adjacent nonneoplastic tissues obtained from patients were preserved in liquid nitrogen within 5 minutes of excision and then were transported frozen to the laboratory and stored at -80°C. The study was approved by the Ethics Committee of Harbin Medical University and informed consent was obtained.

qPCR

Total RNA was extracted from cancerous or noncancerous specimens and Hep-2 cells. The expression level of miR-19a and TIMP-2 were determined by qPCR, as described previously [6]. The primers for miR-19a were 5'-CCTCTCGTTATTTTGTCAAGGC-3' and 5'-CATAGTTGCATAGTTGC-3'. The primers for TIMP-2 were primer: 5'-TCTGGATGGACTGGGTCACA-3' and 5'-CTTGATGCAGGGAAGGAATTC-3'. The relative expression level was calculated using the 2-ΔΔCt method, with the CT values normalized using 18S rRNA as internal control.

Lentivirus vectors for ASO-miR-19a

Lentivirus Vectors for ASO (Antisense Oligonucleotides) of human miR-19a carrying GFP sequence was provided by Genechem (Shanghai, China). The recombinant lentivirus of miR-19a ASO and the control lentivirus (GFP lentivirus) were prepared and titered to 10^8 TU/mL.

Cell culture and virus transduction

Hep-2 cells of human LSCC were kindly provided by the Harbin Medical University Laboratory of Cell Pathology. Cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Gibco; Life Technologies, Carlsbad, CA) in a humidified incubator (37°C, 5% CO_2). Hep-2 cells were plated in 24-well plates (2×10^4 cells/well) overnight. The lentiviruses were diluted in 0.2 mL [10^7 transduction units (TU)/mL] complete medium containing hexadimethrine bromide (Polybrene; 8 mg/mL) and were incubated with the cells for 1 hour at 37°C. Next, the cells were incubated with 0.3 mL fresh prepared Polybrene-Dulbecco’s modified Eagle’s medium for another 24 hours; the medium was then replaced with fresh Dulbecco’s modified Eagle’s medium and the cells were cultured for 48 hours.

MTT assay

After transfection of the Hep-2 cells with mir-206 for varying time-periods: 20, 44, 68 and 92 h, 20 μL of sterile MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye (5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for another 4 h at 37°C. Then, 150 μL of dimethyl sulfoxide was added to each well and the plates were thoroughly mixed for 10 min. Spectrometric absorbance at a wavelength of 492 nm was measured on an enzyme immunoassay analyzer (model 680; Bio-Rad Laboratories, Hercules, CA, USA). The cell proliferative rate was calculated using the following formula: Cell proliferative rate (%) = (mean absorbance in six wells of the treatment group/mean absorbance in six wells of the cells control group)×100%

Tumor growth in nude mice

Sixteen BALB/c mice, age 5 to 6 weeks, were provided by Vital River Laboratories (Beijing, China). They were bred in aseptic conditions and kept at a constant humidity and temperature according to standard guidelines under a protocol approved by Harbin Medical University. All mice were injected subcutaneously in the dorsal scapula region with 100 µl suspension (1×10^6) of Hep-2 cells. The size of the tumor was measured twice a week with calipers, and the volume of tumor was determined using the simplified formula of a rotational ellipsoid (length×width^2×0.5). Once tumors reached approximately 0.5-0.6 cm^3, the mice received an injection into the tumor once a week for 3 weeks. The 9 mice in the experimental group were treated with 100 µl ASO-miR-19a lentivirus, while 7 mice in the control group received
miR-19a in LSCC

TUNEL stain

Apoptosis in vivo was detected using the terminal deoxytransferase-mediated dUTP nick end labeling (TUNEL) method in situ apoptosis detection kit (Roche) according to the manufacturer’s instructions. After deparaffinization and dehydration and inactivation of intrinsic peroxidase activity, 20 paraffin sections of each tumor specimen were incubated with 2 μg/mL proteinase K at 37°C for 15 minutes. Then, the sections were treated with terminal deoxynucleotidyl transferase and biotinylated dUTP. After stopping the reaction with TB buffer (30 mmol/L sodium chloride, 30 mmol/L sodium citrate), the samples were investigated by microscopy. Controls for the TUNEL procedure were treated in the same manner as the test samples except that the TdT enzyme was omitted from the reaction mixtures in both kits and was replaced with dH$_2$O. No labeling was found in the controls.

Transmission electron microscope examination

Apoptotic morphological changes of LSCC xenograft were detected by transmission electron microscope examination. Tumor tissues were fixed (1 h) with 2.5% glutaraldehyde using 0.1 mol/L Na cacodylate buffer, pH 7.3, washed in a buffer (1 h), and then postfixed (1 h) with 1% osmium tetroxide (OsO$_4$). Fixed samples were rinsed and dehydrated in a graded ethanol series. Ultrathin sections were cut, mounted on copper grids, and stained with uranyl acetate and lead citrate by standard methods. Stained grids were examined and photographed in a JEM 1,200EX transmission electron microscope (Tokyo, Japan).

Western blot analysis

Hep-2 cells were collected and analyzed using Western blot to assess TIMP-2 expression, as described previously [7]. Antibody against TIMP-2 was purchased from Boster, Wuhan, China. TIMP-2 antibody was diluted to 1:400. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control on the same membrane.

Statistical analysis

Data are expressed as means±SD of three independent experiments, each performed in triplicate. Differences between groups were assessed by unpaired, two-tailed Student’s t-test. Survival curves were estimated by using Kaplan-Meier method and were compared by using the log-rank test. Multivariate Cox proportional hazards analysis was performed with survival as the dependent variable. P<0.05 was considered significant.

Results

MiR-19a is overexpressed in LSCC

qPCR analysis showed that miR-19a expression was significantly higher (9.39 fold) in 83 LSCC tissues (2.920±0.876) than that in adjacent non-neoplastic tissues (0.305±0.172) (P<0.001). Next, the miR-19a expression was found to be statistically related with T grade, differentiation, neck nodal metastasis and clinical stage (Table 1, Figure 1).

Table 1. Relationship between miR-19a expression level and clinicopathologic parameters of LSCC

<table>
<thead>
<tr>
<th>Characteristics (n)</th>
<th>miR-19a level</th>
<th>P</th>
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<tbody>
<tr>
<td>Sex</td>
<td></td>
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</tr>
<tr>
<td>Male (57)</td>
<td>2.945±0.894</td>
<td>0.427</td>
</tr>
<tr>
<td>Female (26)</td>
<td>2.853±0.849</td>
<td></td>
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<tr>
<td>Age</td>
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<td></td>
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<tr>
<td>≥56 (42)</td>
<td>2.874±0.876</td>
<td>0.314</td>
</tr>
<tr>
<td>&lt;56 (41)</td>
<td>2.966±0.884</td>
<td></td>
</tr>
<tr>
<td>T classification</td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>T1-2 (52)</td>
<td>2.711±0.839</td>
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</tr>
<tr>
<td>T3-4 (31)</td>
<td>3.270±0.835</td>
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<tr>
<td>Differentiation</td>
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<tr>
<td>G1 (59)</td>
<td>2.805±0.851</td>
<td></td>
</tr>
<tr>
<td>G2 (24)</td>
<td>3.202±0.891</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td>&lt;0.01</td>
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<tr>
<td>Negative (54)</td>
<td>2.722±0.854</td>
<td></td>
</tr>
<tr>
<td>Positive (29)</td>
<td>3.288±0.807</td>
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<tr>
<td>Primary location</td>
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<tr>
<td>Supraglottic (35)</td>
<td>2.949±1.031</td>
<td></td>
</tr>
<tr>
<td>Glottic (48)</td>
<td>2.898±0.755</td>
<td></td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
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<tr>
<td>I-II (46)</td>
<td>2.686±0.872</td>
<td></td>
</tr>
<tr>
<td>III-IV (37)</td>
<td>3.211±0.800</td>
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an injection of 100 μl GFP-lentivirus. Tumors were harvested 1 week after the end of treatment.

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miR-19a in LSCC

Kaplan-Meier analysis showed that patients with high miR-19a expression level had significantly shorter overall survival than those with low miR-19a expression level ($X^2=6.140, P=0.013$, Figure 2). The results of the multivariate Cox analysis showed that miR-19a expression ($P=0.034; HR\ 2.260$), pathological differentiation ($P=0.043; HR\ 2.042$) and neck nodal metastasis ($P=0.035; HR\ 9.204$) were independent prognostic factors of overall survival rate of LSCC patients. Taken together, these results suggest that miR-19a plays an important role in the progression of LSCC.

**ASO-miR-19a inhibits proliferation of Hep-2 cells**

By quantitative real-time PCR analysis, we observed that miR-19a was expressed at a significantly higher level in the Hep-2 cells infected with GFP-lentivirus control than in the cells infected with ASO-miR-19a lentivirus, thus demonstrating that ASO-miR-19a down-regulates miR-19a and can be used for further experiments (Figure 3). Next, we performed an MTT assay to examine the effect of downregulation of miR-19a on the

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**Figure 1.** The expression level of miR-19a in LSCC tissues. Tumors with advanced clinical stages, with poor differentiation, with T3-4 grade or with lymph node metastasis expressed higher levels of miR-19a. *: p<0.05; **: P<0.01.

**Figure 2.** The Kaplan-Meier overall survival curve for LSCC patients (n=83). The higher curve represented the patients with tumors that weakly expressed miR-19a, and the lower curve represented the patients with tumors that highly expressed miR-19a (P=0.013).

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**MiR-19a correlates with prognosis of LSCC**

Kaplan-Meier analysis showed that patients with high miR-19a expression level had signifi-
miR-19a in LSCC

proliferation of Hep-2 cells in vitro. After ASO-miR-19a transfection, the viability of the Hep-2 cells was evidently decreased at each different time-point (24, 48, 72, and 96 h) (Figure 4). However, proliferative rates of the Hep-2 cells in the control groups did not show any obvious alteration during the time course (Figure 4) (P<0.01).

ASO-miR-19a suppresses tumor growth in vivo

All of the 16 mice developed detectable tumors after they were subcutaneously injected with Hep-2 cells. The growth of the LSCC xenografts was significantly inhibited in mice treated with ASO-miR-19a lentivirus compared with those treated with GFP-lentivirus. The average tumor weight (1.419±0.261 g) in the ASO-miR-19a treated LSCC xenografts was statistically lower (P<0.001) than the tumors in control group (2.173±0.229 g) (Figure 5).

ASO-miR-19a induces apoptosis of LSCC cells

The extent of DNA fragmentation was determined using TUNEL stain in LSCC xenografts, showing many apoptotic cells in ASO-miR-19a Lentivirus treated Hep-2 xenografts only (Figure 6A). No TUNEL-positive staining was detected in the control xenograft sections (Figure 6B).

The tumor cells in the ASO-miR-19a treated group showed typical apoptotic morphology under transmission electron microscopy (Figure 6C), characterized by homogeneous condensation of chromatin to one side or the periphery of the nuclei. The inner matrix of some mitochondria showed increased electron density as is typically observed with apoptotic cells. These ultrastructural changes were unique in tumor cells of the ASO-miR-19a treated group but were absent in tumor cells of the control group, which showed intact membranes and intact morphology of organelles (Figure 6D).

ASO-miR-19a regulates TIMP-2 expression of in LSCC cells

To determine the effect of miR-19a on TIMP-2 expression, the level of TIMP-2 was detected by realtime PCR and western blot. The results showed that both mRNA and protein level of TIMP-2 was increased in Hep-2 cells transfect- ed with ASO-miR-19a lentivirus compared with control. While similar levels of TIMP-2 expression were found between the control Hep-2 cells and cells without any treatment (Figure 7).

Discussion

It is clear that microRNAs could contribute to cancer development and regulate many biological processes, including cell proliferation and apoptosis by acting as oncogenes or tumor suppressor genes. MiR-19a was found to be up-regulated in a variety of cancers, including gli-
miR-19a in LSCC

In this study, we examined the expression pattern of miR-19a in LSCC tissues using quantitative PCR and results showed that miR-19a was overexpressed in primary LSCC compared with adjacent non-cancerous tissues. Moreover, high miR-19a expression level was correlated with poor differentiation, lymph node metastasis or advanced clinical stages of LSCC. These data suggest that miR-19a may promote the malignant progression of LSCC. A previous study revealed that miR-19a expression was a novel marker for identifying good and poor prognostic factor of rhabdomyosarcoma [13]. In this study, we demonstrated that the overexpression of miR-19a had significant association with decreased overall survival in LSCC. Cox multivariate analysis showed that miR-19a expression was an independent prognostic factor of overall survival rate. To further understand the biological function of miR-19a in LSCC progression, both in vitro and in vivo assays were performed. Our in vitro data demonstrated that ASO mediated downregulation of miR-19a led to significant decrease of proliferation in Hep-2 cells. Furthermore, we injected Hep-2 cells into mice to make xenografts which were treated by ASO-miR-19a lentivirus. The average tumor weight was significantly lower in these mice compared with control, suggesting that miR-19a downregulation could effectively suppress the progress of LSCC in vivo. Moreover, by TUNEL stain and transmission electron microscopy, we found increased apoptotic cells in the ASO-miR-19a treated LSCC xenografts. Collectively, these results indicate that high level of miR-19a promotes the malignant phenotypes of LSCC cells such as apoptosis resistance.

Figure 6. In vivo TUNEL staining of the ASO-miR-19a Lentivirus treated Hep-2 xenograft tumour sections also showed numbers of apoptotic cells (A), whereas no obvious apoptotic cell was found in tumors of the control group (B). Ultrastructure of LSCC cells in ASO-miR-19a treated Hep-2 Xenografts showed apoptotic changes (C) and in control Hep-2 Xenografts showed normal membrane, organelles, and nuclear morphology (D).
More and more genes have been identified as targets of members of the miR-19a, such as IMPDH1 and NPEPL1 [14], CUL5 [12], PTEN [15], TNF-α [16]. Therefore, the oncogenic functions of the miR-19a are probably regulated by multiple genes. TIMP-2 plays an important role in regulating the activity of matrix metalloproteinases and suppresses tumor growth, angiogenesis, invasion and metastasis [17, 18]. The TIMP-2 gene is known to be expressed in normal human tissues, whereas its expression is downregulated in head neck cancers[19]. Furthermore, TIMP-2 gene therapy enhanced the therapeutic effects of HNSCC as both an MMP inhibitor and an anti-angiogenic agent in mouse xenografts [20]. In the TargetScan database (www.targetscan.org), we found TIMP-2 is the putative target of miR-19a. To further demonstrate the effect of miR-19a on TIMP-2 gene, by realtime PCR and western blot analysis, we found increased TIMP-2 expression after miR-19a down regulation in LSCC cells. This suggests that the oncogenic function of miR-19a in LSCC is correlated with TIMP-2 modulation.

In summary, our data suggest that miR-19a is overexpressed in LSCC tumor tissues and associated with progression and prognosis of LSCC. Moreover, we found that downregulation of miR-19a can induce apoptosis of LSCC cells. These oncogenic effects of miR-19a are related to the regulation of TIMP-2 gene. Taken together, these results suggest that miR-19a can serve as a marker for LSCC prognosis and downregulation of miR-19a may be a useful therapeutic strategy for LSCC.

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Disclosure of conflict of interest

None.

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References

miR-19a in LSCC


