Downregulation of miR-21 regulates MMP-2 expression and suppresses migration of laryngeal squamous cell carcinoma

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Abstract

MicroRNA-21 has been demonstrated to play an important role in the pathogenesis of cancers. In this study, we measured cell migration and tumourigenicity in ASO-miR-21-transfected Hep-2 cells in nude mice. We also employed Western blot and immunohistochemistry to detect matrix metalloprotease-2 expression in laryngeal squamous cell carcinoma after microRNA-21 transfection. Our data showed that the migration ability of ASO-miR-21-transfected Hep-2 cells was significantly decreased when compared with that of the control. Downregulation of microRNA-21 markedly suppressed the tumourigenicity of Hep-2 cells in nude mice. Furthermore, ASO-miR-21 could inhibit the expression of matrix metalloprotease-2 when compared with the control both in vitro and in vivo. Our findings suggest that microRNA-21 is involved in cell migration and tumourigenicity of laryngeal squamous cell carcinoma by regulating the matrix metalloprotease-2 expression. This further implicates microRNA-21 as an oncologic microRNA and a potential therapeutic target in laryngeal squamous cell carcinoma.

Introduction

Laryngeal squamous cell carcinoma (LSCC) is a common malignant neoplasm of the head and neck. Despite extensive application of many different treatment modalities, the invasion and metastasis are still considered as the main causes of death in patients with LSCC. The five-year overall survival rate for LSCC is still unsatisfactory¹. Therefore, a better understanding of the molecular mechanisms associated with LSCC progression is potentially important for improving the therapeutic approaches. MicroRNAs (miRNA), a class of small non-coding RNA molecules of about 18-25 nucleotides in length, are endogenously expressed in animal and plant cells. They regulate the expression of protein-coding genes at the translational level. Dysregulation of miRNA expression has been identified in various cancers, and accumulating data indicates that some miRNAs can function as oncogenes or tumour suppressors and play an important role in cancer progression. miR-21 is a unique miRNA in that it is overexpressed in most tumour types analysed so far². Downregulation of miR-21 can suppress tumour growth and invasion in breast, glioma and colon cancer cells³⁻⁵. Recently, tumour suppressors ANP32A and SMARCA4 were demonstrated to be the direct targets of miR-21⁶. Our previous study has shown that miR-21 is overexpressed in LSCC and that antisense oligonucleotide (ASO) of miR-21 can suppress invasion and growth of LSCC both in vitro and in vivo⁷. In the present study, we further investigated the effects of miR-21 on migration of LSCC cells. As per our results, downregulation of miR-21 can inhibit migration of LSCC cells through regulating matrix metalloprotease-2 (MMP-2) expression.

Materials and methods

Cells and ASO-miR-21 transduction

Hep-2 cells of human LSCC were kindly provided by the Laboratory of Cell Pathology, Harbin Medical University. Dulbecco’s modified Eagle’s medium (DMEM) and foetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). ASOs of human miR-21 lentivirus gene transfer vector harbouring green fluorescent protein (GFP) sequence were constructed by Genechem (Shanghai, China). The sequence of the ASO was 5’-TCAACATCAGTCGTAAGCTA-3’. The recombinant lentivirus of ASO-miR-21 and the control lentivirus (GFP-lentivirus) were prepared and tittered to 10⁶ transfection unit/mL. Cell culture and virus transduction were done as previously described [7].

Wound-healing migration assays

Cells were seeded in 6-well plates and grown to about 90% confluency before being wounded using a 200-µL plastic tip across the monolayer cells. Debris was removed by washing three times with phosphate-buffered saline (PBS), and then cells were cultured with fresh DMEM containing 5% FBS. Wound healing was observed at different time points within the scrape line, and representative scrape lines for each cell line were photographed. The migration ability of the cells was evaluated by measuring the width of the wounds (measured at x400 magnification). Images were captured immediately after 24 h post-wounding. Each assay was performed in triplicate in at least two independent experiments. Data

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Materials and methods (cont.)

analysis was performed using Image J software.

Transwell assays
To evaluate the migration ability of the Hep-2 cells, a modified version of the standard transfilter assay was conducted. Transwell filters (pore size, 8 μm; Falcon; BD Biosciences) were coated on the lower side with 8 μg/μL Matrigel and placed on a 24-well plate containing DMEM. Hep-2 cells (1×10⁵) were added to the upper compartment of a transwell chamber and allowed to migrate for 24 h at 37°C. The cells were then harvested and suspended in DMEM with 1% bovine serum albumin. After 24 h, Matrigel and cells remaining on the upper side of the membrane were wiped off with PBS-rinsed cotton swabs, and invading cells that had migrated to the bottom surface of the membrane were fixed in 3.7% paraformaldehyde in PBS. Once fixed, the cells were stained with crystal violet for 10 min at room temperature. Cell migration was quantified by counting the number of cells in three inserts. Data are expressed as the average number of cells per insert.

Western blot analysis
Hep-2 cells were collected and analysed using Western blot to assess MMP-2 expression, as described previously. Antibody against MMP-2 was purchased from Boster, Wuhan, China. MMP-2 antibody was diluted to 1:400. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control on the same membrane. The intensity of the respective signals in these blots was determined by image analysis using Image J software.

Enzyme-linked immunosorbent assay (ELISA)
MMP-2 protein levels were determined in cell culture medium by using the human MMP-2 Biotrack ELISA kit (Boster). MMP-2 ELISA was performed according to the manufacturer's protocol.

Tumourigenicity assays in nude mice
Sixteen female BALB/c mice (provided by The Central Animal Facility of Harbin Medical University) of 5–6 weeks were prepared for tumour implantation. They were bred in aseptic conditions and kept at constant humidity and temperature (25°C–28°C), according to standard guidelines under a protocol approved by Harbin Medical University. Eight mice were injected subcutaneously in the dorsal scapula region with a 100-μL suspension (1×10⁵) of ASO-miR-21 lentivirus-transfected Hep-2 cells. The other eight mice were injected subcutaneously with GFP-lentivirus-transfected Hep-2 cells (control). The tumour size was measured twice a week with callipers for 4 weeks, and the tumour volume was determined using the simplified formula of a rotational ellipsoid (length × width² × 0.5).

Immunohistochemistry
Tumour specimens were fixed in formalin overnight and embedded in paraffin. Series sections of 4 μm thickness were prepared for immunohistological staining. Tissue sections were quenched for endogenous peroxidase with freshly prepared 3% H₂O₂ with 0.1% sodium azide and then placed in an antigen retrieval solution (0.01 mol/L citrate buffer, pH 6.0) for 15 min in a microwave oven at 100°C and 600 W. After incubation in the casein block, rabbit monoclonal anti-MMP-2 antigen (1:400) was applied to the sections for 1 h at room temperature, followed by incubation with biotinylated anti-rabbit IgG as a secondary antibody (Zhongshan, Beijing, China) and ExtrAvidin-conjugated horseradish peroxidase (Maixin Bio Co., Fuzhou, China). The immune reaction was revealed with haematoxylin and eosin.

Figure 1: Effect of ASO-miR-21 on migration of Hep-2 cells. (A and B) Effect of ASO-miR-21 on cell migration was determined using scratch-wound healing migration assays, 24 h post-wounding. Cell migration was significantly repressed in Hep-2 cells by transfecting them with ASO-miR-21. (a) Hep-2 cells without any treatment, (b) Hep-2 cells transfected with GFP vector control, (c) Hep-2 cells transfected with ASO-miR-21 (*P<0.01). (C) Effect of ASO-miR-21 on cell migration was determined using transwell assay. In comparison with the control, the migration of ASO-miR-21 is significantly decreased. (a) Hep-2 cells without any treatment, (b) Hep-2 cells transfected with GFP vector control, (c) Hep-2 cells transfected with ASO-miR-21 (*P<0.01).

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Materials and methods (cont.)
diaminobenzidine tetrachloride, and slides were counterstained with haematoxylin, dehydrated and mounted. Consistent negative control was obtained by replacing the primary antibody with PBS.

Statistical analysis
Data are expressed as means ± SD of three independent experiments, each performed in triplicate. Differences between groups were assessed using unpaired two-tailed Student’s t-test; \( P < 0.05 \) was considered significant.

Results
Downregulation of miR-21 inhibits the migration of Hep-2 cells
To examine whether ASO-miR-21 could affect the migration of Hep-2 cells, we performed wound-healing assays. Our results showed that ASO-miR-21 reduced the Hep-2 cell migration rate when compared with that in the control (Figure 1A and B). To validate our results, we further analysed cell migration using transwell migration assays. Consistent with the results of the wound-healing assay, ASO-miR-21 significantly reduced Hep-2 cell migration rate in vitro (Figure 1C, \( P < 0.01 \)).

Figure 2: ASO-miR-21 downregulated MMP-2 expression in Hep-2 cells. (A and B). MMP-2 expression level was detected by Western blot analysis and standardized against the level of GAPDH, MMP-2 protein level was downregulated after ASO-miR-21 infection. (C) ELISA showed that ASO-miR-21 also decreased MMP-2 protein levels in Hep-2 cells. (a) Hep-2 cells without any treatment, (b) GFP-lentivirus-infected Hep-2 cells, (c) ASO-miR-21-infected Hep-2 cells (\( P < 0.01 \)).

Downregulation of miR-21 regulates MMP-2 expression in LSCC cells
MMP-2 expression in Hep-2 cells after ASO-miR-21 transfection was identified by Western blotting. Our results showed that in comparison with the control, MMP-2 levels were downregulated in Hep-2 cells infected with ASO-miR-21 lentivirus (Figure 2A and B, \( P < 0.01 \)). ELISA showed that ASO-miR-21 also decreased MMP-2 levels in Hep-2 cells. However, challenge with GFP vector control at 72 h did not significantly affect MMP-2 levels in Hep-2 cells (Figure 2C).

Downregulation of miR-21 suppresses tumourigenicity in vivo
To test whether ASO-miR-21 could inhibit the growth of LSCC in vivo, we established an experimental model of Hep-2 tumour-bearing BALB/c nude mice. As anticipated, in comparison with the control, growth of the tumour was markedly suppressed in mice injected with ASO-miR-21-transfected Hep-2 cells. Tumour growth was expressed as volume, and tumour growth curves were determined. The average tumour volume was found to be significantly lower in the experimental group than in the control group (Figure 3).

Discussion
MiRNAs play an important role in gene regulation. Among the miRNAs already identified as regulators of neoplastic transformation, invasion and metastasis, miR-21 has emerged as key miRNA that is dysregulated in many cancers. The first indication of miR-21 aberrant expression came from the miRNA profiling of human
Discussion (cont.)

![Image](https://example.com/image1.png)

Figure 4: ASO-miR-21 downregulated MMP-2 expression in tumour xenografts. (A) Tumours in control GFP-lentivirus-transfected group exhibited strong MMP-2 staining (×400). (B) Tumours in ASO-miR-21-lentivirus-transfected group exhibited weak MMP-2 staining (×400).


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