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Downregulation of miR-21 modulates Ras expression to promote apoptosis and suppress invasion of Laryngeal squamous cell carcinoma

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ABSTRACT

MiRNAs are small, noncoding RNA molecules that emerge as important regulators of cancer-related processes. The miR-21 microRNA is overexpressed in a wide variety of cancers and has been causally related to cellular proliferation and apoptosis. In this study, we found that miR-21 is overexpressed in Laryngeal squamous cell carcinoma (LSCC) and correlated with advanced stage. Inhibition of miR-21 by antisense oligonucleotides (ASO) led to decreased protein level of Ras and profound suppression of cell proliferation and invasion. Hep-2 cells exposed to miR-21 ASO exhibited cell cycle arrest at G1 phase and increased apoptosis. Furthermore, growth of LSCC xenograft tumours was significantly suppressed by repeated injection of ASO-miR-21 lentivirus and the Ras protein expression in LSCC xenograft tumours was also downregulate by ASO-miR-21. Taken together, our data suggest that miR-21 may play an oncogenic role in the cellular processes of LSCC and represent a novel target for effective therapies.

1. Introduction

Laryngeal squamous cell carcinoma (LSCC) is very common malignant neoplasm of the head and neck. Current treatments including surgical intervention, radiation therapy and chemotherapy have a moderate effect on early stage cases, but are less effective for more advanced cases. As a result, five-year overall survival for LSCC patients is still poor.[1] Therefore, a better understanding of the molecular mechanisms underlying LSCC progression is important to improve diagnosis, therapy and prevention of LSCC.

MiRNAs are a class of small, noncoding RNAs that are endogenously expressed in animal and plant cells. They regulate the expression of protein-coding genes at the translational level. One strand of the mature double-stranded miRNA is incorporated into the RNA-induced silencing complex, which down-regulates target mRNAs either by degradation or by translational inhibition.[2,3] MiRNAs play important roles in the regulation of gene expression for developmental timing, cell proliferation and apoptosis. Recent evidence has shown that abnormal expression of miRNAs is associated with the progression of various cancers. MiRNA-21 has been identified to be over-expressed in many types of solid tumours, including stomach, prostate, oesophagus, glioblastoma, breast, colorectal and head and neck cancers.[4–10] It has been reported that downregulation of miR-21 can suppress tumour growth and invasion in breast, glioma and colon cancer cells.[7–9] Furthermore, inhibition of miR-21 could regulate the expression of PTEN, TPM1 and PDCD4 in cancer cells[11–13], suggesting its potential role as a tumour suppressor.

However, the biological roles of miR-21 in LSCC are still poorly understood. Therefore, in this study we first examined

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the expression of miR-21 in LSCC by real-time PCR and observed a significant upregulation of miR-21 in LSCC cancer tissue and cell line. Next we investigated the effects of miR-21 on cell proliferation, invasion, cell cycle and apoptosis. Moreover, we found that miR-21 modulates Ras expression, and thereby is involved in Ras network. Our findings demonstrate that miR-21 plays an oncogenic role and may serve as a target for effective therapy of LSCC.

2. Materials and methods

2.1. Samples

Patients were enroled in the period between June 2008 and December 2009. Included in the study were 39 patients with laryngeal cancer who underwent partial or total laryngectomy at the Department of Otorhinolaryngology, the Second Affiliated Hospital of Harbin Medical University, under an approved protocol of Harbin Medical University. The patients had not received any therapy before admission. After surgery, the matched specimens of LSCCs and the corresponding adjacent non-neoplastic tissues obtained from patients were preserved in liquid nitrogen within 5 min of excision, then transported frozen to the laboratory and stored at -80°C. All tissues obtained from the neck dissection were marked according to their regions, and the lymph nodes were serially sectioned, stained with haematoxylin–eosin and observed microscopically.

2.2. Real-time RT-PCR

For LSCC samples, total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instruction. For Hep-2 cells, total RNA was extracted from the cells with RNA rose (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. About 200 ng of total RNA was reverse transcripted using All-in-OneTM miRNA Q-PCR Detection Kit (Genecopoeia, Germantown, Maryland, USA) according to the manufacturer's manual. Real-time PCR was performed using SYBR-Green PCR Master Mix (ABI, Foster, CA, USA) on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). To estimate the expression of miR-21, the Ct values were normalised using 18S rRNA as internal control. The relative miRNA expression was calculated using the 2^{-DDCt}. The primers for miR-21 detection were 5'-TAGCTTATCAGACTGATGTTGAAA-3'. The hsRNA-U6 primer (CAAATTCGTGAAGCGTTCCATAT) was near the 30-end of 18S rRNA and was used with the universal primer supplied in the NCode™ miRNA First-Strand cDNA Synthesis Kit.

2.3. Lentivirus vectors for ASO-miR-21

Antisense Oligonucleotides (ASO) 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 is as follows: 5'-TCAACAT CAGTCTGATAAGCTA-3'. The recombinant lentivirus of ASO-miR-21 and the control lentivirus (GFP-lentivirus) were prepared and tittered to 10^9 TU/mL (transfection unit).

2.4. Cell culture and virus transduction

The Hep-2 cells of human LSCC were kindly provided by the laboratory of cell pathology, Harbin Medical University. Cells were cultured in DMEM medium containing 10% foetal bovine serum (Gibco) and incubated in a humidified (37°C, 5% CO2) incubator. Hep-2 cells were plated in 24-well plates (2×10^4 cells/well) overnight. The lentiviruses were diluted in 0.2 mL (10^8 TU/mL) complete medium containing polybrene (8 mg/mL) and added to the cells for 1 h incubation at 37°C, followed by incubation in 0.3 mL of freshly prepared polybrene-DMEM for another 24 h, which was replaced with fresh DMEM medium and the cells were cultured for next 48 h.

2.5. MTT assay

After transduction of Hep-2 cells by ASO-miR-21 lentivirus 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, a tetrazole) dye (5mg/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 growth rate was calculated using the following formula:

Cell growth rate (%) = (mean absorbance in six wells of the treatment group/mean absorbance in six wells of the cells control group) × 100

2.6. Cell Cycle Analysis

To determine cell cycle distribution, cells were collected after trypsinisation into a single-cell suspension, fixed with cold ethanol at 4°C for 1 h before being stored at -20°C until required for analysis. On analysis, fixed cells were washed and resuspended in 1 mL of phosphate-buffered saline (PBS) containing 50 Kg/mL RNase A and 50 Kg/mL ethidium bromide. After incubating for 20 min at 37°C, cells were analysed for DNA content by flow cytometry (FACS Calibur; Becton Dickinson Immunocytometry Systems, San Jose, Calif). For each sample, 20,000 events were acquired, and cell cycle distributions were determined using cell cycle analysis software (Modfit; LT for mac V 3.0).

2.7. Apoptosis assay

The cells were washed twice with cold 10 mM PBS and resuspended in 1 × binding buffer (BD Biosciences, San José, CA, USA) at a concentration of 1×10^6 cells/ml. Cells were stained with Annexin V/ APC and propidium iodide (PI), using the Annexin V apoptosis detection kit (KeyGen Biotech, Nanjing, china). Hep-2 cells without any treatment were used as an internal control, and the experiments were repeated at least three times. A dot plot of the X-axis (FL4), being the log of Annexin V fluorescence, and the Y-axis (FL2), which reflects the PI fluorescence.

Apoptotic morphological changes in the nuclear chromatin of cells were detected by staining with the DNA-binding fluorochrome Hoechst 33258 (bisbenzimide). Treated cells were washed in PBS (pH 7.4) and then fixed with 4% paraformaldehyde for 10 min at room temperature. Following fixation, cells were rinsed 3 times in PBS (pH 7.4) and then stained with Hoechst 33258 staining solution according to the manufacturer's instruction (Beyotime, Jiangsu, China). Stained nuclei were observed under a fluorescence microscope (IX70, Olympus, Japan) and five fields were randomly sampled from each coverslip.

2.8. Western blot analysis

Hep-2 cells were collected and analysed by Western blot to assess Ras expression as described previously.[14] Antibody against Ras was purchased from Boster (Wuhan, China). The dilution of Ras antibody was 1:200. Western blot of GAPDH on the same membrane was used as a loading control. The intensity of the respective signals in these blots was determined with an image analysis using Image J program.

2.9. Cell invasion assay

For invasion assay, modified Boyden chambers with filter inserts (pore size, 8 μm) coated with Matrigel (Becton Dickinson Labware) in 24-well dishes were used. Approximately 2.5×10^4 cells in 100 μL of complete RPMI-1640 medium were placed in the upper chamber, and 1 mL of the same medium was placed in the lower chamber. After 48 h in culture, cells were fixed in methanol for 15 min and then stained with 0.05% crystal violet in PBS for 15 min. Cells on the upper side of the filters were removed with cotton-tipped swabs. Cells that had invaded into the lower side of the filter were then stained with H&E and microscopically observed and counted in 5 fields of view at 200×magnification. The invasive activity of cancer cells was expressed as the mean number of cells that invaded to the lower side of the filter and the results were presented as mean ± SD of cells per field of view. Experiments were repeated at least three times.

2.10. Animal experiments

Sixteen BALB/c mice (provided by The Central Animal Facility of Harbin Medical University) were 5-6 weeks old and 20 g in weight. They were bred in aseptic conditions and kept at a constant humidity and temperature (25-28°C) 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 tumour was measured twice a week with calipers, and the volume of tumour was determined using the simplified formula of a rotational ellipsoid (length × width^2 × 0.5). Once tumours reached approximately 0.5-0.6 cm^3, the mice received an injection into the tumour once a week for 3 weeks. 8 mice in the experimental group were treated with 100 μl ASO-miR-21 lentivirus, the remaining eight mice, the control group, received an injection of 100 μl GFP-lentivirus. Tumours were harvested 1 week after the end of treatment.

2.11. Immunohistochemistry

Tumour specimens were fixed in formalin overnight and embedded in paraffin. Series sections of 4 μm thick were prepared for immunohistological staining. Tissue sections were quenched for endogenous peroxidase with freshly prepared 3% H_2O_2 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 multiclonal anti-Ras antigen (1/150, Boster Biological Technology, Ltd. Wuhan, China) was applied to the sections for 1 h at room temperature, followed by incubation with biotinylated anti-rabbit IgG as a second antibody (1/200, Sigma, Shanghai, China) and ExtrAvidin-conjugated horseradish peroxidase (1/30, Maixin Bio Co., Fuzhou, China). The immune reaction was revealed with diaminobenzidine tetrachloride, and slides were counterstained with haematoxylin, dehydrated and mounted. Consistent negative control was obtained by replacement of primary antibody with PBS.

2.12. 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, $P < 0.05$ was considered significant.

3. Results

3.1. MicroRNA-21 is overexpressed in LSCC

The clinicopathologic findings of 39 patients were shown in Table 1. Total RNA was isolated from matched adjacent non-

Table 1 – Relationship between miR-21 expression level and clinicopathologic parameters of LSCC.

Characteristics (n)	miR-21 (T/N expression ratio)	P
Sex		0.80
Male (27)	17.69 ± 10.21	
Female (12)	18.65 ± 10.82	
Age		0.86
⩾62 (20)	18.26 ± 10.92	
<62 (19)	17.69 ± 9.82	
T classification		0.0001
T1-2 (24)	10.83 ± 7.37	
T3-4 (15)	22.45 ± 9.32	
Differentiation		0.004
G1 (28)	11.08 ± 7.75	
G2 (11)	20.69 ± 9.96	
Lymph node metastasis		0.0008
Negative (25)	11.38 ± 7.39	
Positive (14)	21.67 ± 9.89	
Primary location		0.60
Supraglottic (17)	19.00 ± 10.75	
Glottic (22)	17.20 ± 10.06	
Clinical stage		0.0000
I-II (22)	10.50 ± 6.94	
III-IV (17)	23.75 ± 8.59	

neoplastic tissues (N) and LSCC tumour tissues (T), and the miR-21 levels were determined by real-time PCR. The miR-21 expression was significantly higher (18 folds) in LSCC tissues than that in adjacent non-neoplastic tissues ($P < 0.001$). Next, the correlation of T/N ratios for miR-21 expression with the clinicopathologic factors was examined. The T/N ratios of miR-21 expression were found to be statistically related with T grade, differentiation, neck nodal metastasis and clinical stage (Figure W1). Tumours with advanced clinical stages, with poor differentiation, with T3-4 grade or with lymph node metastasis expressed higher levels of miR-21. These data suggest that miR-21 up-regulation is correlated with tumour staging and may play a role in the progression of LSCC.

3.2. *ASO-miR-21 downregulates miR-21 in Hep-2 cells*

As the first step to address the biological function of miR-21, we used a lentiviral vector system that incorporates GFP as a reporter gene to express ASO-miR-21 to downregulate miR-21. A high percentage (more than 90%) of Hep-2 cells expressed GFP at 72 h after the lentivirus transduction (Fig. 1A), indicating a high and stable transduction of lentiviral vector system. Furthermore, by quantitative real-time PCR analysis, we observed that miR-21 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-21 lentivirus, thus demonstrating that ASO-miR-21 downregulates miR-21 and can be used for further experiments (Fig. 1B).

3.3. *Downregulation of miR-21 inhibits the viability of Hep-2 cells*

After ASO-miR-21 transduction, the viability of Hep-2 cells was evidently decreased at each different time point (24, 48, 72, and 96 h, respectively). However, survival rates of Hep-2 cells in control groups did not show any obvious alteration during the time course (Fig. 1C). These results indicated that downregulation of miR-21 could inhibit the viability of Hep-2 cells in vitro.

3.4. *Downregulation of miR-21 inhibits the invasion of Hep-2 cells*

Next, we performed an invasion assay in a modified Boyden chamber to examine the effect of downregulation of miR-21 on the invasion of Hep-2 cells in vitro. Movement of cells through Matrigel-coated Boyden chambers mimics the early steps of tumour invasion. We observed that invasive cells per field of view through the porous transwells were significantly reduced in ASO-miR-21 lentivirus-infected cells than in control lentivirus-infected cells ($P < 0.01$, Fig. 1D).

3.5. *Downregulation of miR-21 induces G1 phase arrest of Hep-2 cells*

A 72 h exposure of GFP-lentivirus-infected Hep-2 control produced 58.9% of G1, 16.9% of G2, 24.2% of S phase; how-

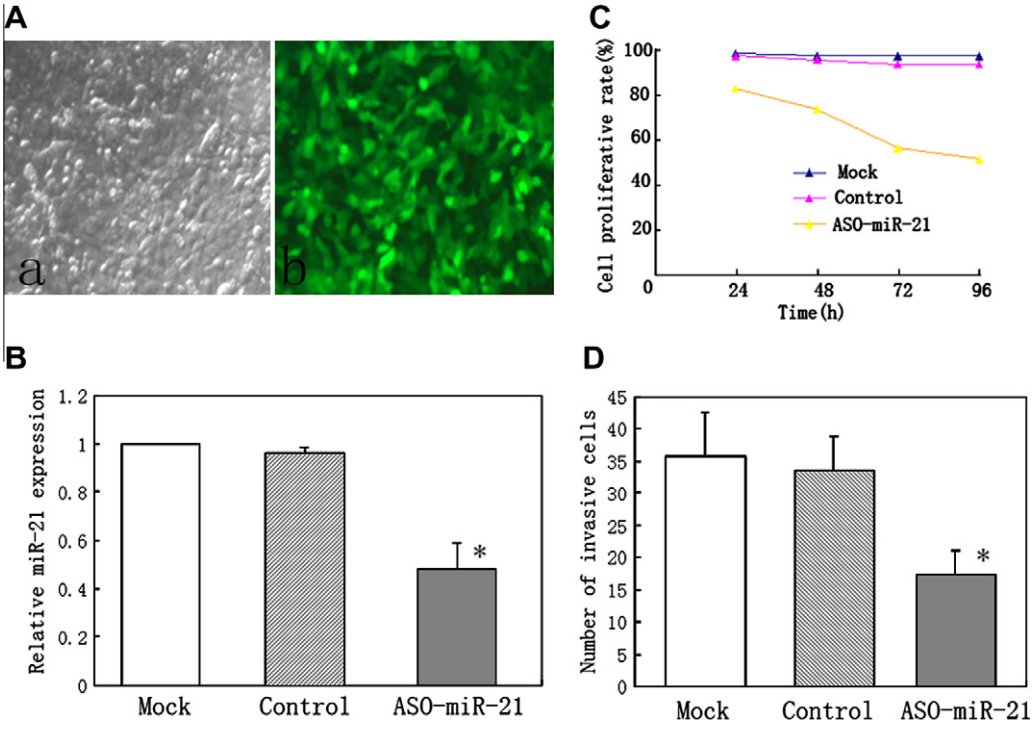

Fig. 1 – ASO-miR-21 inhibits the viability and invasion of Hep-2 cells. A: Images of Hep-2 cells 72 h after transduction with ASO-miR-21 lentivirus. Image a: light microscope images (200×), b: Fluorescence microscope images (200×). B: miR-21 expression in Hep-2 cells. The relative expression level of miR-21 in Hep-2 cells was significantly decreased by ASO-miR-21 lentivirus compared with the control as detected by real-time RT-PCR. *: $p < 0.01$. C: Curve of cell survival rate. After ASO-miR-21 lentivirus transduction, the survival rate of Hep-2 cells was evidently decreased at each different time point (24, 48, 72, and 96 h, respectively) compared with the controls. D: Cell numbers through Matrigel-coated Boyden chambers. Inhibition of miR-21 resulted in a significant reduction of invasive cells through the porous transwells compared with the controls. *: $p < 0.01$.

ever, it produced no net effect on the cell cycle. In contrast, MiR-21 ASO resulted in 75.1% of G1, 13.1% of G2 and 11.8% of S phase at Hep-2 cells, and the cells in G1 phase were significantly increased. This demonstrates that miR-21 inhibition produced a G1-phase arrest because the number of cells in the G1 phase increased by more than 10% and the number of cells in the S phase decreased by more than 10% (Fig. 2).

3.6. *Downregulation of miR-21 induces apoptosis of Hep-2 cells*

The flow cytometric analysis demonstrated that transduction of Hep-2 cells with ASO-miR-21 for 72 h resulted in a significant increase in the percentage of apoptotic cells, compared with cells infected with the GFP-lentivirus vector or cells uninfected. As shown in Fig. 3 (A, B and C), the apoptosis rate

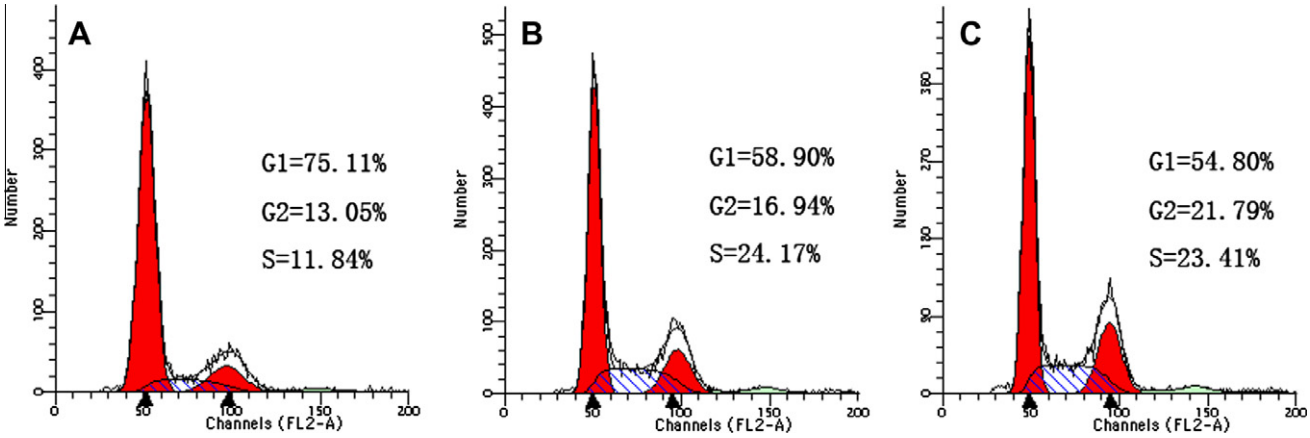

Fig. 2 – Flow cytometric analysis of the effect of ASO-miR-21 on the cell cycle of Hep-2 cells. A: Cell cycle of ASO-miR-21 lentivirus infected Hep-2 cells for 72 h. B: Cell cycle of control lentivirus infected Hep-2 cells for 72 h. C: Blank control.

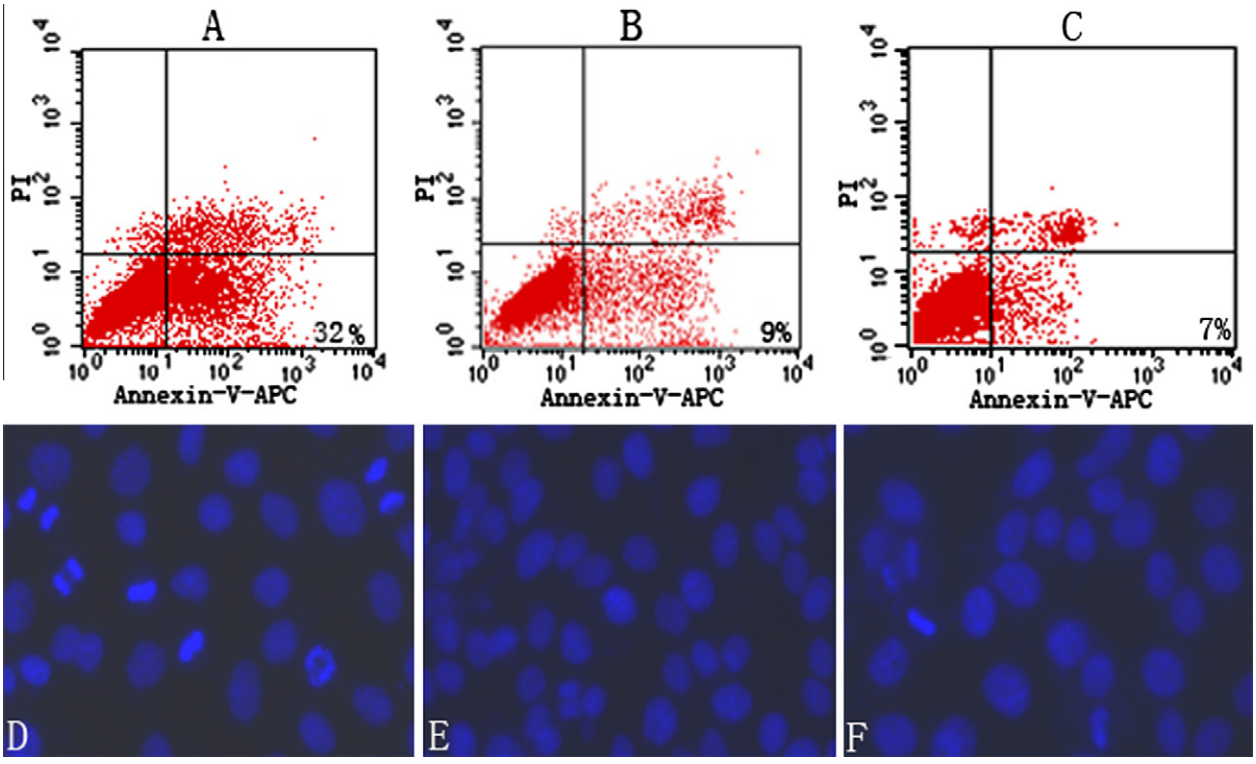

Fig. 3 – ASO-miR-21 induces apoptosis of Hep-2 cells. The dot plot of the X-axis (FL4) of the log of Annexin V fluorescence and the Y-axis (FL2), which reflects the PI fluorescence, was obtained by flow cytometry. A: representative picture of Hep-2 cells infected with ASO-miR-21 lentivirus; B: representative picture of Hep-2 cells infected with GFP-lentivirus control; C: representative picture of Hep-2 cells without any treatment. Morphological analysis of nuclear chromatin by Hoechst 33,258. D: Hep-2 cells displayed apoptotic nuclei 72 h after transduction with ASO-miR-21 lentivirus. E: Hep-2 cells 72 h after transduction with control GFP-lentivirus. F: Hep-2 cells without any treatment.

of Hep-2 cells infected with ASO-miR-21 was higher than that infected with the control lentivirus. To observe the morphological changes of ASO-miR-21-infected Hep-2 cells, we performed morphological examination of cells by Hoechst 33258 staining. As shown in Fig. 3(D, E and F), typical apoptotic morphological changes, such as cell shrinkage, chromatin compaction, condensation of cytoplasm and nuclear fragmentation, were frequently observed in these cells. In contrast, only few apoptotic cells were observed in the control with the same apoptotic morphological changes. Both flow cytometric analysis and Hoechst 33258 staining confirmed the strong anti-apoptotic effect of miR-21.

3.7. *Downregulation of miR-21 regulates Ras expression of in LSCC cells*

To determine the effect of *miR-21* on Ras expression, the protein level of Ras was detected by western blot. The results showed that the protein level of Ras was reduced in Hep-2 cells infected with ASO-miR-21 lentivirus compared with control. Whilst similar levels of Ras expression were found between the control Hep-2 cells and cells without any treatment (Fig. 4).

3.8. *Downregulation of miR-21 suppresses Tumour Growth in vivo*

Of the 16 mice that were injected subcutaneously with 1×10^6 Hep-2 cells, all developed detectable tumours at the termination of this experiment. However, the growth of tumour was significantly suppressed in mice treated with ASO-miR-21 Lentivirus compared with those treated with GFP-Lentivirus (Fig. 5). The average tumour volume and weight in former group was significantly lower than those in the later group. The average tumour weight (1.19 ± 0.22 g) was much lower ($P = 0.005$) than that in control group (1.99 ± 0.57 g). The rate of tumour inhibition was 40.2%.

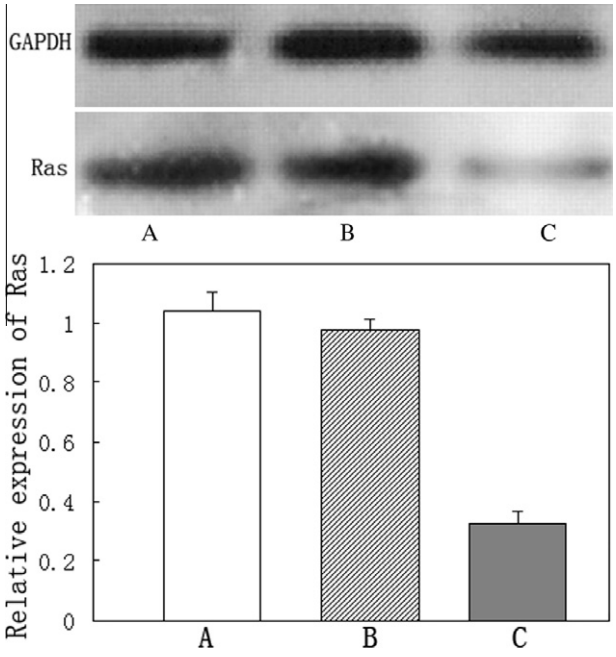

Fig. 4 – ASO-miR-21 downregulates Ras expression in Hep-2 cells. The protein level of Ras was detected by Western blot analysis and standardised against the level of GAPDH and presented as the relative intensity. A: blank control; B: GFP-lentivirus control infected Hep-2 cells; C: ASO-miR-21 infected Hep-2 cells; *: $p < 0.01$.

3.9. *ASO-miR-21 downregulates Ras expression in vivo*

To examine the Ras protein expression in vivo after intratumoural administration of miR-21 Lentivirus or GFP-Lentivirus, immunohistochemistry using anti-Ras antibody was performed. The positive staining of Ras was localised in the cytoplasm. The tumours (8 cases) in ASO-miR-21-treated mice showed weak staining for Ras whilst strong staining of Ras was found in all 8 case tumours of GFP-lentivirus control-treated mice (Fig. 6). These data suggest that ASO-miR-21 can downregulate Ras expression in vivo.

4. Discussion

Accumulating evidence has confirmed miRNAs as a regulatory network with post-transcription regulatory efficiency for almost one third of coding genes in human. MiR-21 has been identified as one of the most overexpressed microRNAs in a number of profiling experiments for the detection of miRNAs in human cancers. MiR-21 was also reported to be expressed at high level in head and neck cancer cell lines.[10] Furthermore, it has been shown that overexpression of miRNA-21 was associated with poor survival in patients with colon cancer, pancreatic cancer and non–small cell lung cancer.[15–17] In agreement with these findings, high level of miR-21 expression was detected in LSCC tissues and Hep-2 cells by real-time PCR in the present study. Moreover, we used the T/N ratio of the miR-21 expression to determine any associations with clinicopathologic features and found that increased miR-21 correlates with poor differentiation, T3-4 grade, lymph node metastasis and advanced clinical stages. These results suggest that miR-21 up-regulation may play an important role in the progression of LSCC. Although the observation period was too short to evaluate the prognosis of 39 LSCC patients enroled in the present study, we presumed that a high expression level of miR-21 may be associated with a poor survival in LSCC patients. Previous studies have found that miR-21 promotes tumour growth, proliferation and invasion in various cancers.[7,13] Recent report suggested that miR-21 targets PDCD4 at the posttranscriptional level and regulates cell proliferation and invasion in oesophageal squamous cell carcinoma.[18] Our previous studies showed that matrix metalloproteinases were significantly related with invasion and metastasis of laryngeal cancer.[19–21] Furthermore, it was reported that ASO-miR-21 inhibits glioma invasion by targeting matrix metalloproteinase regulators.[7] In this study, our data demonstrated that lentivirus can efficiently deliver ASO-miR-21 into Hep-2 cells leading to obvious downregulation of miR-21 expression in vitro. Next, by a modified Boyden chamber and MTT assays, significant decrease of invasive ability and viability of Hep-2 cells was observed after ASO-miR-21-mediated miR-21 downregulation. Furthermore,

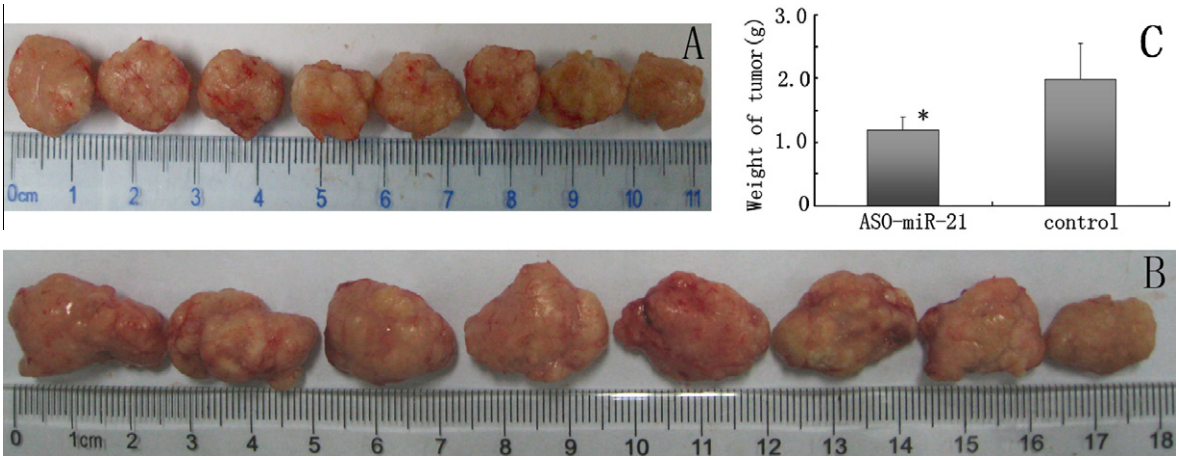

Fig. 5 – Downregulation of miR-21 suppresses Tumour Growth in vivo. Tumours from animals were dissected 1 week after the treatment. A: tumours in ASO–miR-21 lentivirus treated group; B: tumours in the control group. C: difference of tumour weight between ASO –miR-21 lentivirus treated-group and the control. *: $p < 0.01$.

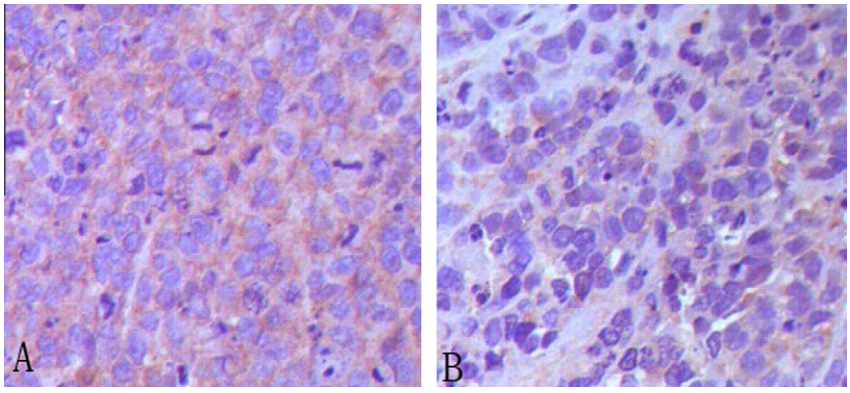

Fig. 6 – ASO-miR-21 downregulates Ras expression in tumour xenografts. A: tumours injected with control GFP-lentivirus exhibited strong Ras protein staining (×400). B: tumours injected with control ASO miR-21 lentivirus exhibited weak Ras protein staining (×400).

to investigate the role of miR-21 in LSCC in vivo, BALB/c-nu mice with induced tumour of Hep-2 cells were treated by ASO-miR-21 lentivirus. Both the average tumour weight and volume were significantly lower in these mice compared with control, and the rate of tumour inhibition was about 40%, thus providing the evidence that miR-21 downregulation could partially inhibit the growth of LSCC in vivo. Cancer invasion and metastasis involve the alteration of multiple gene expression. Given that miRNAs may regulate multiple coding genes that are implicated in tumour growth and invasion, miRNAs are more likely to regulate invasion and growth of tumour effectively.

Apoptosis plays a crucial role in cancer development. In the present study, ASO to miR-21 resulted in G1-phase arrest and induction of apoptosis in the LSCC cells. RAS oncogenes, activated in about one third of human cancers resulting from mutations, had been demonstrated to induce apoptosis in laryngeal cancer cells through caspase and mitochondria-dependent pathways.[22] In the present study, our data showed that Ras expression in LSCC cells was significantly decreased after ASO-miR-21 transduction both in vitro and in vivo. Similarly, Talotta and colleagues reported that miR-21 expression is necessary to achieve the maximal induction of AP-1 in response to Ras.[23] These findings suggest that ASO-miR-21 may induce apoptosis of LSCC through regulating Ras pathway. Interestingly, Tao and colleagues found that miR-21 directly targets MARCKS and promotes apoptosis resistance in prostate cancer cells[9], whilst Zhou and colleagues demonstrated that downregulation of miR-21 induced apoptosis of human glioblastoma cells through inhibiting EGFR pathway.[24] In the MCF-7 breast cancer cell line, apoptosis induced by ASO-miR-21 was due to the down-regulation of Bcl-2.[25] Therefore, our results together with those studies indicate that miR-21 has multiple functions in diverse cancers and downregulation of miR-21 may suppress tumour proliferation and induce apoptosis through multiple mechanisms.

However, further studies are necessary to elucidate the molecular mechanism by which miR-21 modulates Ras pathway to promote LSCC tumourigenesis. On the other hand, intrigued by our interesting finding that downregulation of miR-21 by ASO led to reduced invasion of Hep-2 cells in vitro invasion assay, we are currently exploring the role of miR-21 in tumour metastasis by analysis of the metastasis of Hep-2 xenograft in mice model.

In conclusion, here we show that miR-21 is overexpressed in LSCC tumour tissues, and downregulation of miR-21 inhibits cell proliferation and induces apoptosis of LSCC. These effects are possibly related with regulation of the Ras pathway by miR-21. Thus, miR-21 may play an oncogenic role in LSCC and gene therapy targeting miR-21 should be investigated further as a potential alternative strategy for LSCC therapy.

Conflict of interest statement

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled "Downregulation of miR-21 modulates Ras expression to promote apoptosis and suppress invasion of Laryngeal squamous cell carcinoma".

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2010.07.047.

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