Down-regulation of MiR-206 Promotes Proliferation and Invasion of Laryngeal Cancer by Regulating VEGF Expression

TIANHONG ZHANG[1,2], MING LIU[2], CHUNRUI WANG[1], CHAO LIN[1], YANAN SUN[2] and DEJUN JIN[2]

Department of Otorhinolaryngology, Head and Neck Surgery,
[1]The First Affiliated Hospital, [2]The Second Affiliated Hospital,
Harbin Medical University, Harbin, P.R. China

Abstract. *MicroRNAs (miRNA) are a class of small non-coding RNAs that regulate the expression of their target genes. The aim of the present study was to explore the effects of miR-206 on laryngeal suamous cell carcinoma (LSCC) cells. The expression level of miR-206 was quantified by qRT-PCR in primary LSCC tissues and corresponding adjacent non-neoplastic tissues. MTT, Matrigel invasion assays and flow cytometry methods were used to test the proliferation, invasion and apoptosis of MiR-206 transfection LSCC cells and a mouse model was used to investigate tumorigenesis. MiR-206 was significantly down regulated in the LSCC tissues. Inverse correlation of miR-206 expression was found with the T grade, nodal metastasis and clinical stage of LSCC. Cell proliferation, migration, invasion and tumorigenesis in the LSCC cells were significantly inhibited and apoptotic cells were also increased after miR-206 tansfection. Furthermore, miR-206 transfection down-regulated the expression of vascular endothelial growth factor (VEGF) in the LSCC cells. The loss of miR-206 may play an important role in the progress of LSCC and miR-206 may function as a novel tumor suppressed miRNA.*

Laryngal squamous cell carcinoma (LSCC) is an aggressive head and neck malignancy. A recent meta-analysis showed that the overall 5-year survival rate of LSCC was 64.2% (1). 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. The understanding of the molecular pathways of carcinogenesis or progression would be helpful in improving diagnosis, therapy, and prevention of the disease. MicroRNAs (miRNAs) are a large family of highly conserved short 20- to 25-nucleotide long single-stranded noncoding RNAs which regulate the translational inhibition of target messenger RNAs by binding to their 3'-untranslated region (2). The dysregulation of miRNAs is common in various carcinomas and plays an important role in cancer progression by altering the normal gene expression. MiR-206, which is specifically expressed, plays an important role in skeletal muscle development (3, 4). Recent studies have suggested that miR-206 may act as a suppressor of rhabdomyosarcoma, lung and breast cancer cell proliferation and metastasis (5-8). However, the biological roles of miR-206 in LSCC are still poorly understood. Therefore, in this study the expression of miR-206 in LSCC was examined by real-time PCR and the effects of miR-206 on cell proliferation, invasion and apoptosis were also investigated.

Correspondence to: Prof. Yanan Sun and Prof. Dejun Jin, Department of Otorhinolaryngology, Head and Neck Surgery, The Second Affiliated Hospital, Harbin Medical University. Harbin 150081, PR China. Tel: +86 45186605832, Fax: +86 45153625108, e-mail: syn2767@yahoo.com.cn

Key Words: Laryngal squamous cell carcinoma, microRNA, proliferation, apoptosis.

Materials and Methods

Samples. Patients were enrolled in the period between December 2008 and June 2010. Included in the study were 35 patients with laryngeal cancer who underwent partial or total laryngectomy at the Department of Otorhinolaryngology, the First Affiliated Hospital of Harbin Medical University, under an approved protocol of Harbin Medical University. The patients had not received any therapy before admission. The matched specimens of LSCC and the corresponding adjacent non-neoplastic tissues obtained from the patients were preserved in liquid nitrogen within 5 min of excision, then transported frozen to the laboratory and stored briefly at –80˚C.

Cells. Human Hep-2 cells of human LSCC were kindly provided by the central laboratory, Harbin Medical University, Harbin, China. The Hep-2 cells were grown in RPMI 1640 containing 10% fetal bovine serum (FBS), 2 mmol/L l-glutamine and penicillin-streptomycin (50 IU/mL and 50 mg/mL, respectively), at 37˚C with 5% CO_2.

Real-time RT-PCR. The total RNA was isolated from laryngeal carcinoma and corresponding adjacent non-neoplastic tissues or Hep-2 cells using Trizol reagent (Invitrogen, Carlsbad CA, USA). Approximately 200 ng of the total RNA was reverse transcripted using an All-in-One™ miRNA Q-PCR Detection Kit (Genecopoeia, Germantown, ML, USA) according to the manufacturer's manual.

Real-time PCR was performed using SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). To estimate the expression of miR-206, the Ct (cycle threshold) values were normalized using 18S rRNA as internal control. The relative miRNA expression was calculated using 2^{-DDCt} (delta Ct). The primers for miR-206 detection were 5'-TGGAATGTAAGGA AGTGTGTGG-3'. The hsRNA-U6 primer (CGCTTCGGCAGCA CATATACTA) 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.

Cell culture and transfection. The U6-hsa-mir-206 plasmid harbouring green fluorescent protein (GFP) sequence was constructed by Genechem (Shanghai, China). Hep-2 cells were transfected with either 100 nM control RNA oligonucleotides or 100 nM U6-hsa-mir-206 for varying time-periods. Transfections were performed with Lipofectamine 2000 (Invitrogen), and assessed for subsequent changes in mRNA levels and other functional assays as described below.

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 growth rate was calculated using the following formula:
Cell survival rate (%)=(mean absorbance in six wells of the treatment group/mean absorbance in six wells of the cells control group) ×100%

Apoptosis assay by flow cytometry. 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. The cells were stained with Annexin V/APC and propidium iodide (PI), using the Annexin V apoptosis detection kit (KeyGen Biotech, Nanjing, China) which can successfully separate GFP, APC and PI fluorescence. 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, was constructed.

Invasion assay. To evaluate the invasive ability of the Hep-2 cells, a modified version of the standard transfilter assay was conducted. Transwell filters (pore size, 8 μm; BD Biosciences) were coated on the lower side with 8 μg/μL Matrigel and placed on a 24-well plate containing medium. Hep-2 cells (1×10^5) were added to the upper compartment of a transwell chamber and allowed to migrate for 24 h at 37˚C, then harvested and suspended in medium with 1% bovine serum albumin. After 24 h, Matrigel and cells remaining on the upper side of the membrane were wiped off with phosphate-buffered saline-rinsed cotton swabs and invading cells which had migrated to the bottom surface of the membrane were fixed in 3.7% paraformaldehyde in PBS. Once fixed, the cells on the membrane were stained with crystal violet for 10 min at room temperature. Cell migration was quantified by counting the number of cells in three inserts. The data are expressed as the average number of cells per insert.

Western blot. Cells were transfected with miR-206 for 72 h and cell extracts were prepared in radioimmunoprecipitation assay buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, and 50 mM Tris, pH 8.0), with the addition of 2 mM phenylmethylsulfonyl fluoride. Lysis buffer was freshly prepared and added to transfected cells in six-well plates (100 μL/well) on ice, which were then incubated for 10 min. Protein concentrations were determined by protein assay kit. Equal amounts of proteins (10 μg per condition) were boiled for 10 min in loading buffer before being separated on 15% SDS-PAGE gels. Separated proteins were transferred to polyvinylidene difluoride membranes at 100 V for 1 h before membrane blocking in 5% skim milk powder in PBS with 0.1% Tween 20. Anti-Vascular endothelial growth factor (VEGF) (Boster, Wuhan, China) and secondary antibodies were diluted 1/1,000 in PBS with 0.1% Tween 20 and incubated for 60 min at room temperature with three washes between each step. Protein bands were visualized by enhanced chemiluminescence. Western blotting of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) on the same membrane was used as a loading control.

Tumorigenesis in nude mice. Eighteen 5-6 weeks old BALB/c mice (provided by The Central Animal Facility of Harbin Medical University) approximately 20 g in weight were used. They had been bred in aseptic conditions and were kept at constant humidity and temperature (25-28˚C) according to standard guidelines under a protocol approved by Harbin Medical University. Nine mice were injected subcutaneously in the dorsal scapula region with 100 μl suspension of (1×10^6) miR-206 transfected Hep-2 cells. The other 9 mice were injected subcutaneously with normal Hep-2 cells as control. The size of the tumor was measured twice a week with calipers for 4 weeks, and the volume of the tumor was determined using the simplified formula of a rotational ellipsoid (length × width^2 × 0.5).

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.

Results

MiR-206 expression in LSCC. The clinicopathologic findings of 35 patients were shown in Table I. As determined by real-time PCR, the miR-206 expression was significantly higher (11 folds) in the adjacent non-neoplastic tissues than that in the LSCC tissues ($p<0.001$). The T/N ratios of miR-206 expression were found to be statistically related to the T grade, differentiation, neck nodal metastasis and clinical stage. The tumors with advanced clinical stage, with poor differentiation, with T3-4 grade or with lymph node metastasis expressed lower levels of miR-206.

Expression of miR-206 in Hep-2 cells. As the first step to address the biological function of miR-206, GFP was used as a reporter gene of up-regulated miR-206 expression. More than 50% of the Hep-2 cells expressed GFP at 24 h after the

Table I. *Relationship between miR-206 expression levels and clinicopathological parameters of LSCC patients.*

Characteristics (n)	miR-206 (T/N expression ratio)	*P*-value
Gender		0.792
Male (24)	0.091167±0.0337	
Female (11)	0.0878±0.0347	
Age		0.860
≥58 (18)	0.0831±0.0370	
<58 (17)	0.0966±0.0295	
T classification		0.016
T1-2 (22)	0.100±0.028	
T3-4 (13)	0.073±0.037	
Differentiation		0.013
G1 (25)	0.099±0.029	
G2 (10)	0.069±0.035	
Lymph node metastasis		0.008
Negative (23)	0.101±0.027	
Positive (12)	0.070±0.036	
Primary location		0.221
Supraglottic (15)	0.0952±0.034	
Glottic (20)	0.086±0.033	
Clinical stage		0.002
I-II (20)	0.104±0.027	
III-IV (15)	0.071±0.033	

T/N: Tumor/normal.

transfection (Figure 1A), indicating stable transfection of the vector system. By quantitative real-time PCR analysis, miR-206 was found to be expressed at a significantly higher level in the Hep-2 cells transfected with U6-hsa-mir-206 than in the cells transfected with control oligonucleotides, thus demonstrating that U6-hsa-mir-206 up-regulated miR-206 and could be used for further experiments (Figure 1B).

Effect of miR-206 on the viability of Hep-2 cells. After miR-206 transfection, the viability of the Hep-2 cells was evidently decreased at each different time-point (24, 48, 72, and 96 h). However, survival rates of the Hep-2 cells in the control groups did not show any obvious alteration during the time course (Figure 1C).

miR-206 reduces invasive phenotypes of Hep-2 cells. In the transwell assay, the invasive cells per field of view through the porous transwells were significantly reduced in the miR-206 transfected cells compared to the control cells (118±12 versus 227±15, $p<0.01$, Figure 1D).

Effect of miR-206 on apoptosis of Hep-2 cells. The flow cytometric analysis demonstrated that transfection of Hep-2 cells with miR-206 for 24 h resulted in a significant increase in the percentage of apoptotic cells, compared with the control transfected cells ($p<0.01$), as shown in Figure 2.

miR-206 regulation of VEGF expression in LSCC cells. Western blot showed that the protein level of VEGF was reduced in the Hep-2 cells transfected with miR-206 compared with the control. While similar levels of VEGF expression were found between the control Hep-2 cells and cells without any treatment (Figure 3).

Effect of miR-206 transfection on tumorigenesis of LSCC. Out of the 18 mice that were injected subcutaneously with 1×10^6 Hep-2 cells, all developed detectable tumors by the termination of this experiment. However, the tumor growth was significantly suppressed in the mice injected with miR-206 transfected Hep-2 cells compared with the control (Figure 4). The average tumor volume in former group (0.708 ± 0.108 cm^3) was significantly lower ($p<0.01$) than in the later group(1.574 ± 0.211 cm^3).

Discussion

The expression of miR-206 was significantly decreased in the LSCC tissues compared with the corresponding adjacent non-neoplastic tissues, which suggested that the loss of miR-206 may correlate with the carcinogenesis of LSCC. Moreover, the miR-206 level was inversely correlated with poor differentiation, T3-4 grade, lymph node metastasis and advanced clinical stages, suggesting that miR-206 down-regulation may play an important role in the progression of LSCC. Although the observation period was too short to evaluate the prognosis of the 35 LSCC patients enrolled in the present study, the loss of miR-206 may be presumed to be associated with poor survival in LSCC patients. Previous studies have found that the expression level of miR-206 was inversely correlated with metastatic potential of lung cancer (5). In the present study, mir-206 plasmid transfection led to obvious up-regulation of miR-206 expression and a significant decrease of invasive ability and viability of the Hep-2 cells. Furthermore, the tumorigenesis in nude mice of the miR-206 transfected Hep-2 cells was significantly reduced compared to the control. MiR-206 may thus have a suppressor function in LSCC.

A previous meta-analysis showed that VEGF protein overexpression is associated with worse overall survival in patients with head and neck squamous cell carcinomas (9). In the present study, miR-206 transfection significantly down-regulated VEGF expression in LSCC. By targets scan analysis (miRGen Targets) (data not shown), miR-206 was found to target VEGF gene directly. Thus, miR-206 transfection appears to inhibit proliferation and invasion of LSCC at least partly by regulating VEGF expression. Furthermore, consistent with the findings of Kapral and colleagues (10), our previous study demonstrated that cyclin D1 plays an important role in the proliferation of laryngeal cancer (11). While a report showed that low mir-206

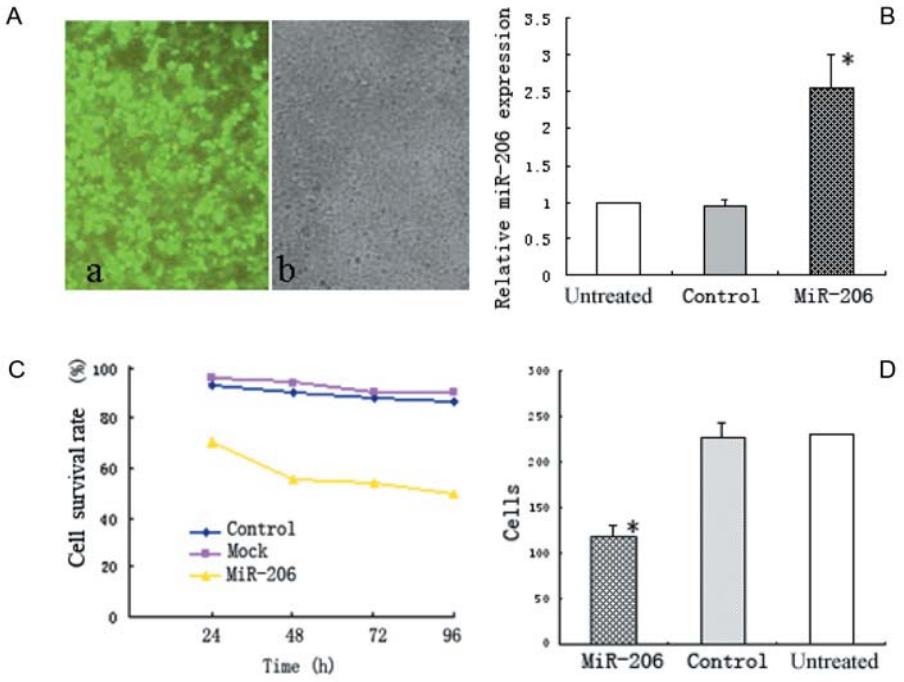

Figure 1. *A: Hep-2 cells 72 h after transfection with miR-206; a: light microscope (×100), b: fluorescence microscope (×100). B: miR-206 expression in Hep-2 cells. The relative expression level of miR-206 in Hep-2 cells 72 h after U6-hsa-mir-206 or control RNA oligonucleotides transfection as detected by real-time RT-PCR. *p<0.01. C: Cell survival after U6-hsa-mir-206 or control RNA oligonucleotides transfection D: Cell number on lower membrane surface 24 h after 1×10^5 cell added to upper chamber (mean of three). *p<0.01.*

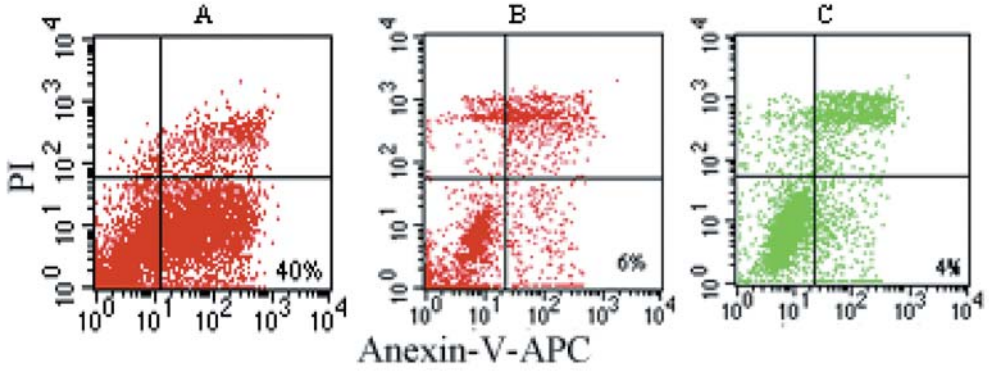

Figure 2. *Apoptosis of Hep-2 cells. The dot plot of the X-axis (FL4) of the log of Annexin V fluorescence and the Y-axis (FL2), which reflects the PI fluorescence, was obtained by flow cytometry. Representative plots of A: Hep-2 cells transfected with miR-206; B: Hep-2 cells transfected with control RNA oligonucleotides; C: Hep-2 cells without any treatment.*

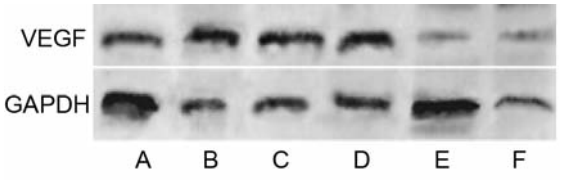

Figure 3. *VEGF expression in Hep-2 cells. VEGF protein detected by Western blot analysis. A and B: untreated control Hep-2 cells; C and D: Hep-2 cells transfected with control RNA oligonucleotides; E and F: Hep-2 cells transfected with miR-206.*

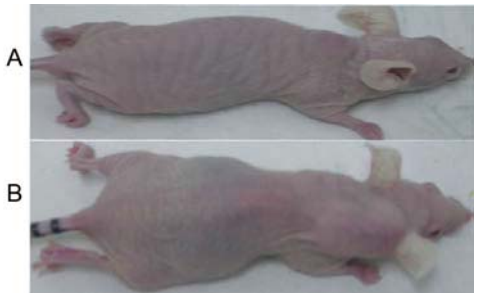

Figure 4. *Representative mice bearing tumors 4 weeks after injection with A: miR-206 transfected Hep-2 cells; B control normal Hep-2 cells.*

expression was associated with cyclin D1 amplification in breast cancer (12). Given that miRNAs may regulate multiple coding genes that are implicated in tumor proliferation and invasion, miR-206 is likely to regulate tumor invasion and proliferation of tumor by different pathways.

In the present study mir-206 transfection induced apoptosis of LSCC cell *in vitro*. Increasing miR-206 expression has also been shown to induce apoptosis in rhabdomyosarcomas (6), to induce apoptosis of cardiomyocytes through regulating Hsp60 expression (13) and to block the anti-apoptotic activity of Notch3 (14). Therefore, miR-206 has multiple functions in diverse neoplasms and may suppress tumor proliferation and induce apoptosis through multiple mechanisms.

In conclusion, the loss of miR-206 plays an important role in the proliferation, invasion and apoptosis of LSCC, effects that are possibly related to regulation of the VEGF pathway. MiR-206 may function as a novel tumor suppressor in LSCC, but more research is needed.

Conflict of Interest

We declare that we have no financial or personal relationships with other people or organizations that could 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 this report.

Acknowledgements

This study was supported by grants from the Natural Science Foundation of China (30540010), the Doctoral Fund of the Ministry of Education of China (20102307110007), the Postdoctoral Foundation (LRB06-294), the Youth Foundation (QC06C054) and the Scientific Research Foundation (1151hz029) of Heilongjiang Province, China.

References

1 Rudolph E, Dyckhoff G, Becher H, Dietz A and Ramroth H: Effects of tumour stage, comorbidity and therapy on survival of laryngeal cancer patients: a systematic review and a meta-analysis. Eur Arch Otorhinolaryngol *268*: 165-179, 2011.

2 Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*: 281-297, 2004.

3 Townley-Tilson WH, Callis TE and Wang D: MicroRNAs 1, 133, and 206: critical factors of skeletal and cardiac muscle development, function, and disease. Int J Biochem Cell Biol *42*: 1252-1255, 2010.

4 Gambardella S, Rinaldi F, Lepore SM, Viola A, Loro E, Angelini C, Vergani L, Novelli G and Botta A: Overexpression of microRNA-206 in the skeletal muscle from myotonic dystrophy type 1 patients. J Transl Med *8*: 48, 2010.

5 Wang X, Ling C, Bai Y and Zhao J: MicroRNA-206 is associated with invasion and metastasis of lung cancer. Anat Rec (Hoboken) *294*: 88-92, 2011.

6 Missiaglia E, Shepherd CJ, Patel S, Thway K, Pierron G, Pritchard-Jones K, Renard M, Sciot R, Rao P, Oberlin O, Delattre O and Shipley J: MicroRNA-206 expression levels correlate with clinical behaviour of rhabdomyosarcomas. Br J Cancer *102*: 1769-1777, 2010.

7 Miyachi M, Tsuchiya K, Yoshida H, Yaqyu S, Kikuchi K, Misawa A, Lehara T and Hosoi H: Circulating muscle-specific microRNA, miR-206, as a potential diagnostic marker for rhabdomyosarcoma. Biochem Biophys Res Commun *400*: 89-93, 2010.

8 O'Day E and Lal A: MicroRNAs and their target gene networks in breast cancer. Breast Cancer Res *12*: 201, 2010.

9 Kyzas PA, Cunha IW and Ioannidis JP: Prognostic significance of vascular endothelial growth factor immunohistochemical expression in head and neck squamous cell carcinoma: a meta-analysis. Clin Cancer Res *11*: 1434-1440, 2005.

10 Kapral M, Strzalka-Mrozik B, Kowalczyk M, Paluch J, Gola J, Gierek T and Weglarz L: Transcriptional activities of histone H3, cyclin D1 and claudin 7 encoding genes in laryngeal cancer. Eur Arch Otorhinolaryngol *268*: 709-714, 2011.

11 Xiao Y, Wang J, Lu J, Liu Y, Wang Y, Gao Y and Jin D: Down-regulation of cyclin D1 by small interfering RNA inhibits cell growth and induces apoptosis of laryngeal squamous cell carcinoma. Am J Otolaryngol 2011 Feb 10. [Epub ahead of print].

12 Ahnström Waltersson M, Karlsson E, Nordenskjöld B, Fornander T, Skoog L and Stål1 O: MiR-206 expression is down-regulated in cyclin D1 amplified breast tumours. Cancer Res *69*: 1127, 2009.

13 Shan ZX, Lin QX, Deng CY, Zhu JN, Mai LP, Liu JL, Fu YH, Liu XY, Li YX, Zhang YY, Lin SG and Yu XY: miR-1/miR-206 regulate Hsp60 expression contributing to glucose-mediated apoptosis in cardiomyocytes. FEBS Lett *584*: 3592-3600, 2010.

14 Song G, Zhang Y and Wang L: MicroRNA-206 targets notch3, activates apoptosis, and inhibits tumor cell migration and focus formation. J Biol Chem *284*: 31921-31927, 2009.

Received July 19, 2011
Revised September 14, 2011
Accepted September 15, 2011