Overexpressed miR-128a inhibits the proliferation of laryngeal cancer cells

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Background: To identify the expression of miR-128a in laryngeal tumor tissues and to clarify the function of this miR on the proliferation capability of laryngeal cancer cells.

Methods: Forty pairs of laryngeal tumor and normal tissue were collected and subjected to quantitative PCR analysis. MiR-128a was overexpressed using lentiviral transfection, and the function of miR-128a was evaluated by proliferation assay, colony formation and apoptosis assay. Tumorigenesis assay was performed *in vivo*.

Results: MiR-128a expression was lower in laryngeal tumor samples. MiR-128a overexpression cells exhibited reduced proliferation, presented enhanced apoptosis, while the reduced tumor formation was observed in miR-128a overexpression cells.

Conclusions: MiR-128a suppressed proliferative capability of laryngeal cancer cells and promoted apoptosis *in vitro* and *in vivo*.

Keywords: MiR-128a; proliferation; laryngeal cancer

Introduction

Laryngeal squamous cell carcinoma is one of the most malignant tumors of the head and neck, which originates from laryngeal epithelial tissue (1,2). Although significant advances have been achieved in the comprehensive treatment of head and neck cancer, numerous cases remain incurable; thus, it is crucial to pursue novel therapies with improved effects. A profound understanding of the underlying molecular mechanisms of laryngeal carcinoma will assist in the development of better strategies.

MicroRNAs (miRNAs or miRs) are newly recognized, noncoding, regulatory RNA molecules with a length of ~22 nucleotides, have been identified in all Metazoa studied thus far, and it is estimated that the human genome may include 800–1,000 miRNAs (3). Although miRNAs account for a minor fraction of the expressed genome, they are pivotal regulators of development and cellular processes, including cell differentiation, proliferation, morphogenesis, motility, survival and apoptosis (4). The molecules are known to control gene expression through association with the 3'-untranslated region of genes, as well as inhibit protein translation (5). In addition, miRNAs are able to destabilize and mediate the degradation of RNA transcripts (6). They also serve an important role in cancer by controlling the expression levels of certain tumor suppressor genes and oncogenes (7). MiRNA profiling has revealed the distinct expression signatures and important roles of these molecules

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Table 1 Clinicopathological characteristics of 40 patients with laryngeal squamous cell carcinoma

Characteristic	No. of cases
Age (years)	
≤50	6
>50	34
Sex	
Male	36
Female	4
Primary tumor	
T1–2	10
T3–4	30
Lymph node metastasis	
N0	28
N1–4	12
Location	
Supraglottic	22
Glottic	18
Histology	
SCC I–II	36
SCC II–III	4

SCC, squamous cell carcinoma.

in various types of human cancer, including glioma (8), lung cancer (9), gastric cancer (10) and prostate cancer (11). Previous studies (12-14) have reported the function of significant miRNAs in head and neck cancer, such as in tongue cancer, oral squamous cell carcinoma and laryngeal squamous cell carcinoma. Furthermore, it has been observed that miR-128 expression was reduced in prostate cancer cells (15), and suppressed prostate cancer by inhibiting BMI-1 and consequently inhibiting tumor-initiating cells (16). Based on our previous research, BMI-1 promotes the progression of laryngeal squamous cell carcinoma (17) and maintains the proliferation of laryngeal cancer stem cells (18), thus suggesting the tumor-suppressive function of miR-128 in laryngeal cancer. However, the role of miR-128 in this carcinoma requires further verification.

In the present study, the aim was to report for the first time the expression of miR-128a in laryngeal squamous cell carcinoma, as well as to investigate whether miR-128a affected the proliferation and apoptosis of laryngeal Hep2

cells following hsa-miRNA-mediated overexpression. By performing a spectrum of functional experiments, the current study provided substantial evidence that miR-128a serves a suppressive role in laryngeal Hep2 cells *in vitro* and *in vivo*.

Methods

Primary laryngeal squamous cell carcinoma specimens

In total, 40 pairs of primary laryngeal squamous cell carcinoma samples and adjacent normal tissue were obtained from patients who had provided informed consent, following the resection of laryngeal carcinoma. The tumor samples were collected under the guidance of pathologists and surgeons. All the margins of the specimens were negative, and the tumor samples used in the experiments of the present study were obtained from center of the specimens. All surgical procedures were required to ensure that the tumor margins remained intact. Adjacent normal tissues used as controls were obtained at least 1 cm away from the margins and clinically considered as normal, given the negative margins. All procedures were approved by the Ethics Boards at the Eye and ENT Hospital of Fudan University (Shanghai, China). The clinical and histological characteristics of the patients are presented in *Table 1*, using the TNM classification system.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

All the tissue samples were homogenized in TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and total RNA was extracted according to the protocol suggested by the manufacturer. The total RNA concentration was measured using spectrophotometry at OD260/280. RT was subsequently performed using 1 µg total RNA and M-MLV Reverse Transcriptase (Promega Corp., Madison, WI, USA). PCR amplification was conducted using *Taq* polymerase (Direct PCR kit, Sangon Biotech Co., Ltd., Shanghai, China), and the miRNA expression was evaluated using the Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). TaqMan probes for the hsa-miR-128a were purchased from Thermo Fisher Scientific, Inc., and RNU6B was used as an endogenous control to standardize the miRNA expression. Thermocycling took place at 95 ℃ for 2 min, followed by 40 cycles of 95 ℃ for 15 s and 55 ℃ for 1 min. All reactions were performed in triplicate. The $2^{-\Delta\Delta Ct}$ formula (19) was

used to calculate the miRNA level.

Cell culture

The laryngeal squamous cell carcinoma Hep2 cell line was purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cell line was cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator with 5% CO_2 at 37 ℃.

Vector construction and lentiviral transfection

The transfer vector pLenO-RIP was used to transfer target gene to lentiviruses, and hsa-miR-128 that mimics the mature miR-128a was synthesized. The pre-miRNA sequence was as follows: 5'-UGAGCUGUUGGAUU CGGGGCCGUAGCACUGUCUGAGAGGUUUACA UUUCUCACAGUGAACCGGUCUCUUUUUCAG CUGCUUC-3'. The synthesized pri-miRNA sequence was as follows: 5'-CGACGCGTGATTTTAGGTT TACAAAGCCCTAGCTGTTTTCTGTGTAGC TTTTATTATTCTTATGACTCTTGACAAGTTT GTAGCTTCACCATATACATTTAATATTTT CAATAATTGGCCTTGTTCCTGAGCTGTTGGA TTCGGGGCCGTAGCACTGTCTGAGAGG TTTACATTTCTCACAGTGAACCGGTCTCTT TTTCAGCTGCTTCCTGGCTTCTTTTTACTC AGGTTTCCACTGCTTTTTTGCTTTTTTTA ATGCTGTATGAAGGTGTTAACATTTGTTTA TATTTTTCATTAATTGTAATACCTTTAAAT CATGCATCATACTCAGAAATAGGGATTAGG CGGCCGCAA-3'.

*Not*I and *MIu*I cloning sites were used for enzyme digestion (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequent to processing enzyme digestion, the lentiviral vector and the target sequences were ligated together. The lentiviral plasmids were then extracted according to the protocol of the AxyPrep Plasmid Miniprep Kit (Axygen; Corning Incorporated, Corning, NY, USA). Next, the hsa-miR-128a lentiviral plasmid was transfected along with packaging plasmids into 293T cells (Gaining Biological, Shanghai, China), and cultured with DMEM with 10% FBS and 1% Glutamax, and then lentiviral titers were measured by flow cytometry following 293T cells transfection. The lentiviruses harboring hsa-miR-128a were then transfected into the laryngeal Hep2 cell line, and the transfection

efficiency was evaluated by flow cytometry. Since the transfected cells showing red fluorescence, flow cytometry can recognize the cells and count the percentage of the transfection. Wild-type Hep2 cells served as the control group, and cells transfected with non-targeting plasmids were termed the red fluorescent protein (RFP) group and served as a negative control. Cells transfected with targeting-plasmids were termed the hsa-miR-128a-RFP group. The miR-128a transcription level was confirmed by qPCR.

Cell proliferation assay

The evaluation of the cell proliferation capacity was performed using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. Cultured wild-type Hep2 cells, RFP group cells and hsa-miR-128a-RFP group cells were plated in 96-well plates in 0.2 mL RPMI 1640 with 10% FBS at a density of $2×10^3$ cells/well. Each group had six replicates. Proliferation assays were performed each day for 9 days through the addition of 20 μL CCK-8 dye to the wells and incubation for 2 h. The visible light absorbance at 450 nm was measured using a microplate reader (Bio-Rad model 680; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The cell proliferation capacity was calculated according to the measured optical density (OD), as follows: Cell proliferation (%) = [(test group OD value – culture medium OD value)/(wild-type control group OD value – culture medium OD value)] ×100.

Colony formation assay

Following transfection, the miR-128a overexpression, RFP control and wild-type Hep2 cells were trypsinized with 0.25% trypsin. Cells were seeded into 9.62 cm^2 (diameter, 3.5 cm) culture plates at a density of 250 cells per well. Next, 1 mL RPMI 1640 medium with 10% FBS was added to the plates to allow for colony formation and incubated at 37 ℃ for 7 days. The cultures were then stained with Toluidine blue (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), and the colony formation rate was calculated as follows: Colony formation (%) = number of colonies/number of transplanted cells.

Apoptosis assay

An apoptosis assay was performed to assess the apoptosis capacity of hsa-miR-128a-RFP and RFP cells. Briefly, cells

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were centrifuged at 200 ×g for 10 min in 4 ℃ and suspended in 0.01 M PBS. For flow cytometry analysis, the suspended cells were collected and resuspended in 1× Annexin V Binding Buffer at a concentration of 1×10^6 cells/mL. A total of 100 µL solution was transferred to a 5 mL culture tube, and stained with 5 µL FITC-Annexin V and 5 µL PI (FITC Annexin V Apoptosis Detection kit I; BD Pharmingen; BD Biosciences, Franklin Lakes, NJ, USA). The solution was cultured for 15 min at room temperature, and then apoptosis was analyzed by a flow cytometry (Beckman Coulter, Inc., Brea, CA, USA). For terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis, 2×10^6 cells/mL cells were suspended in 200 µL PBS and then stained with 50 µL TUNEL reaction mixture (In Situ Cell Death Detection kit, Fluorescein; Roche Diagnostics, Basel, Switzerland). DAPI was used to counterstain the nuclei, and the apoptotic cells were observed under a fluorescence microscope (Leica DM IRB; Leica Microsystems GmbH, Wetzlar, Germany).

In vivo tumorigenicity assay in mice

An *in vivo* tumorigenicity assay was also performed in the present study using 10 male non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice with an age of 6–8 weeks, which were obtained from Shanghai SLAC Laboratory Animals (Shanghai, China). The NOD/SCID mice were kept in a specific pathogen-free animal center at 24 ℃ in a humidity of 50%. The weight of the mice ranged from 18–26 g. RFP and hsa-miR-128a cells were suspended in a volume of 200 µL RPMI 1640 medium. Next, the cells were collected and a concentration of 1×10^5 cells was injected into the subcutaneous space of the axillary fossa of NOD/SCID mice under anesthesia with ketamine/xylazine. A total of 10 mice were injected with RFP cells and 10 were injected with hsa-miR128a cells. During a period of 4 weeks, the mice were examined every 3 days for tumor formation by palpation. Subsequently, all mice were sacrificed by cervical dislocation and tumors were measured in two dimensions with calipers and weighted. The tumor volumes were calculated according to the formula described by Chinnaiyan *et al.* (20), as follows: Volume = length × width^2 × π/6 The experimental protocols were processed according to the guidelines of Shanghai Medical Experimental Animal Care Commission (Shanghai, China).

Statistical analysis

All the results are presented as the mean ± standard deviation of at least three independent experiments. Statistically significant differences were evaluated by independent-samples t-test, analysis of variance and Kruskal-Wallis test. Stata version 12.0 (StataCorp LP, College Station, TX, USA) and Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA, USA) were used for data processing and statistical comparison. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Low expression of miR-128a is observed in laryngeal squamous cell carcinoma

Although miR-128a has been reported to be associated with multiple types of cancer (6-9), it has not been investigated in laryngeal squamous cell carcinoma, to the best of our knowledge. Thus, the present study attempted to determine the association between miR-128a and laryngeal squamous cell carcinoma. Primary tumors were dissected from 40 patients immediately following a laryngectomy. The clinical and histological characteristics of the included patients are presented in *Table 1*.

To quantitatively determine the expression of miR-128a in laryngeal squamous cell carcinoma, qPCR was performed on primary tumor specimens and adjacent normal tissues. The results revealed that significantly lower miR-128a expression was observed in laryngeal tumors in comparison with that in normal tissues (*Figure 1A*). Subsequently, miR-128a expression in different tumor stages and locations was compared. As shown in *Figure 1B*, the expression of miR-128a was significantly higher in stage 1 and 2 tumor specimens as compared with that in stage 3 and 4 tumors (P<0.05). Regarding the tumor location, there was no evident difference in miR-128a expression between the supraglottic and glottic tumors (*Figure 1C*).

MiR-128a overexpression by lentiviral plasmids

In order to determine the biological role of miR-128a in laryngeal cancer, a stable hsa-miR-128a overexpression cell line was established. Lentiviruses harboring hsa-miR-128a were constructed and transfected into laryngeal Hep2 cell line, while a non-targeting negative control RFP cell line was also established, with wild-type Hep2 cells considered as the control. All the transfected cells exhibited red fluorescence, and the transfection rate was evaluated
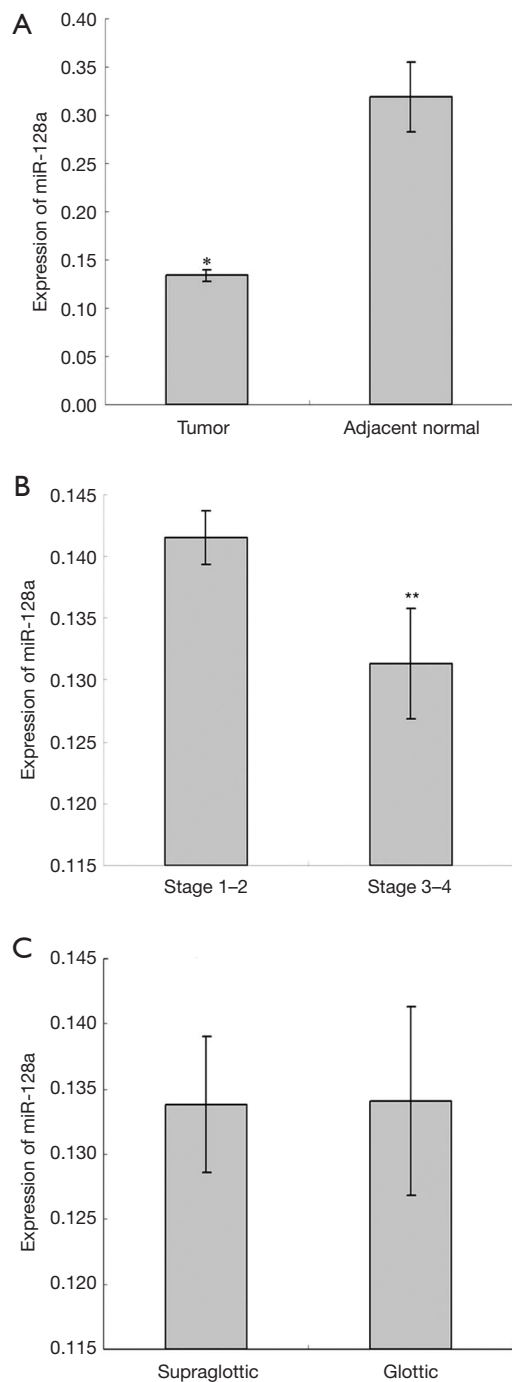
Figure 1 MiR-128a expression in laryngeal cancer tumors. (A) The expression of miR-128a in 40 pairs of laryngeal squamous cell carcinoma specimens and adjacent normal tissues was examined by quantitative polymerase chain reaction (n=40); (B) miR-128a expression in stage 1/2 (n=10) and stage 3/4 (n=30) tumors; (C) comparison of miR-128a expression in 22 supraglottic and 18 glottic tumor samples. *, P<0.05 *vs.* adjacent normal tissue and **, P<0.05 *vs.* stage 1/2 tumors. miR, microRNA.

by flow cytometry, as shown in *Figure 2A*. The RFP cells transfected with non-targeting plasmids were observed to have a transfection rate of 80.92%, while the hsa-miR-128a-RFP cells transfected with target plasmids had a transfection rate of 78.84%. The miR-128a transcription level was confirmed by qPCR and the expression of miR-128a in the hsa-miR-128a cells was markedly higher as compared with that in the negative control RFP cells (*Figure 2B*).

MiR-128a overexpression suppresses cell proliferation in vitro

To evaluate the proliferation capacity of miR-128a, Hep2 and hsa-miR-128a cells were cultured in 96-well plates, and the absorbance of the individual cells was measured by a CCK-8 assay on days 1, 3, 5, 7 and 9. As shown in *Figure 3* and *Table 2*, the proliferation capacity of RFP cells was evidently stronger in comparison with that in hsa-miR-128a cells. The negative control cells presented an increasing trend in their proliferation capacity with each day of incubation, while the growth of hsa-miR-128a cells was gradually decreasing. On day 1, there was no significant difference in the proliferation rate between the two groups; however, from day 3, the difference was statistically significant (P<0.05).

MiR-128a overexpression inhibits laryngeal cancer cell colony formation in vitro

In order to confirm the results of the proliferation assay and to establish a more profound understanding of the effect of miR-128a overexpression, a colony formation assay was performed. Following transfection, hsa-miR-128a and negative control RFP cells were incubated 7 days and then stained with Toluidine blue to examine their colony formation ability. As shown in *Figure 4A*, increased blue staining of colony spheres was observed in the culture of RFP cells. However, in the culture of hsa-miR-128a cells, colony sphere formation was rare, which was consistent with the results of the proliferation assay. In *Table 3*, the colony formation capability of the two groups is compared at a quantitative level. The number of colonies formed/cm^2 of RFP cells was 13.96±0.83, whereas this value was 7.00±0.67 in the hsa-miR-128a cells, with a significant difference observed (P<0.05; *Figure 4B*). The percentage of colonies formed compared with the free cells (colony %) further confirmed the results of previous assays. The colony % of RFP cells was 54.66±0.94, while that of hsa-miR-128a cells was 24.98±1.01, revealing a significantly weaker colony

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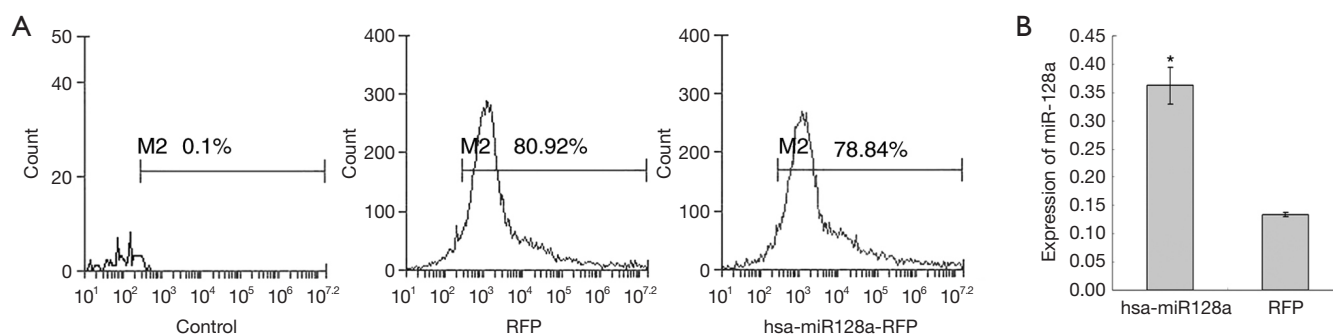

Figure 2 MiR-128a was overexpressed by hsa-miR-128a lentiviral vector transfection, and a stable hsa-miR-128a cell line was established. (A) The transfection rate was measured by flow cytometry in control (rate, 0.1%), negative control RFP (rate, 80.92%) and hsa-miR-128a-RFP (rate, 78.84%) cells; (B) miR-128a transcription level was confirmed by quantitative polymerase chain reaction, and the results revealed that the expression of miR-128a was significantly higher in hsa-miR-128a cells (n=3). *, P<0.05 *vs.* RFP group. miR, microRNA; RFP, red fluorescent protein.
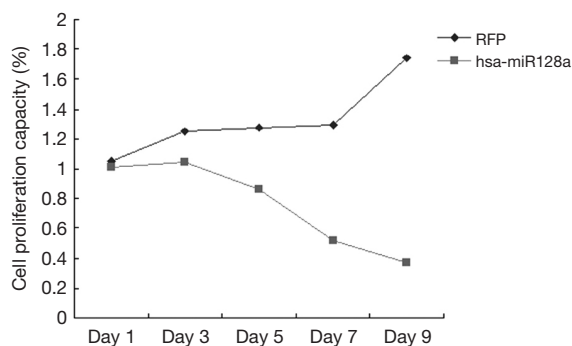

Figure 3 MiR-128a upregulation suppressed the proliferation of laryngeal Hep2 cells. A proliferation assay was performed in hsa-miR-128a and RFP cells by the cell counting kit-8 method. The absorbance was measured on days 1, 3, 5, 7 and 9 to determine the cell proliferation capacity. miR, microRNA; RFP, red fluorescent protein.

formation ability in cells with miR-128a overexpression (P<0.05; *Figure 4C*).

MiR-128a overexpression promotes laryngeal cancer cell apoptosis

As observed in the earlier proliferation assay, miR-128a was capable of inhibiting the growth of laryngeal Hep2 cells. Thus, the study further attempted to examine whether a correlation exists between proliferation and apoptosis in hsa-miR-128a cells by performing apoptosis assays by flow cytometry and TUNEL staining. As observed in the flow cytometry results shown in *Figure 5A,B*, the percentage of apoptotic cells in the hsa-miR-128a group was 38.73%±0.73%, while the RFP cells demonstrated

Table 2 Cell proliferation capacity of RFP and hsa-miR-128a cells

Day	RFP	hsa-miR-128a	P value
1	1.056±0.121	1.005±0.069	0.392
3	1.248±0.024	1.046±0.172	0.034
5	1.276±0.179	0.866±0.129	0.001
7	1.289±0.037	0.516±0.097	<0.001
9	1.738±0.144	0.374±0.043	<0.001

RFP, red fluorescent protein.

a significantly reduced apoptosis rate at 4.81%±0.25% (P<0.05). As shown in *Figure 5C*, the hsa-miR-128a and RFP cells were stained by TUNEL and DAPI, and then observed under a fluorescence microscope. It was observed that hsa-miR-128a cells exhibited enhanced apoptosis as compared with the RFP cells.

Overexpression of miR-128a inhibits transplanted tumor growth in vivo

To evaluate whether overexpression of miR-128a is able to inhibit the tumorigenic capacity of Hep2 cells *in vivo*, RFP and hsa-miR-128a cells were injected into the subcutaneous space of the axillary fossa of mice, and tumor formation was examined after 4 weeks. A significant reduction in the tumor volume was observed in the miR-128a overexpression group compared with that in the RFP negative control group (P<0.05), as shown in *Figure 6A*. The volume of tumors in the RFP group was 0.83±0.45 cm³, while that in the hsa-miR-128a group was 0.015±0.009 cm³. Furthermore, as presented
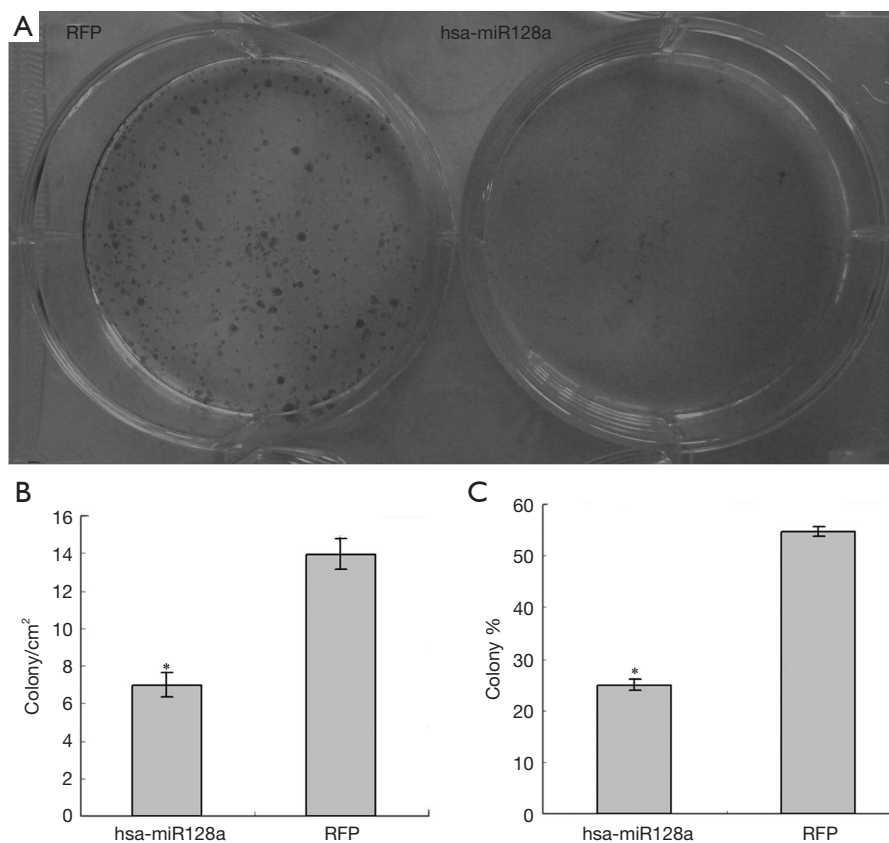
Figure 4 MiR-128a overexpression inhibited the colony formation ability of laryngeal Hep2 cells. (A) A colony formation assay was performed in hsa-miR-128a and RFP cells. Cells were allowed to proliferate for 7 days, and then colonies were stained with Toluidine blue; (B) The percentage of the colony area (colony/cm^2) formed by the hsa-miR-128a and RFP cells was calculated, and the results are expressed as the mean ± standard deviation (n=3); (C) The colony formation percentage of hsa-miR-128a (24.98%±1.01%) and RFP cells (54.66%±0.94%) is shown (n=3). *, P<0.05 *vs*. RFP group. miR, microRNA; RFP, red fluorescent protein.

Table 3 Colony formation assay in RFP and hsa-miR-128a cells

Type	hsa-miR-128a	RFP	P value
Cells (mL)	269.33±19.50	245.67±10.69	0.1391
Cells (cm^2)	28.00±2.00	25.67±1.53	0.1836
Colonies (plate)	67.33±6.43	134.33±8.02	0.0004
Colony (cm^2)	7.00±0.67	13.96±0.83	0.0003
Colony (%)	24.98±1.01	54.66±0.94	<0.001

RFP, red fluorescent protein.

in *Figure 6B*, a significant decrease in the tumor weight was also confirmed in the hsa-miR-128a group as compared with the RFP group (P<0.05). The tumor weight of the RFP group tumors was 88.00±8.92 mg, while the weight in the hsa-miR-128a group tumors was 10.70±2.75 mg.

Therefore, overexpression of miR-128a was able to suppress the tumorigenic ability of Hep2 cells *in vivo*.

Discussion

As one of the most common and severe malignancies of the head and neck, laryngeal squamous cell carcinoma remains incurable at advanced stages; however, promising comprehensive treatment strategies are being developed. With advances in the understanding of pathogenesis at the DNA level, more profound investigations at the miRNA level should be conducted to obtain novel insight that will assist in the disease treatment. miRNAs have been reported to function as tumor suppressors and oncogenes, and are also referred to as oncomiRs (7). Abnormalities in miRNA expression are considered to serve significant roles
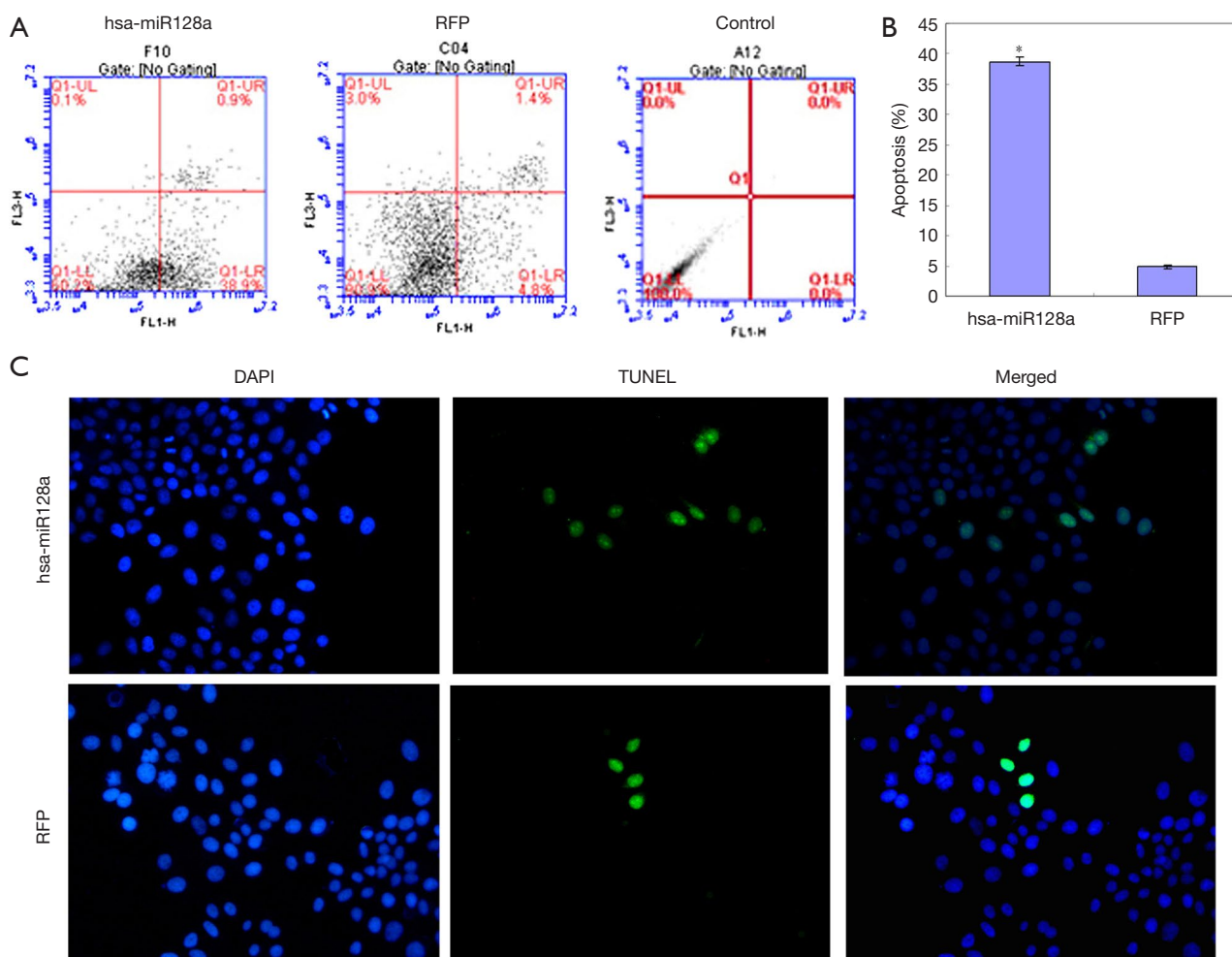
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Figure 5 MiR-128a overexpression induced the apoptosis of laryngeal Hep2 cells. (A) Representative flow cytometry results and (B) apoptosis rate (%) are shown. The apoptosis rate of hsa-miR-128a cells was 38.73%±0.73% and that of RFP cells was 4.81%±0.25% (n=3); (C) cells were stained by TUNEL and observed under a fluorescence microscope (magnification, ×400). Apoptotic cells were stained green (TUNEL), while the nuclei were stained blue (DAPI). *, P<0.05 *vs*. RFP group. miR, microRNA; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

in carcinogenesis, which is supported by investigations on the expression of miRNAs in clinical specimens (21,22). For instance, previous studies revealed that let-7 was downregulated in lung tumors (23) as compared with its expression in normal tissue, and miR-127 and miR-128 were downregulated in human bladder cancer (24) and miR-128 in glioma (8), respectively. However, the expression and function of miR-128a remains unclear in laryngeal squamous cell carcinoma. To the best of our knowledge, the present study demonstrated for the first time that miR-128a is downregulated in primary laryngeal tumors, and that overexpression of endogenous miR-128a suppresses the cell

proliferation and promotes apoptosis. Therefore, targeting miR-128a may be a novel method to promote the treatment of laryngeal squamous cell carcinoma.

In the present study, 40 primary laryngeal squamous cell carcinoma specimens along with 40 adjacent normal epithelial tissue samples were collected. The majority of the included patients were males with an age of >50 years, with an advanced stage of laryngeal cancer and tumors mainly located in the supraglottic region, without lymph node metastasis. qPCR indicated that miR-128a exhibited a significantly lower expression in the tumor samples as compared with the adjacent normal tissue, which is
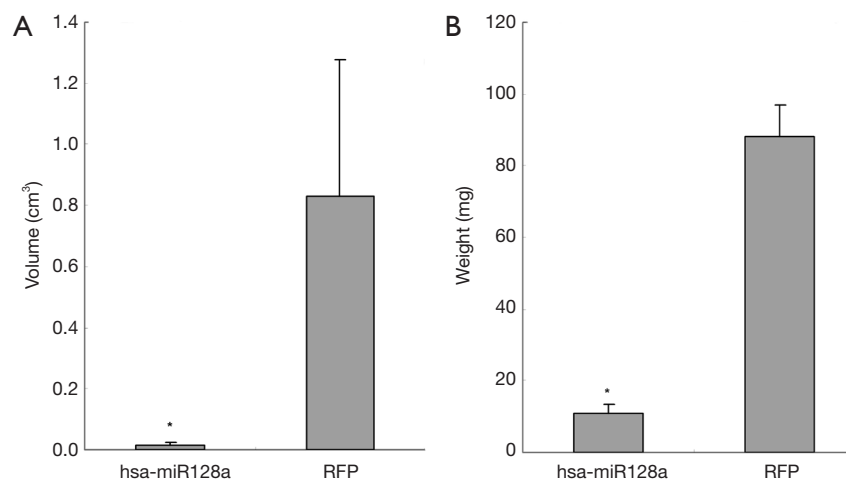
Figure 6 MiR-128a overexpression suppressed the tumor formation *in vivo*. hsa-miR-128a and RFP cells were injected into the axillary fossa of mice, and the tumor (A) volume and (B) weight were measured after 4 weeks. The tumor volume in the hsa-miR-128a group was 0.02 ± 0.01 cm^3, and in the RFP group was 0.83 ± 0.45 cm^3. (n=10). The tumor weight in the hsa-miR-128a group was 10.70 ± 2.75 mg and in the RFP group was 88.00 ± 8.92 mg (n=10). *P<0.05 *vs.* RFP group.

consistent with previous findings on the expression of this miRNA in glioma tissues by Ciafrè *et al.* (8) and Zhang *et al.* (25). However, according to the study by Lu *et al.* (26), miR-128a was upregulated in oral cancer cell lines, which may be caused by the primary tumor location difference. Further research on the signal pathways of the miR-128a is required to better understand this observation. The current study also observed that the expression of miR-128a in stage 1 and 2 tumors was significantly higher in comparison with that in stage 3 and 4 tumors; however, there was no statistically significant variation between the supraglottic and glottic tumors. These results indicated that the expression of miR-128a may be associated with the survival of the laryngeal cancer patients, although further investigations are required to confirm this.

A cell proliferation assay was also performed in the current study to investigate whether the overexpression of miR-128a affected the growth of Hep2 cells *in vitro*. Using the CCK-8 method, it was observed that the proliferation of hsa-miR-128a cells presented a declining trend, while the proliferation of negative control RFP cells appeared to increase each day. This result indicated that hsa-miR-128a transfection not only inhibited the proliferation of Hep2 cells, but also promoted the apoptosis. In the study by Godlewski *et al.* (27), it was observed that a short hairpin oligonucleotide mimic of pre-miR-128-1 led to high levels of mature miR-128 expression, and significantly reduced the proliferation of U87 and U251 glioma cell lines as compared

with a control oligonucleotide. Furthermore, other previous studies (25,28) revealed that miR-128 presented the capacity to suppress the proliferation of cancer cells. The findings of the present study are consistent with the aforementioned previous observations, in particular regarding the cell growth. In order to confirm the results of the proliferation assay, a colony formation assay was also performed in the current study. The colony/cm^2 and colony % of the hsa-miR-128a cells were markedly reduced in comparison with the RFP cells, which also confirmed the observations of the proliferation assay. Apoptosis assays, including Annexin V and TUNEL staining, were subsequently conducted to investigate whether hsa-miR-128a was able to promote apoptosis. It was demonstrated that the apoptosis rate of hsa-miR-128a cells was almost 10-fold greater compared with that of the RFP cells, which indicated that miR-128a not only affected the pathway of proliferation and apoptosis, but also that there may be a crosslink between the two pathways. These findings are novel, and no relevant previous reports exist on this function of miR-128a. In subsequent studies, the signaling pathways of proliferation and apoptosis will be further investigated.

In order to prove the function of miR-128a on the proliferation of laryngeal cancer cells, a tumorigenicity assay was also conducted in the present study. A total of 10 male NOD/SCID mice (6–8 weeks old) were injected with RFP and miR-128a overexpression cells at the subcutaneous space of their axillary fossa under anesthesia. Mice were

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examined every 3 days for tumor formation by palpation for 4 weeks after injection. After sacrifice, the tumor volume of the RFP group tumors was markedly larger in comparison with that of the hsa-miR-128a group tumors. It was also revealed that the weight of tumors in the hsa-miR-128a group was significantly lower compared with that in the RFP group. According to the study by Jin *et al.* (16), miR-128 overexpression inhibited Du145 tumor growth and reduced the tumor incidence rate, providing the first *in vivo* evidence that miR-128 possesses prostate cancer-suppressive effects. The present study results were consistent with these previous observations, further suggesting that miR-128a suppressed the tumor growth of laryngeal squamous cell carcinoma.

In conclusion, the current study reported for the first time that miR-128a is downregulated in laryngeal squamous cell carcinoma. In addition, it was demonstrated that overexpression of miR-128a inhibited the proliferation capacity and promoted the apoptosis of laryngeal Hep2 cells. Furthermore, upregulation of miR-128a suppressed the tumor formation *in vivo*. Taken together, these results suggest that upregulation of miR-128a is a novel possible target for laryngeal cancer treatment.

Acknowledgments

Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2018.06.13). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Informed consent was obtained from the patients. All procedures were approved by the Ethics Boards at the Eye and ENT Hospital of Fudan University (Shanghai, China).

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