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Original Article

miR-18a downregulates DICER1 and promotes proliferation and metastasis of nasopharyngeal carcinoma

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Abstract: Nasopharyngeal carcinoma, common in Southeast Asia and the southern provinces of China, often has metastasized by the time of diagnosis; thus there exists the need for improved diagnosis and treatment. Accumulating evidence indicates that microRNAs (miRNAs), which post-transcriptionally regulate protein expression, contribute to the processes of tumorigenesis, including metastasis and cellular invasion. Here, we studied the effect of one miRNA, miR-18a—which is believed to target the miRNA-processing enzyme DICERI—on nasopharyngeal carcinoma. *In situ* hybridization revealed that miR-18a was more highly expressed in nasopharyngeal carcinoma tissues than in control tissues ($P < 0.05$), and the overexpression correlated with clinical stage and lymph node metastasis ($P < 0.05$), but not with age and gender ($P > 0.05$). *In vitro* analysis of HK1 nasopharyngeal carcinoma cells transfected with miR-18a exhibited significantly decreased expression of DICER1 mRNA and protein but significantly increased proliferation and invasion properties compared to control cells ($P < 0.05$). Finally, nude mice injected with miR-18a transfected-HK1 cells displayed significantly increased tumor growth and lung metastasis *in vivo* ($P < 0.05$). These findings suggest that miR-18a expression can promote proliferation and metastasis of nasopharyngeal carcinoma cells and that these activities may occur through its regulation of DICER1.

Keywords: Nasopharyngeal carcinoma, HK1 cells, microRNA-18a, Dicer1, proliferation, metastasis

Introduction

Nasopharyngeal carcinoma is one of the most common cancers in Southeast Asia and the southern provinces of China, but is rare in northern China, Europe, and the United States [1, 2]. The occurrence, development, and metastasis of nasopharyngeal carcinoma are complex multi-factorial processes that lead to variation in the manifestation of disease as successive magnitude changes [3, 4]. More than 70% of newly diagnosed cases have metastasized to cervical lymph nodes and ~20-35% of these cases have distant metastases; therefore, it is important to investigate mechanisms of invasion and metastasis during nasopharyngeal carcinoma treatment [5].

microRNAs (miRNAs) are small non-coding RNAs that are about 20-22 bp in length. miRNAs undergo several intracellular processing steps by RNase enzymes such as DICER1. Once mature, miRNAs post-transcriptionally regulate protein expression by guiding silencing complexes to degrade mRNA or inhibit mRNA translation [6-8]. Much miRNA research has focused on the function and expression of tumor-related miRNAs [9]; recent studies show that miRNA expression is associated with a variety of cancers and plays a vital role in tumorigenesis [10, 11]. Further, miRNA regulation is associated with each hallmark of tumorigenesis, including sustained cell proliferation, escape of growth inhibition, resistance to cell death, invasion and metastasis, angiogenesis, and immortalization [12-15]. By using a stem-loop real-time-PCR method, 35 miRNAs were identified for significant alteration in nasopharyngeal carcinoma (NPC) samples; several known oncogenic miRNAs, the miR-17-92 cluster and miR-155, were among the miRNAs upregulated in NPC, and tumor-suppressive miRNAs, including miR-34

family, miR-143, and miR-145, were significantly downregulated in NPC [16].

c-Myc and the miR-17-92 family are overexpressed in lymphoma cells characterized by stronger proliferation and lower cell death than cells that overexpress only c-Myc [17]. Many additional studies also suggest that the miR-17-92 family contributes to cellular proliferation to induce tumorigenesis. The miR-17-92 family consists of seven miRNAs, including of miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92a. This family has an established role in tumorigenesis; however, the specific roles of individual miRNAs within the family, such as miR-18a, remain largely unknown. Other miRNAs, such as miR-103/107, can downregulate DICER1 expression to reduce overall miRNA synthesis and promote cellular invasion and metastasis [18]. Bioinformatics analysis suggests that DICERl is a target of miR-18a [19], but biological studies have not yet confirmed the *in silico* findings.

To examine the potential role of miR-18a in tumorigenesis, we assessed miR-18a expression in clinical nasopharyngeal carcinoma samples *in situ* and analyzed the relationship between expression and clinicopathological features. Further, we directly tested the effect of miR-18a on nasopharyngeal carcinoma cell lines and the relationship between miR-18a and DICERl expression *in vitro*. Finally, we used nude mice to test the ability of miR-18a to mediate cell proliferation and metastasis of nasopharyngeal carcinoma cells *in vivo*.

Materials and methods

Clinical data

Archived paraffin blocks of nasopharyngeal biopsy specimens were collected from our hospital the First Affiliated Hospital, Nanjing Medical University (Nanjing, China) between January 2010 and December 2012. All 38 cases were pathologically confirmed as nasopharyngeal carcinoma. Cases included 30 males and 8 females aged 22 to 72 years, with a mean age of 50.6 ± 10.2 years. According to Fuzhou staging criteria for nasopharyngeal carcinoma, there were 11 stage I + II and 27 stage III + IV cases; 7 cases were negative and 31 cases were positive for lymph node metastasis. All pathological specimens were obtained before radiotherapy and chemotherapy. Tissues from 26 cases of chronic nasopharyngeal mucosa inflammation were also collected as controls. All specimens were fixed in 10% neutral formalin and embedded in paraffin within 3 days.

In situ hybridization

Paraffin sections were dewaxed in water, incubated in 3% H_2O_2 for 5-10 min to inactivate endogenous enzymes, and then washed thrice in DEPC-treated ultrapure water for 1 min each. Tissue sections were digested in pepsase (1 mL) diluted with 3% citric acid for 20 min at 37°C and washed thrice in PBS for 5 min each and once in DEPC-treated ultrapure water for 1 min. Tissue sections were fixed in 1% paraformaldehyde/0.1 M PBS pH 7.2-7.6 at room temperature for l0 min and washed with DEPC-treated ultrapure water thrice for 1 min each. Tissue sections were placed in a humidified hybridization chamber (Wuhan Boster, Wuhan, China) containing 20 mL 20% glycerol and prehybridized with 20 μL at 59°C for 2-4 h. Tissue sections were then hybridized with 20 μL at 59°C overnight and washed with 2X SSC (saline sodium citrate) twice for 5 min, 0.5X SSC for 15 min, and 0.2X SSC for 15 min, all at 37°C. Blocking serum was applied for 30 min at 37°C, then excess liquid was shaken off. Biotinylated rat anti-digoxin was dropped onto tissue sections, incubated at room temperature for 2 h, washed with PBST (Phosphate Buffered Saline with Tween-20) four times for 5 min then each. One drop of streptavidin-biotin-peroxidase complex (SABC) was added for 30 min at room temperature and then washed with PBST thrice for 5 min each. One drop of chromogenic agents A, B, and C, respectively, were mixed into 1 mL of distilled water and added to tissue sections. Sections were washed with water, counterstained with hematoxylin, and again washed with water. Tissues were dehydrated in alcohol, vitrified with dimethylbenzene, and sealed with neutral gum. Slides were analyzed semi-quantitatively according to both staining intensity and distribution. Scores were given for the proportion of positive-stained cells as follows: 1 = -; 2 = +; 3 = ++; 4 = +++. Scores were given for the number of positive cells as follows: 1 = 0-25%; 2 = 26-50%; 3 = 51-75%; 4 = 76-100%. The two scores were multiplied together; a product less than eight was considered low expression,

while greater than or equal to eight was considered high expression.

Cell culture

The HK1 nasopharyngeal carcinoma cell line (Cell Bank of Chinese Academy of Sciences, Shanghai, China) was grown in RPMI-1640 (Gibco Company, USA) culture medium containing 10% fetal bovine serum (Hangzhou Evergreen Company, Hangzhou, China) and cultured at 37°C with 5% CO_2. The medium was changed and cells were subcultured with 0.25% trypsin every 2 to 3 days. Cells were in logarithmic growth before each experiment.

For transfection, HK1 cells were cultured in 24-well plates at 5 × 10^4 cells/well for 24 h. Plated cells were then transfected with miR-18a mimetic or negative control oligonucleotides at a final concentration of 200 nM using Oligofectamine™ transfection reagent as directed by the manufacturer (Invitrogen, USA). Mimetic oligonucleotides were synthetized (TaKaRa, Shiga, Japan) as follows: miR-18a, 5'-UAAGGUGCAUCUAGUGCAGAUAG-3'; negative control, 5'-AUCUGCACA UGAUGCACCUUAUU-3'. After 4 h, media was added to each well and cells were cultured for 48 h. Non-transfected and mock-transfected controls were included and for all groups, three duplicate wells were analyzed for each group.

RT-PCR

Primers for *MIR18a* mRNA analysis by RT-PCR were synthesized (Shanghai Bioengineering, China) as follows: upstream (F), 5'-GCAGTGAAGGCACTTGTA GC-3'; downstream (R), 5'-TGCTTGGCTTGAATTATTGG-3'. Total RNA was extracted by one-step Trizol (Invitrogen, USA) and was reverse transcribed to cDNA. The cDNA was used as a template for PCR with miR-18aF and miR-18aR primers. miR-18a was amplified at 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 1 min, 72°C for 20 s; and 72°C for 10 min on a PE cycler.

Primers for *DICER1* mRNA analysis by RT-PCR were synthesized (Shanghai Bioengineering, China) as follows: upstream (F), 5'-AAGGAAGCTGGCAAACA AGA-3'; downstream (R), 5'-AAAACGAACCACCAAGTTGC-3'. Total RNA was extracted and cDNA generated as above. *DICER1* was amplified at 95°C for 2 min; 28 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 20 s; and 72°C for 10 min.

Western blotting

Total protein was collected from HK1 cells with cell lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 100 mg/L PMSF), and protein concentration was quantified with BCA (Bicinchoninic acid) protein assay reagent (Bio-Rad, USA). Protein samples (30 μg) were separated by electrophoresis and Western blotted with anti-DICER1 (Santa Cruz, USA) at 1:1000 and anti-β-actin (Sigma, USA) as a loading control. Proteins were visualized with an HRP-labeled goat anti-mouse IgG secondary antibody (R&D Systems, Inc) at 1:2000 and enhanced chemiluminescence (ECL) (GE, USA).

MTT assay

HK1 cells were cultured to 75%-80% confluence and were washed twice with D-Hanks solution (Shanghai Gongshuo Biotechnology Company, Shanghai, China). Cells were digested with trypsin and counted, then inoculated in 6-8 wells of a 96-well plate at 2.5-5.0 × 10^3 cells/well. Each day for five consecutive days, 20 μL of 5 mg/mL MTT solution (Sigma, USA) were added into each well. After 4 hours, medium was removed from each well and 150 μL dimethyl sulfoxide (Nanjing Kezheng Chemical Company, Nanjing, China) were added. Plates were shaken on a table concentrator at low speed for 10 min to dissolve all crystals. A microplate reader (ELx, Biotek, USA) was used to measure OD490 absorbance values of each well. To standardize the experimental data, OD490 values were divided by the OD490 value of the first day to calculate increase rates.

Transwell migration assay

Matrigel (Becton, Dickinson and Company, USA) was prepared at a 1:1 ratio in serum-free RPMI-1640 medium. Transwell chambers (Coming, USA) were clamped with forceps and 25 μL of diluted Matrigel were added evenly to each well. Transwell chambers were placed into a 24-well plate and dried at 37°C for 2 h. Cell suspensions were prepared at 1 × 10^5 cells/mL in media containing 2% fetal calf serum. Media containing 15% fetal calf serum was added to the lower layer of the transwell chamber, and

Table 1. miR-18a expression and clinicopathological parameters in nasopharyngeal carcinoma

Parameters	*n*	miR-18a low-expression *n* (%)	miR-18a high-expression *n* (%)	χ^2	*P*
Gender					
Male	30	9 (30.0)	21 (70.0)	0.077	0.782
Female	8	2 (25.0)	6 (75.0)		
Age (years)					
< 50	15	5 (33.3)	10 (66.7)	0.232	0.630
≥ 50	23	6 (26.1)	17 (73.9)		
Clinical stage					
I + II	11	6 (54.5)	5 (45.5)	4.932	0.026
III + IV	27	5 (18.5)	22 (81.5)		
Lymph node metastasis					
No	7	5 (71.4)	2 (28.6)	7.529	0.006
Yes	31	6 (19.4)	25 (80.6)		

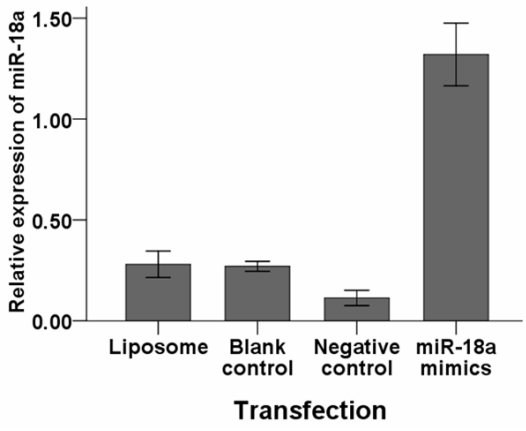

Figure 1. miR-18 expression in transfected HK1 cells. RT-PCR measured miR-18a mRNA expression in HK1 cells mock-transfected with liposome, blank control, transfected with negative control oligonucleotides, or transfected with miR-18a mimetic oligonucleotides ($n = 3$). $P < 0.05$, $F = 748.493$.

200 μL of the cell suspension were added to each transwell chamber and cultured for 48-72 hours. Cells in each chamber were fixed in 10% formalin for 10 min and washed with DEPC-treated ultrapure water three times. Cells were counterstained with crystal violet for 2 min, washed with DEPC-treated ultrapure water, and excess crystal violet was removed with cotton swabs. Cells were photographed and counted by inverted microscopy (Olympus, Japan) and mean values were calculated. Experimental results presented are in triplicate.

Mice

Twenty male SPF-grade BALB/C nude mice (Animal Center, Nanjing Medical University, Nanjing, China) at 4-5 weeks of age (17-18 g in weight) were used for these studies. Mice were given access to sterile water and feed (Animal Center, Nanjing Medical University, Nanjing, China) and all other materials in contact with mice were sterilized. Mice were randomly divided into four groups of five mice each. HK1 cells were intracutaneously injected into the oxter or intravenously injected at 1×10^7 cells per mouse. After injection, subcutaneous tumor growth was assessed by measuring the long tumor diameter and short tumor diameter and tumor volumes were calculated according to this formula: $V = a \times b^2/2$. Mice were euthanized 30 days after injection and tumors were isolated, weighed, and recorded. Lung tissues were also collected, fixed in 10% formalin, and embedded in paraffin. Tissue sections were stained with H&E and analyzed for the size and number of metastases.

Statistical analysis

Data were processed with SPSS17.0 software (IBM, NY, USA) and expressed as mean ± standard deviation ($\bar{x}\pm s$). χ^2 test was used to compare miR-18 expression in tissues. Means between multiple groups were compared with one-way ANOVA and pairwise comparison based on one-way ANOVA (SNK method). Hypothesis tests were two-sided and $P < 0.05$ was considered statistically significant.

Results

miR-18a is expressed in nasopharyngeal carcinoma cases

In situ hybridization of tissues with an miR-18a probe revealed that 27 of 38 (71.1%) nasopharyngeal carcinoma cases had high expression of miR-18a, while none of the 26 cases of chronic nasopharyngeal mucosa inflammation had high expression of miR-18a ($\chi^2 = 8.123$, $P = 0.004$). Clinical data analysis suggested that

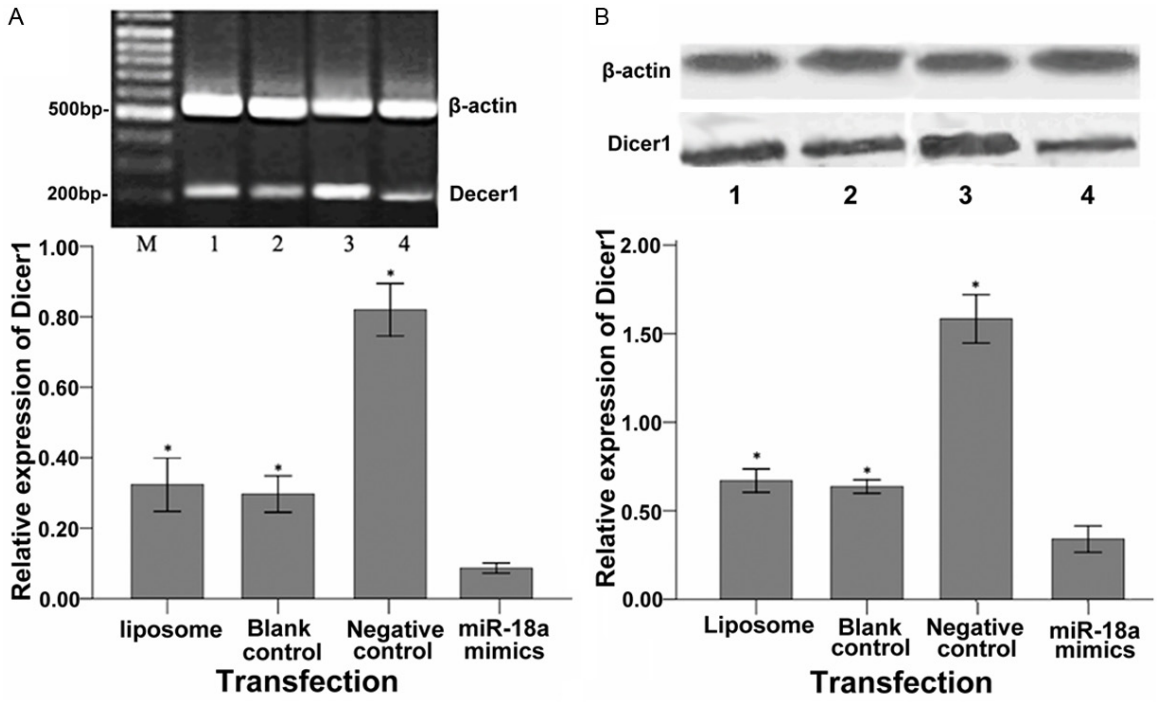

Figure 2. DICER1 expression in transfected HK1 cells. A. RT-PCR measured *DICER1* mRNA. B. Western blotting measured DICER1 in HK1 cells mock-transfected, non-transfected, transfected with negative control oligonucleotides, or transfected with miR-18a mimetic oligonucleotides (n = 3). Gel images depict representative results, while graphs depict quantitation of both A (mRNA expression) and B (protein levels as assessed by densitometry). $^*P < 0.05$ compared to miR-18a mimetic.

Table 2. HK1 cell proliferation rate increases after transfection

	Cell proliferation rate increase			
Transfection (n = 3)	Day 2	Day 3	Day 4	Day 5
Mock	1.29 ± 0.04*	2.49 ± 0.21*	5.60 ± 0.49*	9.57 ± 0.34*
Non-transfected	1.33 ± 0.08*	2.67 ± 0.14*	5.30 ± 0.23*	9.43 ± 0.30*
Negative control oligo	1.33 ± 0.07*	2.65 ± 0.23*	5.10 ± 0.43*	8.34 ± 0.28*
miR-18a mimetic oligo	1.32 ± 0.07	3.45 ± 0.43	7.61 ± 0.27	14.35 ± 0.10
F	0.308	7.286	29.306	289.090
P	0.819	0.011	0.001	0.001

*$P < 0.05$ compared to miR-18a mimetic.

high miR-18a expression in nasopharyngeal carcinoma tissues was correlated with clinical stage and lymph node metastasis (P < 0.05) but not with gender and age (P > 0.05) (**Table 1**).

miR-18a decreases DICER1 expression in nasopharyngeal carcinoma cells

HK1 cells were transfected with miR-18a mimetic or negative control oligonucleotides and mRNA expression was examined by RT-PCR. miR-18a mimetic oligonucleotides significantly increased *MIR18a* expression (1.32 ± 0.06; F = 748.493, P = 0.001) over negative control oligonucleotides (0.11 ± 0.02), non-transfected controls (0.27 ± 0.01), and mock-transfected controls (0.28 ± 0.02) (**Figure 1**).

We then measured *DICER1* mRNA and DICER1 protein expression in HK1 cells transfected with miR-18a mimetic or negative control oligonucleotides and control cells. Cells transfected with miR-18a mimetic oligonucleotides decreased *DICER1* mRNA (0.09 ± 0.01) and DICER1 protein (0.34 ± 0.03) expression as compared to non-transfected cell mRNA (0.30 ± 0.02) and protein (0.64 ± 0.02) or mock-transfected cell mRNA (0.32 ± 0.03) or protein (0.67 ± 0.03) levels ($P < 0.05$) (**Figure 2**). However, transfection with negative control oligonucleotides increased *DICER1* mRNA (0.82 ± 0.03) and protein (1.58 ± 0.06) levels.

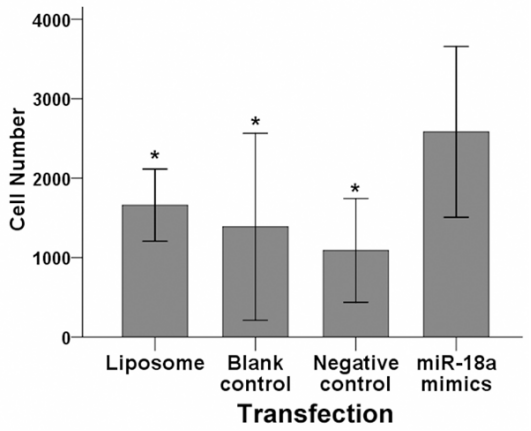

Figure 3. Transwell migration of HK1 cells after transfection. HK1 cells were mock-transfected, non-transfected, transfected with negative control oligonucleotides, or transfected with miR-18a mimetic oligonucleotides, and cell invasion was measured by transwell migration assay on matrigel (n = 3). Panels indicate representative stained migrated cells. Graph indicates quantitation of the number of migrated cells after the indicated transfection. $^{*}P$ < 0.05 compared to miR-18a mimetic.

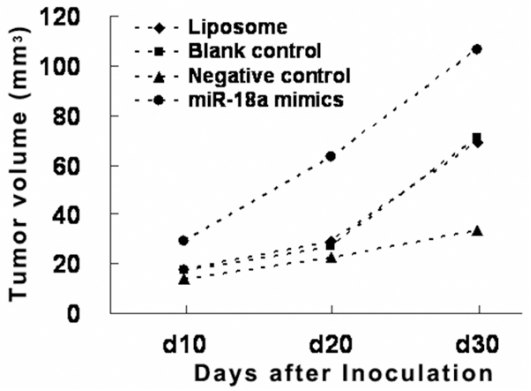

Figure 4. Volume of Subcutaneous tumors in nude mice xenografted with HK1 cells transfected with miR-18a mimics or control HK1 cells. Mice were intracutaneously injected with HK1 cells that were mock-transfected, non-transfected, transfected with negative control oligonucleotides, or transfected with miR-18a mimetic oligonucleotides. After injection, subcutaneous tumor growth was assessed by measuring the long tumor diameter and short tumor diameter, and tumor volumes were calculated according to this formula: V = a × b^2/2. Mice were euthanized 30 days after injection and tumors were isolated, weighed, and recorded among groups.

miR-18a increases proliferation of nasopharyngeal carcinoma cells

MTT assays measured proliferation of control HK1 cells or after transfection with miR-18a mimetic or negative control oligonucleotides. Although cellular proliferation did not differ between any group on day 2, transfection with miR-18a mimetic oligonucleotides significantly increased cell proliferation on days 3, 4, and 5 from all other groups (**Table 2**) (P < 0.05). Transfection with negative control oligonucleotides did not alter cellular proliferation significantly from non-transfected or mock-transfected cells (**Table 2**).

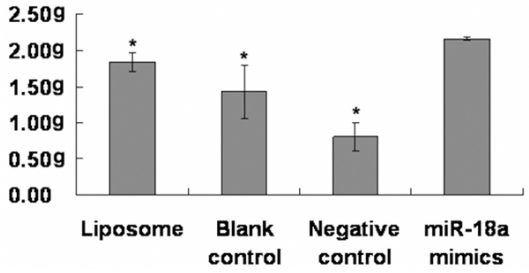

Figure 5. Weight of subcutaneous tumors in nude mice xenografted with HK1 cells transfected with miR-18a mimics or control HK1 cells. Mice were intracutaneously injected with HK1 cells that were mock-transfected, non-transfected, transfected with negative control oligonucleotides, or transfected with miR-18a mimetic oligonucleotides. After injection, subcutaneous tumor growth was assessed by measuring the long tumor diameter and short tumor diameter, and tumor volumes were calculated according to this formula: V = a × b^2/2. Mice were euthanized 30 days after injection and tumors were isolated, weighed, and recorded among groups. $^{*}P$ < 0.05 *vs* miR-18a mimics group.

miR-18a increases invasion of nasopharyngeal carcinoma cells

Transwell migration assays measured invasion of control HK1 cells or after transfection with miR-18a mimetic oligonucleotides. Significantly more migrated cells were counted after transfection with miR-18a mimetic oligonucleotides (2583 ± 433) compared to transfection with negative control oligonucleotides (1091 ± 262), non-transfected cells (1660 ± 183), or mock-transfected cells (1388 ± 474) (F = 9.704, P = 0.005) (**Figure 3**).

miR-18a increases tumor size and tumor weight in nude mice

Control HK1 cells or those transfected with miR-18a mimetic or negative control oligonucleotides were injected into nude mice and tumor size was monitored for 30 days. Injection with cells transfected with miR-18a mimetic oli-

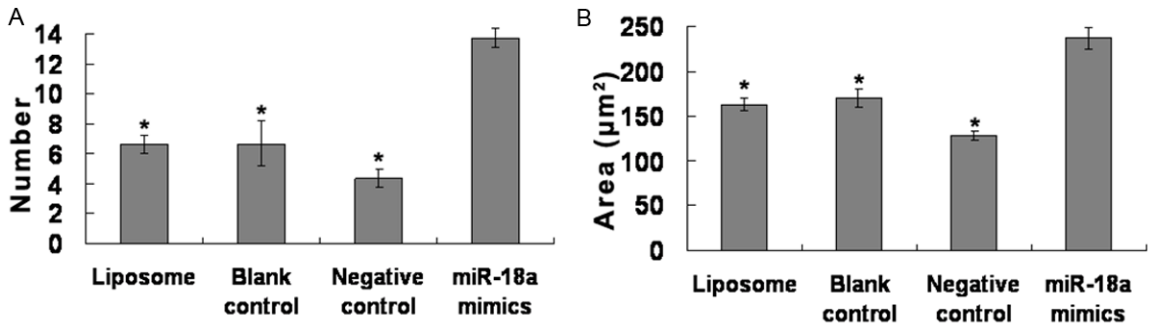

Figure 6. Number (A) and area (B) of metastases in lungs of nude mice xenografted with HK1 cells transfected with miR-18a mimics or control HK1 cells. Mice were intracutaneously injected with HK1 cells that were mock-transfected, non-transfected, transfected with negative control oligonucleotides, or transfected with miR-18a mimetic oligonucleotides. Lung tissues were collected, fixed in 10% formalin, and embedded in paraffin. Tissue sections were stained with H&E and analyzed for the size and number of metastases.

gonucleotides significantly increased tumor sizes on days 10, 20, and 30 after inoculation as compared to non-transfected cells, mock-transfected cells, or cells transfected with negative control oligonucleotides ($P < 0.05$) (**Figures 4** and **5**). After 30 days, tumors were collected and total tumor weight was measured. Tumor weight was also significantly higher in mice injected with cells transfected with miR-18a mimetic oligonucleotides as compared to controls ($P < 0.05$) (**Figures 4** and **5**).

miR-18a promotes metastasis in nude mice

Lung metastases were also analyzed 30 days after injection of nude mice with control HK1 cells or those transfected with miR-18a mimetic or negative control oligonucleotides. The number and area of lung metastases in mice injected with cells transfected with miR-18a mimetic oligonucleotides were significantly increased compared to those in mice injected with non-transfected cells, mock-transfected cells, or cells transfected with negative control oligonucleotides ($P < 0.05$) (**Figure 6**).

Discussion

miRNAs are closely involved in tumorigenesis; many studies have shown differences in miRNA expression between tumors and normal tissues [20]. miRNAs also play important regulatory roles in cellular differentiation [21], proliferation, migration [22], and apoptosis [23]. Therefore, miRNA dysfunction may lead to a variety of human diseases, such as cancer, liver disease, immune dysfunction and metabolic disorders. Likewise, the miR-17-92 family has an established role in tumorigenesis, although the specific roles of its individual family members, including miR-18a, remain largely unknown.

Morimura *et al* [24] detected increased miR-18a expression in plasma of patients with pancreatic cancer as compared to healthy persons, suggesting miR-18a may be involved in pancreatic tumorigenesis. Conversely, Tsang *et al.* found that miR-18a* can suppress K-Ras to inhibit tumor growth [25]. Here, we show that miR-18a expression is increased in human nasopharyngeal carcinoma cases *in situ* and is correlated with clinical stage and lymph node metastasis. Further, *in vitro* studies in HK1 cells suggest that miR-18a can promote proliferation and invasion of nasopharyngeal carcinoma cells and *in vivo* injection of these cells into nude mice forms larger and more metastatic tumors. While the larger tumors formed from miR-18a-transfected xenografts may produce more cells that can subsequently metastasize, our results nonetheless indicate that miR-18a can promote proliferation and metastasis of nasopharyngeal carcinoma cells and suggest that miR-18a is an oncogene.

Consistent with bioinformatics analyses, we also found that miRNA-18a expression decreases DICER1 transcription and translation in HK1 cells. These results indicate that miR-18a may downregulate DICER1 expression. Other studies have demonstrated that dysregulation of DICER1 results in global changes in expression of many miRNAs, and that the disrupted balance in expression of tumor-suppressing versus tumor-promoting miRNAs promotes onco-

genesis [26]. Previous analysis of nasopharyngeal carcinoma indeed identified global disruptions in miRNA expression [16]. Although direct evidence is needed, it is possible that the regulation exerted on DICER1 by miR-18a subsequently produces changes in expression of other miRNAs, which then promote invasion and metastasis of nasopharyngeal carcinoma cells.

Although further analyses are required, our results suggest that compounds that inhibit miR-18a may offer a novel therapy for nasopharyngeal carcinoma.

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Disclosure of conflict of interest

None.

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