MicroRNA-18a high expressed in nasopharyngeal carcinoma facilitates tumor growth by targeting SKT4

Fujun Li¹, Jing Ju², Yi Ling¹, Hua Jiang¹, Yating Peng³

¹Department of Otorhinolaryngology, The Second Affiliated Hospital, School of Medicine, Zhejiang University, China; ²Department of Biochemistry and Molecular Biology, Nanjing Medical University, China; ³Department of Otorhinolaryngology, Integrated Chinese and Western Medicine Hospital of Zhejiang Province, Hangzhou Red Cross Hospital, Hangzhou, China

Received September 21, 2016; Accepted September 29, 2016; Epub February 1, 2017; Published February 15, 2017

Abstract: MicroRNA-18a (miR-18a) is significantly overexpressed in various cancers and facilitates tumor growth and cell proliferation. However, whether miR-18a plays oncogenic roles in nasopharyngeal carcinoma (NPC) is still unknown. Here, we report that miR-18a expression was obviously increased in the NPC samples and cell lines. Clinically, NPC patients with higher miR-18a predicted worse clinical outcome compared with those with lower miR-18a. Furthermore, we found that expression of serine/threonine-protein kinase (STK4) was down-regulated in NPC tumors and inversely correlated with miR-18a expression. Importantly, functional analyses of the effect of miR-18a on cancer phenotypes revealed that transfection of miR-18a inhibitor into NPC cells suppressed cell growth, induced apoptosis, and blocked the cell cycle. Subcutaneous xenografts of transfected cells in nude mice demonstrated that miR-18a inhibitor significantly inhibited tumor growth. Finally, SKT4 was identified as a direct target of miR-18a using a Luciferase reporter assay and Western Blot analysis. Together, these results indicate that miR-18a is an oncogenic miRNA that promotes tumor progression and leads us to propose that miRNAs may serve as key regulatory hubs in NPC development.

Keywords: Nasopharyngeal carcinoma, miR-18a, proliferation, apoptosis, STK4

Introduction

Nasopharyngeal carcinoma (NPC) is a rare tumor that arises from the nasopharyngeal epithelium. Nasopharyngeal carcinoma occurs around the world; however, it is vastly more common in certain regions of Southeast Asia than elsewhere [1]. Despite recent improvements in diagnosis and treatment, frequent tumor recurrence and distant metastases remain major obstacles for long-term patient survival [2, 3]. Previous studies identified many aberrantly expressed protein-coding genes in NPC, but novel molecular markers that can help in early diagnosis and risk assessment are still urgently needed. Thus, identifying more accurate predictive biomarkers is of paramount importance to further understand NPC cell biology and develop novel therapeutic strategies.

MicroRNAs are highly conserved non-coding RNAs of approximately 20-24 nucleotides that inhibit gene expression at the posttranscriptional level via binding to imperfect complementary sites within the 3'-untranslated regions (3'UTR) of messenger RNAs (mRNAs) [4]. Increasing evidence supports that miRNAs play indispensable roles in many biological processes involving in the pathogenesis of diverse cancer, such as proliferation, apoptosis and angiogenesis in lung cancer, breast cancer, ovarian cancer and so on [5-7]. And, kinds of miRNAs have been confirmed to participate in the initiation and progression of nasopharyngeal carcinoma [8-21]. A study from Li T showed that miR-18a was overexpressed in NPC tissues compared to the normal samples [22]. Recently, Luo, Z et al. found that miR-18a promoted cell growth and mobility, at least in part through targeting Dicer in nasopharyngeal carcinoma, suggesting an oncogenic role for miR-18a in NPC [23].

STK4, known as STK4 (mammalian sterile 20-like kinase 1) and Krs-2, is a 56-60-kDa protein
that is highly conserved across different species. The expression of STK4 in different cancers varies such as colon cancer, prostate cancer and hepatic cell carcinoma [24]. Previously, Hsu et al. found that miR-18a exhibited an oncogenic role in prostate cancer by targeting STK4 [25]. In addition, STK4 has been identified as a pro-apoptotic kinase and has acted as inhibitors of endogenous AKT [26]. However, whether STK4 participates in the role and function of miR-18a in NPC is still unknown.

In this study, we aimed to examine the roles of miR-18a in the progression of NPC and, importantly, to clarify the mechanism by which miR-18a promotes malignant tumor progression. Efforts to interpret the association of miR-18a and STK4 are included in this report.

Materials and methods

Cell lines and tissue samples

Immortalized human NPC cell lines (CNE1, HONE1, HNE1, 5-8F and 6-10B) were maintained in our lab. These cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) in humidified 37°C incubator with 5% CO₂. Thirty-five freshly frozen NPC biopsy specimens and thirty-five normal nasopharyngeal epithelium specimens were obtained from the Department of Otolaryngology, Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang Province, PR China. None of the patients with NPC had received radiotherapy or chemotherapy before biopsy. This study was approved by the Institutional Ethical Review Boards of our institute, and written informed consent was obtained from each patient.

RNA extraction, reverse transcription and quantitative RT-PCR

Total RNA was extracted using TRIzol reagent (Life Technologies, Grand Island, NY, USA) and reverse transcribed using Bulge-LoopTM microRNA specific primers (RiboBio Co., Guangzhou, China) and M-MLV reverse transcriptase (Promega, Madison, WI, USA) to quantify the expression of miR-18a or using random primers (Promega) and M-MLV reverse transcriptase (Promega) to quantify the expression of STK4 mRNA. Quantitative RT-PCR reactions were performed in a CFX96Touch™ sequence detection system (Bio-Rad, Hercules, CA, USA) using Platinum SYBR Green qPCR Super Mix-UDG reagents (Invitrogen). RNU6B (U6) or GAPDH were used as controls for normalization; relative expression levels were calculated using the 2-ΔΔCT method. All experiments were performed in triplicate.

Cell proliferation assay

NPC cells were seeded in 96-well plates at a density of 2,000 cells/100 μL/well and incubated at 37°C. Cell proliferation was assessed every 24 h for the indicated duration of time following transfection. Briefly, 10 μL of MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, 5.0 mg/mL; Sigma-Aldrich) was added to each well, and the plates were incubated for 4 h at 37°C. The culture media were removed, 100 μL DMSO was added, and the cells were incubated for 10 min on a shaker. Optical densities were determined at 570 nm on a Bio-Rad model 680 microplate reader, (Bio-Rad Laboratories, Hercules, CA, USA). OD values reflect the relative number of viable cells.

Apoptosis analysis by flow cytometry

To measure apoptosis, expression of Annexin V-FITC and exclusion of propidium iodide (PI) (TACS Annexin V-FITC, Trevigen, Inc., Gaithersburg, MD) were detected by double-label flow cytometry. Seventy-two hours after transfection, cells were collected, washed once with PBS, and washed again with 400 μL binding buffer. Samples were incubated with 100 μL Annexin V-FITC reagent in the dark for 15 min at room temperature, and then the volume was adjusted to 500 μL with binding buffer. Fluorescence was measured on a flow cytometer (BD Biosciences, FACSCanto II, San Jose, CA, USA) within 1 h for maximal signal.

Cell cycle analysis

Cell cycle analysis was performed as described previously [27]. Cells were collected 48 h post-transfection. The cell pellets were rinsed once with phosphate-buffered saline (PBS) and fixed in 70% ice-cold ethanol at -20°C overnight. The cells were then resuspended in PBS, stained
MicroRNA-18a facilitates NPC cells growth by targeting SKT4

with propidium iodide containing RNase A for 30 min at 37°C, and analyzed by flow cytometry. Each assay was conducted in triplicate and repeated at least three times.

In vivo tumor growth assay

All BALB/c nude mice aged 6-7 weeks and weighing 20-22 g were used in the experiment. The animal study was performed at the Zhejiang University with approval from the Institutional Animal Care and Use Committee in accordance with the institutional guidelines. Animals were housed in ventilated caging conditions under a 12-h dark/light cycle at constant humidity and temperature. Animals were permitted free access to sterile water and standard laboratory chow. Subcutaneous xenografts were established by inoculating $2 \times 10^6$ miR-18a inhibitor–transfected cells into the left flank, or an equal number of control inhibitor-transfected cells into the right flank. After 15 or 25 days, animals were sacrificed and tumor tissues were resected. The tumor weight was measured and the tumor volume was calculated according to the formula: Tumor volume ($\text{mm}^3$) = ($wh^2$)/2, where $w$ is the longest axis (mm) and $h$ is the shortest axis (mm).

Cell transfection

MiR-18a mimic, miR-18a inhibitor and miR negative control (NC) were synthesized by Qiagen. CNE-1 and 5-8F cells were plated in six-well plates ($1.5 \times 10^5$ cells per well) for 24 h and transfected with miRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 24 h. Cells transfected with miRNA were harvested 48 h post-transfection.

Luciferase assays

Luciferase reporter assay was performed as described previously. Dual luciferase assays were conducted in a 24 well plate format. pGL3-STK4 3’UTR report/pGL3-STK4 3’UTR Mutant report +TK100 Renilla report were transfected into 70% confluent HEK293 cells, along with miR-18a mimic, miR-18a inhibitor or each control. After 48-h transfection, firefly and renilla luciferase were quantified sequentially using the Dual Luciferase Assay kit (Promega, USA) following the manufacturer’s recommendations.

Western blotting analysis

Cells were harvested and homogenized with cell lysis buffer (Beyotime, China). Then, the homogenates were centrifuged for 30 min at 4°C, 12000 rpm, and the supernatants were collected as protein samples. Protein amounts were measured using BCA Protein Assay Kit (Beyotime, China). Equal amounts of protein samples were separated by denaturing 10% SDS-PAGE and transferred onto polyvinylidene-nedifluoride (PVDF) membranes. Membranes were incubated in a 5% skim milk TBST blocking solution at room temperature (RT) for 1 h. And, membranes were incubated with agitation at 4°C overnight with specific primary antibodies against STK4 and β-catenin. Then, membranes incubated by secondary antibodies conjugated with horseradish peroxidase (HRP) at RT for 50 min. Finally, protein bands were visualized using an enhanced chemiluminescence (ECL) western blotting detection system (GE Healthcare, Amersham, UK).

Statistical analysis

Data are reported as mean ± standard deviation (SD). Statistical significance was determined using Double-sided Student’s t test. Multiple groups were analyzed using ANOVA. A $p$ value of less than 0.05 was considered to be significant.

Results

Aberrant expression of miR-18a in human NPC tissues and cell lines

To determine the expression of miR-18a in NPC, qPCR analysis was conducted in 35 pairs of clinic NPC tissue and matched adjacent normal tissue samples. As shown in Figure 1A, miR-18a was up-regulated in NPC tissue compared with adjacent normal tissues. Subsequently, we also detected the mRNA level of miR-18a in five NPC cell lines including 5-8F, 6-10B, HNE1, CNE-1, and HONE-1 using qRT-PCR. A human immortalized nasopharyngeal epithelial cell line (NP69) used as a negative control. High expression of miR-18a in NPC cell lines were also observed, especially in 5-8F and CNE1 cells (Figure 1B). These data suggest that upregulated expression of miR-18a is related to the progression of NPC.
MicroRNA-18a facilitates NPC cells growth by targeting SKT4

To investigate whether miR-18a expression is associated with clinical outcome in NPC patients, NPC patients were divided into two different groups according to the median miR-18a level. MiR-18a expression above or below the median was considered as high or low expression. As indicated in Figure 1C, NPC patients with higher expression of miR-18a predicted worse OS compared with those with lower miR-18a expression.

As STK4 is an important established regulator of miRNA processing, the expression level of STK4 was evaluated using quantitative real-time PCR in 35 pairs of clinic NPC tissue and matched adjacent normal tissue samples. We found that the expression levels of STK4 decreased (Figure 1D). Moreover, a significant inverse correlation between miR-18a and STK4 was found in these tissues ($R^2=0.8746$, $P<0.001$; Figure 1E), indicating that abnormal miR-18a expression might also lead to STK4 dysregulation due to their interactions.

Knockdown of miR-18a suppresses cell proliferation and cell cycle, and induces apoptosis in NPC cell lines

The significant increase of miR-18a in NPC prompted us to explore the possible biological roles of miR-18a in NPC. To analyze it, we performed in vitro experiments to determine the roles of miR-18a in NPC development including the effects on cell growth, cell cycle and apoptosis. MiRNAs function was tested by transfecting NPC cells using chemically synthesized miR-18a inhibitors. qRT-PCR analysis showed that miR-18a expression was effectively reduced by miR-18a inhibitor (Figure 2A). Knockdown of miR-18a significantly decreased proliferation (Figure 2B and 2C) and induced apoptosis (Figure 2D) in 5-8F and CNE1 cells. Further, we asked whether miR-18a knockdown affected

![Figure 1](Figure 1.png)

**Figure 1.** MiR-18a is highly expressed in NPC tissues and cell lines. A. The expression of level of miR-18a was detected in 35 pairs of NPC tumors and adjacent normal tissues by RT-qPCR. B. The relative level of miR-18a in five NPC cell lines including 5-8F, 6-10B, HNE1, CNE-1, and HONE-1. C. Kaplan-Meier overall survival curve was indicated according to miR-18a expression level. D. The expression level of STK4 was detected in 35 pairs of NPC tumors and adjacent normal tissues by RT-qPCR. E. Pearson’s correlation was performed to analyze the correlations between miR-18a and STK4 in NPC tissues ($R^2=0.8746$, $P<0.001$). All values are the mean ± SD of triplicate measurements, and experiments were repeated 3 times with similar results. *$P<0.05$, **$P<0.01$. 

---

MicroRNA-18a facilitates NPC cells growth by targeting SKT4

As indicated in Figure 2E, we found that most of the miR-18a inhibitor transfected cells were arrested in the G0/G1 phase of the cell cycle, while the percentage of cells in the G2/M phase was markedly decreased. All these results suggested that miR-18a executed an oncogenic effect on NPC cells.
MicroRNA-18a facilitates NPC cells growth by targeting SKT4

To further examine the role of miR-18a in NPC development, we performed a xenograft study in which miR-18a inhibitor or inhibitor negative control (inhibitor NC)-transfected 5-8F and CNE1 cells were transplanted into the flanks of BALB/c nude mice. The tumor weight and tumor growth curve suggested that miR-18a knockdown suppressed effectively tumor growth comparing with the inhibitor NC treated xenograft tumors (P < 0.05, Figure 3A-D). The tumor xenograft experiment indicated that miR-18a downregulation played a role in suppressing NPC cell growth in vivo.

STK4 is involved in the role of miR-18a in the regulation of NPC cell activity

To examine whether miR-18a directly targets STK4, we conducted a luciferase assay (Figure 4A). The luciferase assay used in the present study is to evaluate the effect of miRNA-dependent post-transcriptional regulation of target genes. The luciferase activity was significantly inhibited when STK4-WT was co-transfected with miR-18a mimics compared with that after the mimic NC co-transfection, whereas the inhibitory effect was abolished when the STK4 3'UTR was mutated (Figure 4B).

To evaluate whether miR-18a regulated STK4 expression, we detected the protein expression level of STK4 in miR-18a mimic or miR-18a inhibitor infected cells. Western blot analysis showed that miR-18a overexpression markedly decreased the protein level of STK4, whereas miR-18a inhibition increased the protein expression of STK4 (Figure 4C). Taken together, we showed that the STK4 is directly regulated by miR-18a via conserved seed matching sequences.

Figure 3. miR-18a inhibits NPC tumor growth in vivo. The NPC mouse model in mice was constructed by using 5-8F and CNE1 cells transfected with miR-18a inhibitor and inhibitor-NC. The size of subcutaneous tumors in these two groups was calculated and compared. A, C. Tumor volume (in mm$^3$, recorded every week). B, D. Mice body weight (in grams, recorded every week). Data were expressed as mean ± SD, experiments were repeated three times. *P < 0.05, **P < 0.01 vs. inhibitor NC group.
MicroRNA-18a facilitates NPC cells growth by targeting SKT4

Discussion

In this study, miR-18a was identified to be significantly increased in NPC tissues and cell lines. The clinical correlation analysis confirmed the miR-18a was strongly indicative of poor prognosis. And, a significant inverse correlation between miR-18a and STK4 was found in NPC tissues. In addition, miR-18a was confirmed to target the STK4 3’UTR using luciferase activity and western blot assays. Therefore, STK4 was a functional target of miR-18a. More importantly, the physiological significance of interaction is underscored that miR-18a knockdown suppresses cell proliferation, induces apoptosis and blocks cell cycle, suggesting that miR-18a may act as an oncogenic microRNA in NPC.

More and more emerging evidence indicates that miRNAs, which were frequently dysregulated in diverse human cancers, may play regulatory roles in the cell proliferation and tumor growth [28-30]. Several research studies referring to the relationship between miRNA and cancers had focused on miR-18a, which could be provided as a supplement for the biological mechanism of tumorigenesis and progression [31-34]. For example, microRNA-18a regulates gastric carcinoma cell apoptosis and invasion by suppressing hypoxia-inducible factor-1α.

Figure 4. miR-18a directly binds and downregulates STK4. A. Schema of the firefly luciferase reporter constructs for the STK4, indicating the interaction sites between miR-18a and the 3’-UTRs of STK4. B. Luciferase activities. HEK-293 cells were co-transfected with firefly luciferase constructs containing the STK4 wild-type or mutated 3’-UTRs and miR-18a mimic, mimic NC, miR-18a inhibitor or inhibitor NC, as indicated (n=6). C. Protein expression of STK4 after treatment with miR-18a mimic or miR-18a inhibitor (n=6). All data represent the mean ± SD results of three independent experiments. **P < 0.01.
MicroRNA-18a facilitates NPC cells growth by targeting SKT4

expression [31]. Chung-Wah Wu et al. found that microRNA-18a attenuates DNA damage repair through suppressing the expression of ataxia telangiectasia mutated in colorectal cancer [35]. Recently, miR-18a has been confirmed to promote cell growth and mobility, at least in part through targeting Dicer [34]. In this study, we found that the expression of miR-18a was increased in NPC tissues when compared with adjacent normal tissues. We further demonstrated that miR-18a overexpression was correlated with poor overall survival, thus, miR-18a was hypothesized to play a role in the regulation of NPC cell behavior. In addition, the results demonstrated that the ectopic expression of miR-18a inhibited cell proliferation, induced significantly apoptosis and induced cell arrest in the G0/G1 phase. Tumor growth in vivo was also suppressed by exogenous miR-18a. Therefore, the increased expression of miR-18a may contribute to the development and progression of NPC.

As miRNAs exert their function through direct binding to the 3’-UTRs of their target genes, we determined the possible target genes of miR-18a. A single miRNA can regulate a number of target genes, which play an important role in cancer-related pathways; some of miR-18a target genes that have been identified and validated include HIF-1α [31], Ataxia Telangiectasia Mutated (ATM) [35], Dicer1 [34] and STK4 [25]. Among miR-18a predicted target genes, STK4 acted as a direct target. STK4, a homolog of Hippo (Hpo/hpo) in Drosophila, was originally identified as a pro-apoptotic protein [36, 37]. In addition, STK4 has been identified as a pro-apoptotic kinase and has acted as inhibitors of endogenous AKT [26]. It was previously reported that the upregulation of miR-18a bound directly to the 3’UTR of the STK4 mRNA, down-regulating STK4 at the protein level and thus suppressing apoptosis and promoting tumor survival in prostate cancer [25]. In this present study, we found that the expression of STK4 was regulated by miR-18a and that the level of STK4 protein expression was inversely correlated with miR-18a expression in NPC cells. Taken together, the data indicated that miR-18a acted as a novel oncogene in NPC by targeting STK4.

In conclusion, the results of the present study indicated that miR-18a was upregulated and was able to affect cell proliferation, cell apoptosis and cell cycle in 5-8F and CNE1 cell lines. Furthermore, STK4 was identified as a potential target gene of miR-18a. Thus, miR-18a has significant value in clinical trials and may serve as a prognostic marker and therapeutic target in the future.

Acknowledgements

This study was supported by the Foundation from the Education Department of Zhejiang Province (N20140212).

Disclosure of conflict of interest

None.

Address correspondence to: Yating Peng, Department of Otorhinolaryngology, Integrated Chinese and Western Medicine Hospital of Zhejiang Province, Hangzhou Red Cross Hospital, 208 East Huancheng Road, Hangzhou 310003, China. Tel: +86 571-56109588; E-mail: pengyating66@126.com

References

MicroRNA-18a facilitates NPC cells growth by targeting SKT4


MicroRNA-18a facilitates NPC cells growth by targeting SKT4


