Original Paper

MiR-142-3p Suppresses SOCS6 Expression and Promotes Cell Proliferation in Nasopharyngeal Carcinoma

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Key Words

Nasopharyngeal carcinoma • miR-142-3p • Proliferation • SOCS6

Abstract

Background/Aims: An increasing number of studies show that microRNAs (miRNAs) play crucial roles in nasopharyngeal carcinoma (NPC) tumorigenesis. The aim of our study was to investigate the biological roles and mechanisms of miR-142-3p in NPC. **Methods:** miR-142-3p expression was examined in NPC specimens and nasopharyngitis biopsy samples by quantitative real-time PCR. The biological functions of miR-142-3p were studied using a series of in vitro and in vivo approaches. **Results:** miR-142-3p is over-expressed in NPC tissues and cell lines. Knockdown of miR-142-3p significantly inhibited cell proliferation and cell cycle progression in vitro, and suppressed tumor growth in a mouse model. Suppressor of cytokine signaling 6 (SOCS6) was identified as a direct target of miR-142-3p, and miR-142-3p down-regulated the expression of SOCS6 by directly binding to its 3'untranslated region (UTR). Knockdown of SOCS6 abrogated the effects of miR-142-3p down-regulation. **Conclusion:** These findings indicate that miR-142-3p regulates NPC development by down-regulating SOCS6 expression and suggest that modulation of miR-142-3p expression could be a therapeutic strategy for NPC.

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Introduction

Nasopharyngeal carcinoma (NPC) is a non-lymphomatous squamous cell carcinoma arising from epithelial cells located in the nasopharynx [1]. The prevalence of NPC is low in most parts of the world, whereas it is high in southern China and other Southeast Asian

countries [2]. Despite significant advancements in the treatment of NPC, the average 5-year survival rate remains between 34% and 52% [3]. However, the mechanisms underlying the pathogenesis and progression of NPC remain unclear, underscoring the need for further study to improve our understanding of this disease.

MicroRNAs (miRNAs) are small (20–25 nucleotides) noncoding RNAs that function as negative regulators of gene expression via binding to the 3'-untranslated region (3'-UTR) of their target messenger RNAs (mRNAs) [4]. miRNAs play vital roles in many fundamental biological processes including cell proliferation, differentiation, apoptosis, migration, and invasion by suppressing their target genes [5–10]. Numerous studies have demonstrated that deregulation of specific miRNAs is associated with cancer initiation and progression in many malignancies including NPC, and miRNAs can function as oncogenes or onco-suppressors depending on their targets [11–15]. miR-142-3p is dysregulated in various human cancers, including thyroid carcinoma, hepatocellular carcinoma, osteosarcoma, colon cancer, esophageal cancer, head and neck squamous cell carcinoma, non-small cell lung cancer (NSCLC) and T-cell acute lymphoblastic leukemia [16–23]. Recently, Chen *et al.* reported that miR-142-3p is up-regulated in NPC [24]. However, the biological function of miR-142-3p remains largely unknown.

In the present study, we confirm that miR-142-3p is up-regulated in NPC tissues and cell lines, and show that it acts as an oncogene by promoting cell proliferation and cell cycle progression. Suppressor of cytokine signaling 6 (SOCS6) was identified as a direct target of miR-142-3p. Silencing of SOCS6 partially rescued the inhibitory effect of miR-142-3p down-regulation. Collectively, our data indicate that miR-142-3p may be a potential target for the treatment of NPC.

Materials and Methods

Clinical specimens and cell culture

NPC specimens (n = 26) and nasopharyngitis biopsy samples (n = 11) were obtained from patients undergoing complete or partial surgical resection at the 88th Hospital of PLA (Tai'an, China) and were frozen in liquid nitrogen for further study. All patients provided their written informed consent, and the research protocols were approved by the Ethics Committee of the 88th Hospital of PLA. Human NPC cell lines (HONE-1, C666-1, CNE-2 and SUNE-1) were maintained in RPMI-1640 (Invitrogen, Carlsbad, CA, USA). The normal nasopharyngeal epithelial cell line NP69 was grown in Keratinocyte-SFM (Invitrogen) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 100 units/ml penicillin-streptomycin (Invitrogen). All cells were maintained in a humidified chamber with 5% CO₂ at 37°C.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from fresh frozen tissues and cultured cells using the TRIzol reagent and quantified with Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). miRNAs were isolated with a miRNeasy Mini Kit (Qiagen, Hilden, Germany). The SuperScript III reverse transcription kit (Invitrogen) was used to synthesize the cDNA according to the manufacturer's instructions. qRT-PCR reactions were performed using SYBR Green PCR Master Mix (Takara, Dalian, China) on the Applied Biosystems 7900 (Applied Biosystems, Foster City, CA, USA). U6 or β -actin was used as an internal control for miR-142-3p or SOCS6. The relative expression of target genes was calculated using the $2^{-\Delta\Delta CT}$ equation. The PCR primers for miR-142-3p and U6 were purchased from RiboBio (Guangzhou, China). The PCR primers for SOCS6 and β -actin were as follows: SOCS6-forward: 5'-GCCTTGGTTCACCTT CC-3', SOCS6-reverse: 5'-ATCATCGTGACCCGCTCT-3'; β -actin-forward: 5'-GGAAATCGT GCGTGACAT-3', β -actin-reverse: 5'-AAGGAAGGCTGAAGAGTG-3'. All reactions were run in triplicate.

Oligonucleotide transfection

AntagomiR-142-3p, miR-142-3p mimic, siRNAs targeting SOCS6 and their corresponding controls were purchased from RiboBio. Oligonucleotides were transfected into cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were collected 48 h after transfection.

Plasmid construction

The 3'-UTR of SOCS6 containing the putative binding site for miR-142-3p was PCR-amplified from normal human cDNA. The related mutant construct was generated by mutating the miR-142-3p seed sequence (from ACACUAC to CCAAUGC). The wild-type and mutant 3'-UTRs of SOCS6 were cloned into the psiCHECK2 luciferase vector (Promega, Madison, WI, USA). The constructs were verified by DNA sequencing.

Cell viability assays

Cell viability assays were performed using the Cell Counting Kit-8 (CCK-8, Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's protocols. CNE-2 and SUNE-1 cells were seeded in 96-well cell culture plates at a density of 1.5×10^3 cells per well. CCK8 (20 μ l) was added into each well after cells were maintained for 1, 2, 3, 4 and 5 days. Subsequently, the cells were incubated for another 4 h at 37°C, and the absorbance was measured at 490 nm. The results were averaged among three independent experiments.

Bromodeoxyuridine labeling of cultured cells

Cells (5×10^4) grown on coverslips were incubated with 10 μ M bromodeoxyuridine (BrdU) for 2 h, and then fixed and labeled with an anti-BrdU antibody (Invitrogen) for 12 h according to the manufacturer's instructions. Secondary antibody was added. DAPI was used for nuclear staining. The number of BrdU positive cells was counted in three random microscopic fields using NIH Image J software [25].

Flow cytometry analysis

Cells were harvested, washed and fixed in 75% ethanol at 4°C overnight. Then, cells were pelleted and resuspended in PBS at 1×10^6 cells/ml, and incubated with bovine pancreatic RNase (Sigma, St. Louis, MO, USA) for 30 min at 37°C and propidium iodide for 30 min at room temperature. The cell cycle profiles were analyzed using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Tumor xenograft model

Healthy female BALB/c nude mice (4–5 weeks of age) were obtained from the Center of Experimental Animal of Guangzhou University of Chinese Medicine. All animal experiments were approved by the 88th Hospital of PLA Animal Care and Use Ethics Committee. The nude mice were randomly divided into two groups: negative control (antagomiR-NC) and antagomiR-142-3p (five mice per group). SUNE-1 cells (1×10^6 cells in 200 μ l PBS) transfected with antagomiR-142-3p or antagomiR-NC were subcutaneously injected into the nude mice. Tumor size was monitored by measuring the length (A) and width (B) with a slide caliper every 3 days and tumor volume (V) was calculated as follows: $V = (A \times B^2) \times 0.5$. After 28 days, the mice were sacrificed and tumors were removed and weighed.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues were cut into 4 mm-thick sections. The slides were deparaffinized with xylene, dehydrated with ethanol, boiled in 0.01 M citrate buffer (pH 6.0), and then treated with 3% H_2O_2 for 10 min to inactivate endogenous peroxidase activities and blocked with 10% goat serum in 0.2 M PBS (pH 7.4). Slides were then incubated with anti-Ki67 overnight at 4°C. Secondary antibody was added and incubated for 2 h at room temperature, followed by treatment with DAB for 5–10 min at room temperature and counterstaining with hematoxylin.

Luciferase reporter assay

HEK 293T cells were maintained in 24-well plates and transfected with wild-type or mutant reporter vector together with miR-142-3p mimic or negative control using Lipofectamine 2000 (Invitrogen). After 48 h, cells were harvested and lysed. Firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega) [8]. For each plasmid construct, the transfection experiments were performed in triplicate.

Western blotting

Protein extracts were separated using 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% non-fat milk and

probed with primary antibodies against SOCS6 (1:1000, Abcam, Cambridge, UK) and GAPDH (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. Secondary antibody was added and incubated for 2 h at room temperature. Bands were detected by enhanced chemiluminescence (Pierce, IL, USA).

Statistical analysis

Data were presented as the mean $\pm$ standard deviation (SD), and statistical analysis was performed using SPSS 16.0 software. Student's *t*-test was used to analyze the differences between two groups. The correlation between miR-142-3p and SOCS6 expression was analyzed by Spearman's correlation. $P < 0.05$ was defined as statistically significant.

Results

miR-142-3p is up-regulated in NPC tissues and cell lines

To validate the previously observed pattern of aberrant miR-142-3p expression in NPC, miR-142-3p expression was measured by qRT-PCR in 26 primary NPC tissues and 11 nasopharyngitis samples. As shown in Fig. 1A, the mRNA levels of miR-142-3p were significantly higher in primary NPC tissues than in nasopharyngitis samples. Similarly, miR-142-3p levels were generally increased in NPC cell lines (HONE1, C666-1, CNE-2 and SUNE-1) compared with the normal nasopharyngeal epithelial cell line NP69 (Fig. 1B). These results indicate that miR-142-3p is up-regulated in NPC.

Inhibition of miR-142-3p reduces the proliferation of NPC cells

To explore the biological role of miR-142-3p in NPC progression, CNE-2 and SUNE-1 cells were transfected with antagomiR-142-3p or antagomiR-NC. As shown in Fig. 2A, the viability of cells transfected with antagomiR-142-3p was markedly decreased compared to that of the negative control. To further study the role of miR-142-3p in NPC cell growth promotion, BrdU incorporation was examined in cells subjected to miR-142-3p down-regulation. As observed in Fig. 2B, the percentage of BrdU positive cells was significantly lower in cells transfected with antagomiR-142-3p than in the controls (Fig. 2B). Furthermore, flow cytometric analysis showed that inhibition of miR-142-3p caused an increase in the proportion cells in G0/G1 phase and a decrease in the proportion cells in S phase (Fig. 2C). Collectively, these results suggest that miR-142-3p regulates cell growth and cell cycle progression at the G1/S transition in NPC cells.

Fig. 1. miR-142-3p is up-regulated in NPC tissues and cell lines. (A) Relative miR-142-3p expression in 26 primary NPC tissues and 11 nasopharyngitis samples, as detected by qRT-PCR. U6 was used as internal control. (B) qRT-PCR analysis of miR-142-3p expression in NPC cell lines and NP69 cells. Data represent the mean $\pm$ SD of three independent experiments. * $P < 0.05$.

Fig. 2. Inhibition of miR-142-3p suppresses proliferation and cell cycle progression of NPC cells. CNE-2 and SUNE-1 cells were transfected with antagomiR-142-3p or antagomiR-NC. (A) Cell viability was evaluated by CCK-8 assay. (B) Representative images (left) and quantification of BrdU positive cells. (C) Cell cycle progression was measured by flow cytometry. Data represent the mean $\pm$ SD of three independent experiments. * $P < 0.05$.

Down-regulation of miR-142-3p inhibits NPC tumorigenesis *in vivo*

Since our *in vitro* studies indicated that inhibition of miR-142-3p affected NPC cell proliferation, we investigated whether such an effect would occur in mouse xenograft models. SUNE-1 cells were transfected with antagomiR-142-3p or antagomiR-NC and subcutaneously injected into nude mice. Assessment of tumor growth showed that the average tumor volume in the antagomiR-142-3p group was drastically reduced compared with that of the control group (Fig. 3A). The tumors formed by antagomiR-142-3p-transfected SUNE-1 cells grew slower and had significant lower tumor weights than control tumors (Fig. 3B and C). IHC staining showed fewer Ki-67-positive cells in the tumors formed from SUNE-1-antagomiR-142-3p cells than in those formed from negative control cells. Collectively, these results suggest that inhibition of miR-142-3p represses NPC cell growth *in vivo* (Fig. 3D).

SOCS6 is the direct downstream target of miR-142-3p

Analysis using different databases (TargetScan and miRanda) predicted that SOCS6 is a candidate miR-142-3p target (Fig. 4A). To investigate whether miR-142-3p directly targets SOCS6, 3'-UTR fragments of SOCS6 containing the miR-142-3p binding site and the corresponding mutant fragments were subcloned into the psiCHECK-2 vector and co-transfected with miR-142-3p mimic or miR-NC into 293T cells. Ectopic expression of miR-142-3p significantly decreased the luciferase activity of the reporter containing the SOCS6 3'-UTR, whereas the luciferase activity of the mutant 3'-UTR showed little change (Fig.

Fig. 3. Down-regulation of miR-142-3p inhibits NPC tumorigenesis *in vivo*. (A) Tumor volumes were detected every 3 days after implantation of SUNE-1 cells transfected with antagomiR-142-3p or antagomiR-NC. (B) Representative photograph of tumors formed. (C) The mean weight. (D) H&E- and Ki-67-stained sections of transplanted tumors. Data represent the mean $\pm$ SD of three independent experiments. * P < 0.05.

4B). miR-142-3p over-expression significantly down-regulated SOCS6 mRNA and protein expression in NP69 cells, whereas miR-142-3p knockdown up-regulated such expression in SUNE-1 cells (Fig. 4C and D). In addition, SOCS6 expression was significantly lower in NPC tissues than in nasopharyngitis samples and was negatively correlated with miR-142-3p expression (Fig. 4E and F). These results indicate that miR-142-3p binds directly to the SOCS6 3'-UTR to repress its gene expression.

miR-142-3p promotes NPC cell growth by modulating SOCS6 expression

To further study the functional relationship between miR-142-3p and SOCS6, we explored whether miR-142-3p enhanced NPC growth by down-regulating SOCS6. For this purpose, SOCS6 siRNA and antagomiR-142-3p were co-transfected into SUNE-1 cells. Western blot analysis verified that SOCS6 expression was down-regulated by SOCS6 siRNA (Fig. 5A). Cell viability, BrdU, and cell cycle progression assays showed that down-regulation of miR-142-3p significantly inhibited the growth of NPC cells. However, knockdown of SOCS6 expression by SOCS6 siRNA in antagomiR-142-3p-transfected cells reversed the inhibitory effects of antagomiR-142-3p (Fig. 5B, C and D). Collectively, these data indicate that miR-142-3p inhibits cell growth by suppressing its target gene SOCS6.

Discussion

In the present study, we showed that miR-142-3p was significantly up-regulated in NPC clinical specimens and cell lines. Inhibition of miR-142-3p reduced NPC cell proliferation, induced cell cycle arrest, and inhibited tumor growth in nude mice. miR-142-3p down-regulated SOCS6 expression by directly binding to its 3'-UTR, and knockdown of SOCS6 significantly reversed the suppressive effect of miR-142-3p inhibition. Our study indicates

Fig. 4. SOCS6 is the direct downstream target of miR-142-3p. (A) Schematic showing the SOCS6 3'-UTR-containing reporter construct. (B) Luciferase activity of wild-type or mutant SOCS6 3'-UTR luciferase vectors in the presence of miR-142-3p. qRT-PCR (C) and western blot (D) analysis of SOCS6 mRNA and protein expression in response to over-expression of miR-142-3p in NP69 cells or knockdown of miR-142-3p in SUNE-1 cells. (E) SOCS6 mRNA expression in 26 primary NPC tissues and 11 nasopharyngitis samples. (F) Scatter plot showing the correlation between miR-142-3p and SOCS6 expressions in NPC tissues. Data represent the mean $\pm$ SD of three independent experiments. * $P < 0.05$.

that miR-142-3p acts as a novel proliferation promoter by directly targeting SOCS6 in NPC.

There are currently two opposing views on the role of miR-142-3p in human cancer. miR-142-3p is down-regulated in thyroid carcinoma, hepatocellular carcinoma (HCC), osteosarcoma and colon cancer [16-19]. Chai et al. reported that miR-142-3p over-expression inhibits the ability of CD133-expressing HCC cells to self-renew, initiate tumors, invade, migrate and resist chemotherapy [17]. Xu et al. showed that over-expression of miR-142-3p inhibits osteosarcoma cell proliferation, migration and invasion, whereas knockdown of miR-142-3p has the opposite effects [18]. On the other hand, miR-142-3p is up-regulated in esophageal cancer, head and neck squamous cell carcinoma, NSCLC and T-cell acute lymphoblastic leukemia [20-23]. High levels of miR-142-3p alleviated the suppressive effect of PKA on T-leukemic cell proliferation [23]. MiR-142-3p represses TGF- β -induced growth inhibition by down-regulating TGF β R1 in NSCLC [22]. Consistent with the latter observation, our data showed that miR-142-3p is over-expressed in NPC tissues and cell lines. Knockdown of miR-142-3p inhibited NPC cell proliferation, induced G1 arrest, and repressed the tumorigenesis of NPC cells. These results suggest that deregulation of miR-142-3p plays important roles in promoting carcinogenesis and progression of NPC. However, further study is needed to characterize the underlying molecular mechanisms.

We used online bioinformatics tools to identify SOCS6 as a potential target of miR-142-3p. Luciferase assays demonstrated that miR-142-3p could directly bind to the 3'-

Fig. 5. miR-142-3p promotes NPC growth by down-regulating SOCS6. (A) SUNE-1 cells were transfected with antagomiR-142-3p, SOCS6 siRNA, antagomiR-NC, or co-transfected with antagomiR-142-3p and SOCS6 siRNA. After 48h, SOCS6 protein level was examined by western blotting. CCK-8 (B), BrdU incorporation (C) and cell cycle assays (D) were performed to detect NPC cell growth. Data represent the mean $\pm$ SD of three independent experiments. * P < 0.05.

UTR of SOCS6. SOCS6 is a member of the SOCS family and participates in negative feedback regulation of receptor tyrosine kinase signaling [26, 27]. Recently, SOCS6 was found to play essential roles in tumor development and tumorigenesis in various cancers. SOCS6 is down-regulated in gastric cancer (GC) samples and inhibits GC cell growth and colony formation [28, 29]. SOCS6 is also down-regulated in pancreatic cancer specimens, and was shown to inhibit the proliferation, migration and invasion of pancreatic cancer cells and to increase cell apoptosis [30]. Moreover, the expression of SOCS6 is markedly reduced in HCC, and SOCS6 down-regulation is correlated with aggressive tumor progression and poor prognosis [31]. In our study, we found that SOCS6 was down-regulated in NPC tissues and its expression was inversely correlated with that of miR-142-3p. Furthermore, miR-142-3p significantly suppressed the expression of SOCS6 in NPC cells. siRNA mediated knockdown of SOCS6 in SUNE-1/antagomiR-142-3p cells reversed the inhibitory effect of miR-142-3p silencing. Our results indicate that miR-142-3p up-regulation may be an important mechanism of down-regulation of SOCS6 in NPC cells that contributes to the malignancy of NPC.

In summary, we showed that miR-142-3p is over-expressed in NPC tissues and cell lines, and knockdown of miR-142-3p inhibited NPC cell growth by targeting SOCS6. The miR-142-3p–SOCS6 axis may provide insight into the mechanisms of NPC progression and suggests potential novel strategies for the treatment of NPC.

Disclosure Statement

None disclosed.

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