

MicroRNA-4649-3p inhibits cell proliferation by targeting protein tyrosine phosphatase SHP-1 in nasopharyngeal carcinoma cells

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Abstract. The present study aimed to investigate the influence of microRNA-4649-3p on nasopharyngeal carcinoma (NPC) cell proliferation and how it regulated SHP-1 expression. The online software TargetScan was used to predict the microRNAs targeting SHP-1 and identified that miR-4649-3p was one of the possible miRNAs targeting SHP-1. Subsequently, quantitative polymerase chain reaction (PCR) was used to detect the expression level of miR-4649-3p and SHP-1 mRNA in different NPC cell lines. The miR-4649-3p mimics and inhibitors were transfected into NPC cells and cell proliferation was examined by the MTT assay. The SHP-1 expression level was determined by PCR and western blot analysis. Lentivirus containing the SHP-1 gene and miR-4649-3p mimics was co-transfected into the NPC cells and cell proliferation was detected by the MTT assay. The expression level of miR-4649-3p and SHP-1 mRNA was negatively correlated in the NPC cell lines. miR-4649-3p mimics suppressed NPC cell proliferation whereas miR-4649-3p inhibitors promoted NPC cell proliferation. The SHP-1 expression level was suppressed when transfected with miR-4649-3p mimics in NPC cells. The miR-4649-3p inhibitors increased SHP-1 expression. The luciferase reporter assay showed that miR-4649-3p directly targeted SHP-1 by binding to the 3'-untranslated region of SHP-1 mRNA. Overexpression of SHP-1 inversed the inhibited effect of miR-4649-3p mimics on cell proliferation. In conclusion, miR-4649-3p inhibits cell proliferation by targeting SHP-1 in NPC cells.

Introduction

Nasopharyngeal carcinoma (NPC) is mainly prevalent in Southern China and Southeast Asia (1). Radiation therapy (RT) is the primary treatment for NPC. Intensity-modulated radiotherapy (IMRT), which exhibits fewer side effects compared with conventional two- and three-dimensional conformal radiation, is now widely used and achieves a good local control rate (2). However, for locally advanced or recurrent patients, treatment with IMRT alone often shows a poor prognosis (3). Besides RT, chemotherapy could also be administered as neoadjuvant, concurrent or adjuvant and is beneficial to NPC treatment. Even with the combination of RT and chemotherapy, local failure occurs in 7-13% of patients following primary treatment for NPC (4). In addition to RT and the traditional chemotherapy, targeted therapy has attracted increasing attention for cancer treatment in the past few decades. Despite the success in lung (5), breast (6-8) and colorectal cancer (9,10), phase II studies of targeted therapy showed insufficient results for NPC treatment. Drugs involved in the studies, including gefitinib, sorafenib and cetuximab, did not show a clear benefit to progression-free survival (PFS) or overall survival (OS) (11-14). These results suggested that traditional molecular targets, which are beneficial in other tumors, may not work well in NPC treatment. Therefore, it is necessary to discover new molecular targets in NPC treatment and improve the prognosis.

SHP-1 is an SH2 domain containing protein tyrosine phosphatase (PTP), which consists of 17 exons and 16 introns and spans ~17 kb DNA (15,16). PTP SHP-1 expression increased in NPC tissue and was associated with radiation resistance and local recurrence (17). Knocking down SHP-1 by siRNA resulted in higher radiosensitivity in the NPC and lung cancer cell lines (18,19). According to these studies, SHP-1 may be a potential target in NPC treatment and silencing SHP-1 may help improve the NPC prognosis.

The discovery of microRNAs (miRNAs or miRs) established a new era for targeted therapy. miRNAs are small non-coding RNAs that containing 19-25 nucleotides. These small non-coding RNAs regulate the target gene expression at the post-transcriptional level by binding to the 3' or 5'-untranslated region (UTR) and cause the degradation of target mRNA (20,21). By regulating oncogene and anti-oncogene expression, miRNAs have important roles in tumor initiation

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Abbreviations: NPC, nasopharyngeal carcinoma; RT, radiation therapy; IMRT, intensity-modulated radiotherapy; OS, overall survival; PFS, progression-free survival; UTR, untranslated region; SCR, control scrambled sequence

Key words: miRNA, nasopharyngeal carcinoma, protein tyrosine phosphatase, SHP-1, proliferation

Table I. Primer sequences for PCR.

Genes	Sequences
miR-4649-3p	Forward: 5'-ACACTCCAGCTGGGTCTGAGGCCTGCCTC-3' Reverse: 5'-CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGTGGGGA-3'
U6 snRNA	Forward: 5'-CTTCGGCAGCACATATAC-3' Reverse: 5'-GGCCATGCTAATCTTCTC-3'
SHP-1	Forward: 5'-ACCATCATCCACCTCAAGTACC-3' Reverse: 5'-CTGAGCACAGAAAGCACGAA-3'
β -actin	Forward: 5'-GATGAGATTGGCATGGCTTT-3' Reverse: 5'-CACCTTCACCGTTCCAGTTT-3'

and progression (22,23). For that reason, miRNAs are closely associated with the tumor behaviors, as they can be conveniently detected in blood and other body fluid and they have great potential to become new biomarkers to monitor tumor development and therapeutic effect (24-26). As carcinogenesis is often correlated to abnormal miRNAs expression, reconstructing the normal miRNA network is a novel strategy in tumor treatment (27). A paradigm of miRNA therapeutics has been reported by Duchaine and Slack (28). The paradigm pointed out a 'double strategies' concept: Using miRNAs as an anticancer agent or as cancer therapeutic targets (29).

In the present study, miRNA was used as an anticancer agent in NPC treatment. The miRNAs targeting SHP-1 were used to treat NPC cells, explore how it affects NPC cell proliferation and how it regulates SHP-1 expression.

Materials and methods

Cell culture. The NPC cell lines, CNE-1 and CNE-2, were purchased from the Cell Bank of Sun Yat-sen University (Guangzhou, China). 5-8F was obtained from the Cell Bank of Xiangya University (Changsha, China). C666-1, 6-10B, HNE-2, HNE-1 and HONE-1 were obtained from the Cell Bank of JRDUN Biotechnology (Shanghai, China). Cells were routinely cultured in RPMI-1640 (HyClone, Logan, UT, USA) supplemented with 12% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (HyClone). The cells were maintained at 37°C in a humidified incubator with 5% CO₂.

Lentivirus, miRNA mimics or inhibitor transfection. miRNA mimics or inhibitors were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The control scrambled sequence (SCR) was used as a negative control. The miRNA mimics, inhibitors and SCR were transfected into NPC cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Lentiviruses containing the SHP-1 gene (LP-H1802-Lv201-C0010) and negative oligomers (LP-Neg-Lv201-0200) were purchased from GeneCopoeia (Guangzhou, China; 8.6x10⁹ copies/ml). The two lentiviral stocks (50 μ l; LP-H1802Lv201 and LP-NegLv201) were transfected into the CNE-2 cells. Puromycin (2 μ g/ml) was used to select the lentiviral-transfected cells. Total protein was isolated, and SHP-1 expression was detected by western blotting.

MTT assay. Cells were seeded into 96-well culture plates and incubated for 1, 2, 3, 4 and 5 days, respectively. The serum-containing culture medium was exchanged for normal culture medium not containing serum and 20 μ l MTT (5 mg/ml; Sigma, St. Louis, MO, USA) was added. The samples were incubated in the dark for 4 h. The culture medium was removed, 150 μ l dimethyl sulfoxide (Sigma) was added and the sample was slowly agitated for 15 min. The optical density (OD) at 490/630 nm was tested using a microplate reader system (BioTek Instruments, Inc., Winooski, VT, USA).

Western blotting. Cells were harvested, lysed by the radioimmunoprecipitation assay (Google Biotechnology Ltd. Co., Wuhan, China) and total protein was extracted. Protein concentrations of the lysates were determined by the bicinchoninic acid protein assay system (Google Biotechnology Ltd. Co.). Equal amounts of protein were separated by 8-12% SDS-PAGE, and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% bovine serum albumin and subsequently probed with either anti-SHP-1 (Cat. no. 1606-1; monoclonal, rabbit, targeted against mouse, rat, human; Epitomics, Burlingame, CA, USA) or anti-GAPDH (Cat. no. sc-25778; polyclonal, rabbit, targeted against human; Santa Cruz Biotechnology, Dallas, TX, USA) antibodies. Following washing, the membrane was incubated with the appropriate horseradish peroxidase secondary antibody (Cat. no. A24537; polyclonal, goat, targeted against rabbit; Life Technologies, Carlsbad, CA, USA) and visualized by chemiluminescence using a chemiluminescence kit (Invitrogen), and the specific bands were recorded by a UV transilluminator (Uvitec Ltd., Avebury House, Cambridge, UK). GAPDH protein levels were used as a control to verify equal protein loading.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted by TRIzol (Invitrogen) and reverse transcription was used to generate cDNA, according to the manufacturer's instructions of the Takara RT-PCR kit (Takara Bio, Shiga, Japan). Subsequently, qPCR was performed according to the manufacturer's instructions of SYBR-Green (Applied Biosystems, Foster City, CA, USA) in a PCR amplifier (ABI Prism 7000; Applied Biosystems). StepOne™ software v2.1 was used to analyze the data. Primer sequences are shown in Table I.

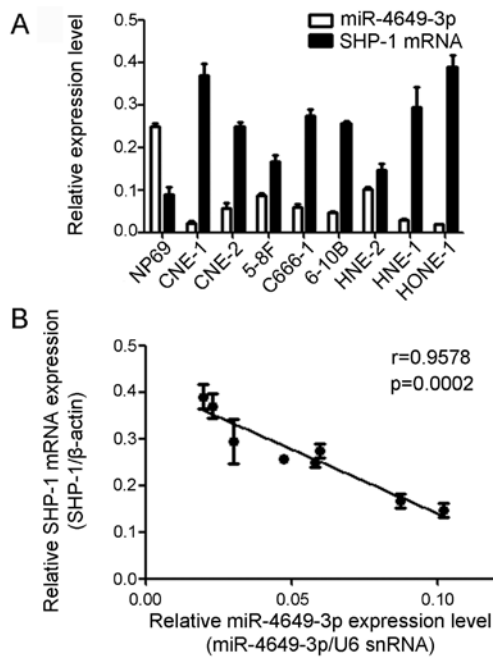


Figure 1. Expression of miR-4649-3p and SHP-1 mRNA in normal nasopharyngeal epithelia cell (NP69) and nasopharyngeal carcinoma (NPC) cells. (A) Compared with the normal nasopharyngeal epithelia cells, expression of miR-4649-3p in NPC cells was significantly decreased. However, SHP-1 mRNA in NPC cells was clearly increased. (B) Correlation between miR-4649-3p and SHP-1 mRNA was analyzed by the Pearson correlation test in NPC cells. The analysis results suggested a Pearson correlation parameter of $r = -0.9578$ ($P = 0.0002$), meaning that miR-4649-3p expression was inversely correlated to SHP-1 expression. The data are presented as mean \pm standard deviation from at least three independent experiments.

Luciferase reporter assay. A 3'UTR fragment of SHP-1 containing the putative miR-4649-3p binding site (1018-1024 nucleotides) was amplified by PCR. The PCR product was subcloned into a p-GL3 vector (Promega, Madison, WI, USA) immediately downstream to the luciferase gene sequence. A p-GL3 construct containing the 3'UTR of SHP-1 with a mutant sequence of the miR-4649-3p binding site was also synthesized. All the constructs were verified by DNA sequencing. Cells were seeded in 96-well plates and subsequently co-transfected with 100 ng constructs with or without miR-4649-3p mimics or inhibitors. At 48 h after transfection, luciferase activity was detected using a dual-luciferase reporter assay system (Promega) and normalized to Renilla activity.

Statistical analysis. Experimental data are expressed as the means \pm standard deviation from ≥ 3 independent experiments. Differences in measured variables between experimental and control groups were assessed using the t-test (SPSS 21.0 software; IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-4649-3p is downregulated in NPC cell lines accompanied with SHP-1 upregulation. qPCR was used to detect the miR-4649-3p and SHP-1 mRNA expression level in the normal nasopharyngeal epithelia cell line and 8 different NPC cell lines. Compared with the normal nasopharyngeal epithelia

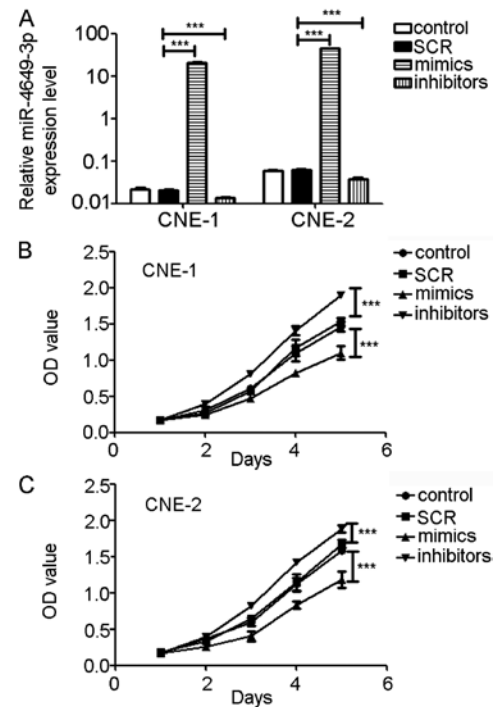


Figure 2. miR-4649-3p inhibited NPC cell proliferation. miR-4649-3p mimics and inhibitors were transfected into the nasopharyngeal carcinoma (NPC) cells CNE-1 and CNE-2. Subsequently, cell proliferation was determined by MTT assays. miR-4649-3p mimics increased miR-4649-3p expression by 900- to 1,000-fold, while miR-4649-3p inhibitors decreased miR-4649-3p expression by 40-50%. (A) miR-4649-3p expression level in CNE-1 and CNE-2. miR-4649-3p mimics inhibited NPC cell proliferation and miR-4649-3p inhibitors promoted proliferation. (B) Cell proliferation in CNE-1 presented by the optical density (OD) value. (C) Cell proliferation in CNE-2 presented by the OD value. The data are presented as mean \pm standard deviation from at least three independent experiments; *** $P < 0.001$.

NP69 cells, the miR-4649-3p expression level was significantly decreased in the 8 NPC cell lines, CNE-1, CNE-2, HNE-1, HNE-2, 5-8F, 6-10B, C666-1 and HONE-1. However, the expression of SHP-1 mRNA was not the same as miR-4649-3p. The SHP-1 mRNA expression level in the NP69 cells was evidently lower compared to the NPC cells. The Pearson's correlation test was used to analyze the association between miR-4649-3p and SHP-1 mRNA expression in the 8 NPC cell lines. The results showed that miR-4649-3p expression was inversely correlated to the SHP-1 expression level. The Pearson correlation parameter was $r = -0.9578$ ($P = 0.0002$) (Fig. 1).

miR-4649-3p inhibits NPC cell proliferation. To evaluate whether miR-4649-3p affects NPC cell proliferation, miR-4649-3p mimics and inhibitors were used to transfect CNE-1 and CNE-2 cells. SCR was used as a negative control. NPC cells transfected with miR-4649-3p inhibitors showed a 40-45% decrease in miR-4649-3p expression. The miR-4649-3p mimic-transfected cells showed a 900- to 1,000-fold increase of miR-4649-3p expression. Three days after transfection, CNE-1 and CNE-2 cells were seeded in 96-wells plates and incubated for different times. Subsequently, cell proliferation, presented by the OD value, was determined. Cell proliferations were significantly inhibited in CNE-1 and CNE-2 cells treated with miR-4649-3p mimics. However, miR-4649-3p inhibitors significantly promoted cell proliferation (Fig. 2).

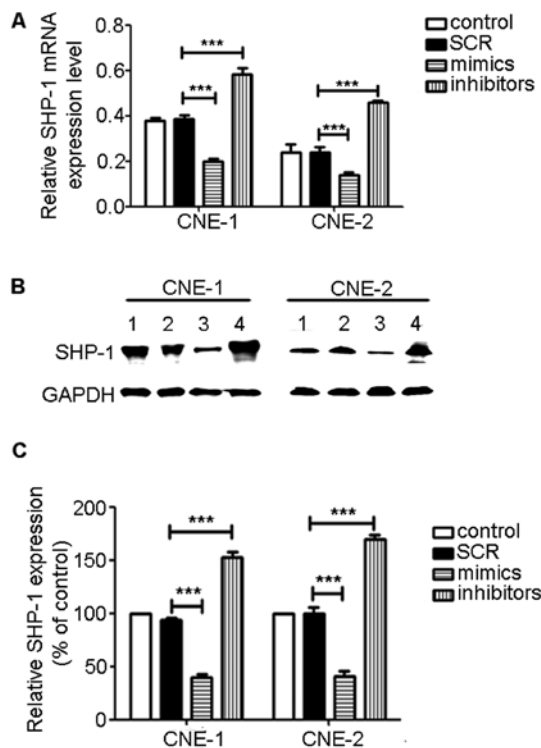


Figure 3. miR-4649-3p downregulates SHP-1 expression. miR-4649-3p mimics and inhibitors were transfected into nasopharyngeal carcinoma (NPC) cells CNE-1 and CNE-2. Subsequently, the SHP-1 expression level was determined by polymerase chain reaction and western blot analysis for the mRNA and protein levels, respectively. miR-4649-3p mimics significantly suppressed SHP-1 expression and miR-4649-3p inhibitors increased SHP-1 expression at the mRNA and protein levels. (A) SHP-1 mRNA expression level in CNE-1 and CNE-2. Data are presented as mean \pm standard deviation (SD) from three independent experiments; *** P <0.001. (B) SHP-1 protein expression in CNE-1 and CNE-2. Lane 1, control; lane 2, control scrambled sequence (SCR); lane 3, miR-4649-3p mimics; lane 4, miR-4649-3p inhibitors. Data are presented as one representative out of three independent experiments. (C) The optical density values were measured and presented as percentages of control. Data are displayed as mean \pm SD from three independent experiments; *** P <0.001.

miR-4649-3p mimics downregulate SHP-1 expression. To investigate how miR-4649-3p affects SHP-1 expression, NPC cells were transfected with miR-4649-3p mimics and inhibitors. After three days, total RNA and protein were extracted and SHP-1 expression was examined by qPCR and western blot analysis. Between the control and SCR group, SHP-1 mRNA and protein expression did not have a significant difference. However, in the miR-4649-3p mimic-transfected group, SHP-1 expression was inhibited at the mRNA and protein level. In the miR-4649-3p inhibitor-transfected group, the SHP-1 expression level was evidently increased (Fig. 3).

miR-4649-3p directly targets SHP-1 3'UTR. To further understand how miR-4649-3p upregulated SHP-1 expression, luciferase reporter assays were performed. TargetScan human6.2 (www.targetscan.org) was used to predict the binding site of SHP-1 (PTPN6) with miR-4649-3p. According to the binding site sequence, a mutant sequence of the 3'UTR of SHP-1 was constructed by changing certain base pairs. Subsequently, the wild-type (wt) and mutant (mt) 3'UTR of SHP-1 were connected to a luciferase reporter gene and the wt-3'UTR and mt-3'UTR vectors were formed. The wt-3'UTR

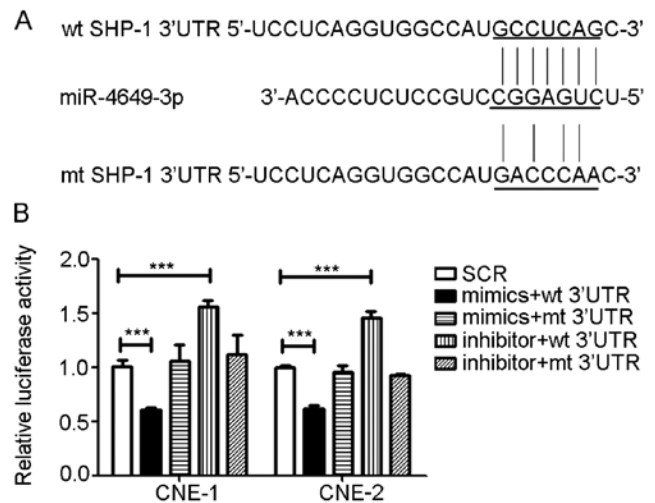


Figure 4. miR-4649-3p directly targets SHP-1 3'UTR. According to the binding site of SHP-1 3'UTR and miR-4649-3p, mt SHP-1 3'UTR was constructed by replacing certain base pairs. Subsequently, the luciferase reporter assay was used to examine the binding of miR-4649-3p and SHP-1 3'UTR in CNE-1 and CNE-2. When miR-4649-3p mimics and wt SHP-1 3'UTR were co-transfected into nasopharyngeal carcinoma (NPC) cells, luciferase activity was significantly inhibited. When co-transfected with miR-4649-3p inhibitors and wt SHP-1 3'UTR, luciferase activity was clearly increased. However, in the mt SHP-1 3'UTR groups, the effect of miR-4649-3p mimics and inhibitors was abolished. (A) Sequence of miR-4649-3p, wt SHP-1 3'UTR and mt SHP-1 3'UTR. (B) Luciferase activity of CNE-1 and CNE-2. Data are presented as mean \pm standard deviation from three independent experiments; *** P <0.001. SCR, control scrambled sequence; UTR, untranslated region; wt, wild-type; mt, mutant.

and miR-4649-3p mimics or inhibitors were co-transfected into the NPC cells. Luciferase activity of the miR-4649-3p mimics group was significantly suppressed while the activity of miR-4649-3p inhibitors groups was clearly increased. However, when the miR-4649-3p mimics or inhibitors was co-transfected with mt-3'UTR, the impact of the miR-4649-3p mimics or inhibitors on luciferase activity was abolished (Fig. 4).

Overexpression of SHP-1 abolishes the inhibition effect of miR-4649-3p. The lentivirus containing the SHP-1 gene (LP-H1802lv201) was used to transfect the NPC cells, CNE-1 and CNE-2, and SHP-1 overexpressed NPC cells were generated. LP-Neglv201 was a negative control. The MTT assay was performed again and an increasing SHP-1 expression level promoted cell proliferation. However, transfection of the miR-4649-3p mimics into NPC cells inhibited cell proliferation. When miR-4649-3p mimics and LP-H1802lv201 were co-transfected into NPC cells, the influence of SHP-1 on cell proliferation was abolished (Fig. 5)

Discussion

miRNAs have attracted increasing attention since being discovered, particularly in the field of cancer diagnosis and therapy. Since they can be detected in peripheral blood, body fluid and even in feces, they are extremely convenient for clinical use. Thus far, a significant number of studies have been performed utilizing miRNAs, from tumor markers to cancer therapy (21,30-37,39). Traditional chemotherapeutic agents have certain limitations, among which severe side effects and drug resistance are the

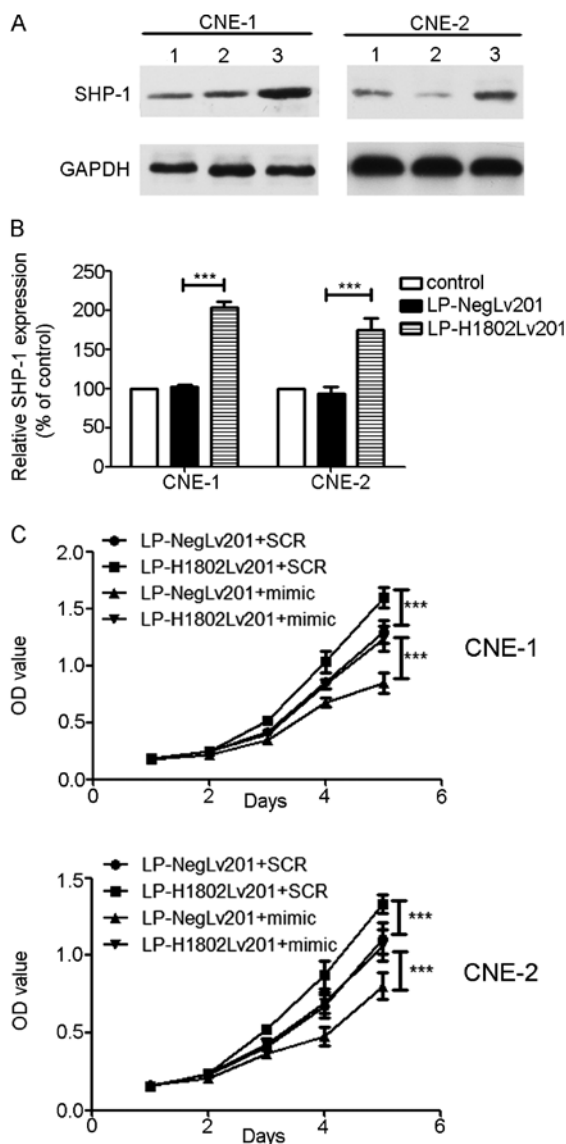


Figure 5. Overexpression of SHP-1 abolishes the inhibition effect of miR-4649-3p. Lentivirus containing the SHP-1 gene (LP-H1802Lv201) was used to transfect nasopharyngeal carcinoma (NPC) cells and obtain SHP-1 overexpressed NPC cells. LP-NegLv201 was a negative control. Western blot analysis was used to detect the transfected efficiency. LP-H1802Lv201 promoted NPC cell proliferation in contrast to LP-NegLv201. When LP-H1802Lv201 and miR-4649-3p mimics were co-transfected into NPC cells, the influence of LP-H1802Lv201 was reversed. (A) SHP-1 expression level in CNE-1 and CNE-2. Lane 1, CNE-1/CNE-2; lane 2, LP-NegLv201; lane 3, LP-H1802Lv201. (B) The optical density values were measured and presented as percentages of control. Data are presented as mean \pm standard deviation from three independent experiments; *** P <0.001. (C) Cell proliferation of CNE-1 and CNE-2, represented by the optical density value.

major ones. Through binding to target gene mRNA, miRNAs can knockdown the oncogene expression. The strategy of using miRNAs to inhibit oncogene expression provides a new therapeutic approach with high selectivity and small side effects. Increasing research has focused on using miRNAs as new therapeutic agents in tumor therapy. Inhibiting NF- κ B activation by miR-31 in breast cancer promoted cell death and increased cell sensitivity to ionizing radiation (38). Zhang *et al* (39) found that miR-451 can increase radiosensitivity of NPC cells by targeting RAB14. Recently, certain investigators have tried to deliver miRNAs in combination with traditional chemotherapeutic

drugs in cancer therapy. They found that the combination of miRNAs and chemotherapeutic drugs was effective and could enhance the sensitivity of cancer cells (40).

In the past few years, studies of miRNAs in NPC have achieved significant advancements. A number of miRNAs have been found to be associated with NPC formation, progression and other malignant behaviors. The majority of these miRNAs have roles in NPC pathology by modulating their target gene expression, including cell proliferation, migration, invasion and radiosensitivity.

SHP-1 is an SH2 domain containing PTP, which has 17 exons and 16 introns and spans ~17 kb (15,16). It is strongly expressed in normal hematopoietic cells and weakly expressed in certain hematological malignancies. However, specific studies have found that SHP-1 is highly expressed in certain epithelial carcinoma cells, such as ovarian and breast cancer cell lines (41-45). It has been reported that SHP-1 was involved in NPC initiation and progression. Knocking down SHP-1 enhances radiosensitivity in the NPC and NSCLC cells (17-19). The present study aimed to find a way in which SHP-1 expression could be downregulated and thus inhibit NPC malignant behaviors. The achievements reached in miRNA research have offered a new insight.

As miRNAs can selectively regulate target gene expression at a post-transcription level, our attention was focused on miRNAs targeting SHP-1. First, the miRNAs that target SHP-1 were predicted using TargetScan human6.2. Ten candidate miRNAs were selected and tested. Among these, miR-4649-3p suppressed SHP-1 expression most significantly (data not shown). Thus, the present study aimed to investigate the influence of miR-4649-3p on NPC cell proliferation and SHP-1 expression. The miR-4649-3p and SHP-1 expression levels were examined in normal nasopharyngeal epithelia NP69 cells and 8 NPC cells. Compared with the normal nasopharyngeal epithelia cells, miR-4649-3p was downregulated whereas SHP-1 was upregulated in NPC cell lines. Increasing miR-4649-3p expression ectopically inhibited NPC cell proliferation. PCR and western blot analysis showed that miR-4649-3p mimics suppressed SHP-1 expression. The luciferase reporter assay indicated that miR-4649-3p suppressed SHP-1 expression by binding to SHP-1 3'UTR and inhibiting SHP-1 mRNA translation. To verify that miR-4649-3p inhibited NPC cell proliferation by targeting SHP-1, we ectopically expressed SHP-1 in NPC cells and found that the effect of the miR-4649-3p mimics was reversed. Therefore, ectopically expressing miR-4649-3p in NPC cells may potentially be a new strategy in NPC treatment.

Acknowledgements

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