

MiR-34a Regulates Nasopharyngeal Carcinoma Radiosensitivity by Targeting SIRT1

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Abstract

Background/Aims: Nasopharyngeal carcinoma is a common head and neck cancer in South China and Southeast Asia. Radiotherapy is the standard treatment for nasopharyngeal carcinoma. Accumulating evidence showed that the expression of miR-34a was abnormal in nasopharyngeal carcinoma. Here, this study investigates the effect of miR-34a on radiosensitivity of nasopharyngeal carcinoma cells and explored the underlying mechanisms.

Methods: Reverse transcription quantitative polymerase chain reaction was used to analyze the expression of miR-34a in nasopharyngeal carcinoma cell lines and NP69 cells. The effect of miR-34a on radiosensitivity of nasopharyngeal carcinoma (CNE-1 cells) was evaluated by Cell Counting Kit-8, flow cytometry, and Transwell migration assays following transfection with miR-34a mimic. Luciferase reporter assay was used to assess the target genes of miR-34a.

Results: In this study, it revealed that miR-34a was downregulated, while silent information regulator 1 was upregulated in nasopharyngeal carcinoma cell lines. The overexpression of miR-34a enhanced radiation-induced proliferation and migration inhibition and apoptosis in CNE-1 cells. Bioinformatics, Luciferase reporter, reverse transcription quantitative polymerase chain reaction, and Western blotting assays indicated that silent information regulator 1 is a direct target of miR-34a in nasopharyngeal carcinoma cells. Knockdown of silent information regulator 1 enhanced radiosensitivity of nasopharyngeal carcinoma cells as evidenced by increasing proliferation and migration inhibition and apoptosis after radiation exposure.

Conclusion: In summary, our results indicated that the overexpression of miR-34a enhanced radiosensitivity of nasopharyngeal carcinoma cells by targeting silent information regulator 1. Further studies are warranted to investigate the potential use of miR-34a in the clinical management and treatment prediction of patients with nasopharyngeal carcinoma.

Keywords

nasopharyngeal carcinoma, miR-34a, radiosensitivity, SIRT1

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**Introduction**

Nasopharyngeal carcinoma (NPC) is a common head and neck cancer, highly endemic in Southeast Asia and with highest incidence, and incidence rate varied from 20 to 50 cases per 100,000 people. Treatment of NPC is primarily based on radiotherapy. Over the past few years, people have witnessed the rapid replacement of 2-dimension conventional radiotherapy (2DCRT) by intensity-modulated radiotherapy (IMRT), which has become the most commonly used radiation option for NPC due to its ability to protect normal tissues while elevating the dosage in the target region. Although radiotherapy for NPC is encouraging, recurrence of NPC is still evident after initial treatment. At present, molecular markers have been shown to be effective in predicting treatment outcome of multiple solid tumors, but their application in monitoring radiation response or prognosis in NPC is still limited.

MicroRNA (miRNA) is a class of endogenous single-stranded small noncoding RNA molecules. Mature miRNA can recognize and bind to the 3′-untranslated regions (3′-UTRs) of target messenger RNA (mRNA) to cause translational repression or mRNA degradation, regulating cell proliferation, cell cycle, migration, apoptosis, and angiogenesis. Increasing research studies have indicated that the expressions of miRNAs are closely associated with the radiosensitivity of tumors. MiR-34a is usually lowly expressed in a variety of tumors including NPC and acts as a tumor suppressor to induce apoptosis. Long et al. reported that hypofractionated radiotherapy can induce miR-34a expression and enhance apoptosis in human NPC cells. Therefore, we speculated that miR-34a overexpression could enhance the radiosensitivity of NPC cells. Silent information regulator (SIRT1) has been reported to be highly expressed in a variety of malignancies and to enhance its radiosensitivity. However, the expression of SIRT1 in NPC and its effect on the radiosensitivity of NPC are still unknown. Previous study has shown that miR-34a plays proapoptotic and pro senescence roles in mesenchymal stem cells (MSCs) by targeting SIRT1. High glucose could upregulate miR-34a-5p to aggravate fibrosis by targeting SIRT1 in HK-2 cells. However, whether miR-34a can enhance the radiosensitivity of NPC by regulating SIRT1 has not been reported. In this study, we addressed the functional role of miR-34a in the responsiveness of NPC cells to radiation treatment. In the end, it revealed that miR-34a was downregulated in NPC cell line. And the overexpression of miR-34a could enhance the radiosensitivity of nasopharyngeal carcinoma (CNE-1 cells) through inhibiting SIRT1.

**Materials and Methods**

**Cell Culture and Transfection**

The human immortalized nasopharyngeal epithelial cell lines NP69 (BNCC338439), CNE-2 (BNCC341794), and HONE-1 (BNCC338405) were purchased from BeNa Culture Collection. The human NPC cell line CNE-1 (CL-0063) was purchased from Procell Life Science & Technology Co., Ltd. Cells were cultured in Dulbecco’s Modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin and streptomycin (Solarbio) in a humidified atmosphere of 5% CO2 at 37°C.

The miR-34a mimic and negative control molecules (NC-mimic) were purchased from Guangzhou RiboBio Co, Ltd. The siRNA against SIRT1 (SIRT1-siRNA) and negative control siRNA (NC-siRNA) were chemically synthesized by Shanghai GenePharma Technology Co, Ltd. Lipofectamine 2000 (Invitrogen) was used to perform siRNA transfection according to the manufacturer’s protocol with 50 pmol/mL miR-34a mimic and negative control molecules or 40 pmol/mL SIRT1-siRNA and NC-siRNA. Transfection was terminated following incubation for 24 hours.

**Reverse Transcription Quantitative Polymerase Chain Reaction**

Relative miR-34a and SIRT1 mRNA expressions were routinely detected by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Briefly, total RNA was isolated from cell lines using TRIzol reagent (Invitrogen), according to the manufacturer’s instruction. The levels of mature miRNAs in the cell lines were determined using the Bulge-Loop miRNA RT-qPCR Primer Set (RiboBio Co, Ltd). U6 was used as the endogenous control. The level of SIRT1 in the cell lines was determined using the SYBR Premix Ex Taq II (TaKaRa). β-actin served as internal control. The specific primer sequences were as follows: SIRT1 forward, 5′-GCC AGA GTC CAA GTT TAG AAGA-3′ and reverse, 5′-CCA TCA GTC CCA AAT ACCAG-3′; β-actin forward, 5′-GAA GAT CAA GAT CAT TGC TCC T-3′ and reverse, 5′-TAC TCC TGG TGT ATC GA-3′; miR-34a forward, 5′-TGG CAG TGT CTT AGC TGG TTG-3′ and reverse, 5′-CAT TGG TGT CGT TGT GCT CT-3′; U6

**Abbreviations**

CCK-8, Cell Counting Kit-8; 2DCRT, 2-dimensional conventional radiotherapy; DMEM, Dulbecco’s Modified Eagle’s medium; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IMRT, intensity-modulated radiotherapy; miRNA, microRNA; mRNA, messenger RNA; NPC, nasopharyngeal carcinoma; PI, propidium iodide; RT-qPCR, reverse transcription quantitative polymerase chain reaction; SIRT1, silent information regulator 1; 3′-UTR, 3′-untranslated region

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forward, 5'-GCT TCG GCA GCA CAT ATA CTA AAAT-3'
and reverse, 5'-CGC TTC ACG AAT TTG CGT GTC AT-3'.

**Western Blotting Assay**

Cells specimens were lysed with RIPA lysis buffer (Boster). Protein concentrations were measured by bicinchoninic acid protein assay (Boster). Equal amounts of total protein were boiled in sample buffer and separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Proteins were then transferred to a polyvinylidene fluoride membrane (Millipore) and blocked with 5% skim milk powder at room temperature for 1 hour. The membrane was incubated with rabbit monoclonal antibody against SIRT1 (#2496; Cell Signaling Technology), matrix metalloproteinase (MMP)-2 (#40994; Cell Signaling Technology), MMP-9 (#13667; Cell Signaling Technology), and goat anti-rabbit immunoglobulin G. An electrochemiluminescence kit was used to visualize the protein bands. Protein levels were calculated relative to β-actin.

**Luciferase Reporter Assay**

CNE-1 cells growing in log-phase were harvested and then seeded into 24-well plates at a density of 3 × 10^4/well. Following culture for 6 hours, the cells were transfected with the Renilla luciferase pRL-TK plasmid plus the recombinant Firefly luciferase pGL3 reporters containing 3'-UTR region of human SIRT1 (GenePharma Technology Co, Ltd) in combination with miR-34a mimic and NC mimic by using Lipofectamine 2000. Luciferase and Renilla signals were measured 24 hours after transfection using a dual-luciferase reporter assay kit (Promega).

**Cell Viability Assay**

The proliferation ability of CNE-1 cells was evaluated using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc). In brief, CNE-1 cells growing in log-phase were harvested and then seeded into 96-well plates at a density of 6 × 10^3/well overnight. Subsequently, cells were transfected with SIRT1-siRNA sequence and/or miR-34a mimic. Following transfection for 6 hours, cells were treated with different doses of X-ray radiation (0-8 Gy). At the indicated time points of incubation for 24 hours, the original culture medium was removed and 100 μL fresh medium mixed with CCK-8 at a ratio of 10:1 was added to each well. The absorbance at a wavelength of 450 nm was recorded using a microplate reader (Thermo Fisher Scientific, Inc).

**Annexin-V/Propidium Iodide Double-Staining and Flow Cytometry Assays**

Apoptotic assays were performed using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BD Biosciences), according to the manufacturer’s protocol. CNE-1 cells growing in log-phase were harvested and then seeded into 6-well plates at a density of 1 × 10^5/well overnight. Subsequently, cells were transfected with SIRT1-siRNA sequence and/or miR-34a mimic. Following transfection for 6 hours, cells were treated with different doses of X-ray radiation (0-8 Gy). At the indicated time points of incubation for 24 hours, cells were stained with Annexin V-FITC and PI for 15 minutes in the dark following washing, trypsin digestion, and centrifugation. The apoptotic cells were analyzed using a flow cytometer (BD Biosciences) within 1 hour.
Cell migration assay was performed using 24-well Transwell chamber (Corning) with 8.0 μm pore membranes. Briefly, CNE-1 cells (1 × 10^5) were suspended in a serum-free DMEM and then plated in the upper chamber (200 μL) of Transwell. The lower chamber was filled with 600 μL DMEM containing 10% FBS. After being incubated for 48 hours at 37 °C under 5% CO₂, nonmigrated cells were wiped out and the migrated
cells on the lower surface were fixed with 70% ethanol for
20 minutes and stained with 0.1% crystal violet for 15 minutes.
Finally, the migratory cells were photographed in 5 randomly
chosen fields at a ×200 magnification and counted using Image
J software (NIH).

Statistical Analysis
All results were expressed as means ± SD from 3 separate
experiments. Statistical analysis was performed using SPSS
20.0 software (SPSS, Inc). Statistical significance between the
3 groups was evaluated by Student t test. Statistical significant-
cance between the 2 groups was evaluated by 1-way analysis
of variance. P < .05 was considered to indicate a statistically
significant difference.

Results
Expression of MiR-34a Is Downregulated, While SIRT1 Is
Upregulated in NPC Cell Lines
To explore the biological function of miR-34a and SIRT1 in
NPC progression, we determined the expression of miR-34a
and SIRT1 in NPC cell lines. As shown in Figure 1C to F, SIRT1 mRNA and protein were significantly
upregulated in NPC cell lines. These results indicated that the
miR-34a and SIRT1 are dysregulated in NPC.

Overexpression of MiR-34a Enhanced Radiosensitivity
of NPC Cells
To explore the impact of miR-34a on the radiosensitivity of
NPC, CNE-1 cells were treated with various doses (0-8 Gy) of
radiation following transfection with miR-34a mimic for
6 hours. The RT-qPCR results showed that the miR-34a expres-
sion was significantly increased in the miR-34a mimic group
compared with the control group, which indicated high trans-
fection efficiency in CNE-1 cells (Figure 2A). The CCK-8
results showed that the overexpression of miR-34a decreased
the proliferation of CNE-1 cells when subjected to different
doses of irradiation (0-8 Gy; Figure 2B). Flow cytometry
results showed that the miR-34a overexpression combined with
radiation significantly increased apoptosis (Figure 2C and D).
In addition, the Transwell migration assay revealed that the
overexpression of miR-34a decreased the migratory cell num-
ber of CNE-1 cells when subjected to different doses of irra-
diation (0-8 Gy; Figure 3). These data indicated that
overexpression of miR-34a enhanced the effect of X-ray radiation on cell proliferation and migration inhibition and apoptosis in NPC cells. In this study, since the 8 Gy radiotherapy dose had the best effect on the proliferation and migration inhibition and apoptosis of NPC cells, the radiotherapy dose we chose in the follow-up study was 8 Gy.

**Silent Information Regulator 1 Is a Direct Target of MiR-34a in NPC Cells**

To investigate the potential mechanisms of miR-34a in NPC, we predicted its target genes according to sequence complementation using TargetScan (http://www.targetscan.org/), mirDB (http://mirdb.org/), and DIANA TOOLS (http://diana.imis.athena-innovation.gr), then we found that SIRT1, whose 3'-UTR was contained a conserved putative target site for miR-34a (Figure 4A). The effect of miR-34a on the translation of SIRT1 mRNA into protein was then assessed by using a luciferase reporter assay. The miR-34a mimic significantly repressed the luciferase activity of the reporter gene with the wild-type construct but not with the mutant SIRT1 3'-UTR construct (Figure 4B). Western blotting revealed that the overexpression of miR-34a reduced the protein expression level of SIRT1 (Figure 4C). The RT-qPCR revealed that the overexpression of miR-34a significantly decreased the mRNA expression level of SIRT1 (Figure 4D). Taken together, we demonstrated that the miR-34a targets SIRT1 and suppresses its expression in NPC cells.

**Overexpression of MiR-34a Enhanced Radiosensitivity of NPC Cells Through Inhibiting SIRT1**

In order to confirm that miR-34a enhanced radiosensitivity by directly targeting SIRT1, the CNE-1 cells were transfected with miR-34a mimic and/or SIRT1-siRNA sequence and measured the cell proliferation, apoptosis, and migration. Our results showed that the expression levels of SIRT1 mRNA and protein were significantly decreased following transfection with SIRT1-siRNA sequence in CNE-1 cells (Figure 5A and B), indicating the expression of SIRT1 was successfully inhibited by SIRT1-siRNA sequence. The CCK-8 results showed that the silencing of SIRT1 could enhance the effect of miR-34a mimic on cell proliferation inhibition when treated with X-ray radiation at doses of 0 or 8 Gy (Figure 5C). Cell apoptosis analysis suggested that the silencing of SIRT1 could enhance the effect of miR-34a mimic on cell apoptosis when treated with X-ray radiation at doses of 0 or 8 Gy (Figure 5D and E). Transwell migration assay suggested that the SIRT1 silencing could enhance the effect of miR-34a mimic on migration inhibition when treated with X-ray radiation at doses of 0 or 8 Gy (Figure 6). In addition, Western blotting results showed that the SIRT1 silencing could enhance the inhibitory effect of miR-34a mimic on the expression of MMP-2 and MMP-9 when
treated with X-ray radiation at doses of 0 or 8 Gy (Figure 7). These results indicated that miR-34a enhanced radiosensitivity of NPC cells through inhibiting SIRT1.

**Discussion**

With the development of technology, IMRT has improved regional control and overall survival of NPC compared with 2DCRT and become the preferred radiation option for NPC. Radioresistance often limits the therapeutic efficacy of NPC; there is about 10% of patients with NPC receiving standard dose of radiation therapy cannot be controlled, while the higher doses of radiation therapy produce severe toxicity. Therefore, clarifying the molecular mechanisms responsible for NPC radioresistance provides a basis for solving this issue. Increasing studies have highlighted the biological roles of deregulated miRNAs in various malignant cell behaviors, including radioresistance.

MiR-34a is a member of the miR-34 family with 2 other members: miR-34b and miR-34c. MiR-34a is located on chromosome 1p36.22 and is ubiquitously expressed at higher levels than miR-34b/c with the exception of the lung tissues. Studies have suggested that miR-34a is an emerging miRNA in recent radiobiology studies and played an important role in the tumor radioresistance, normal tissue radiotoxicity, or as predictive biomarkers to radiation. JiHoon et al reported that the rhamnetin and cirsiliol induce radiosensitization in non-small cell lung cancer by miR-34a-mediated suppression of Notch-1 expression. MiR-34a expression was increased after radiotherapy in NPC cells. In the present study, we found that miR-34a is downregulated in NPC cell lines. The overexpression of miR-34a could enhance radiosensitivity of NPC cells. These data indicated that the miR-34a may act as a tumor suppressor whose downregulation contributed to radioresistance of NPC in vitro.

It is well known that the miRNAs exert significant biological functions by regulating various target genes. Silent
information regulator 1 is the mammalian homolog of yeast Sirt2, which catalyzes NAD$^+$-dependent histone deacetylation and ADP-ribosylation. Increasing research studies have indicated that SIRT1 was aberrantly expressed in a variety of malignant tumors and promoted the tumorigenesis. Moreover, SIRT1 has emerged as a novel target of radiation damage in hepatoma entities. The downregulation of SIRT1 enhanced radiation sensitization and radiation-induced apoptosis in A549 lung cancer cells and glioma CD133-positive cells. Silent information regulator 1 has been identified as a direct target of miR-34a in prostate cancer and gastric cancer. However, whether miR-34a could directly target SIRT1 in NPC is unclear. Using bioinformatics analysis, the SIRT1 is predicted as a potential target of miR-34a. The luciferase reporter assay demonstrated that miR-34a specifically binds to the SIRT1-3’-UTR. In addition, we found that the mRNA and protein levels

![Figure 6](image-url)

Figure 6. Overexpression of miR-34a enhanced the effect of X-ray radiation on cell migration inhibition through inhibiting SIRT1 in CNE-1 cells. The histogram showed the migrating cells per field. Data were obtained from 3 independent experiments and results are shown as the mean ± SD. **P < .01 versus control (0 Gy) group; ##P < .01 versus control (8 Gy) group; %P < .05 and $$P < .01 versus 0 Gy; @@P < .01 versus miR-34a mimic (0 Gy) group; $P < .01 versus miR-34a mimic (8 Gy) group. SIRT1 indicates silent information regulator 1.
of SIRT1 in CNE-1 cells were both decreased following transfection with miR-34a mimic. These results indicated that SIRT1 is a direct target of miR-34a, and miR-34a regulates SIRT1 expression level by targeting the 3′-UTR of SIRT1. Further to clarify whether SIRT1 is involved in miR-34a-regulated radiosensitivity, CNE-1 cells were transfected with SIRT1-siRNA sequence with high transfection efficiency. As expected, SIRT1 gene silencing was markedly reversed miR-34a-mediated enhancement on radiosensitivity of NPC cells, suggesting that miR-34a increased radiosensitivity by targeting SIRT1 in NPC.

In conclusion, our study demonstrated that miR-34a was downregulated in NPC and the overexpression of miR-34a improved radiosensitivity of NPC cells by targeting SIRT1. These findings are encouraging and suggest that miR-34a might be a promising therapeutic target for the treatment of NPC.

Authors’ Note
The data sets used or analyzed during the current study are available from the corresponding author on reasonable request. All authors agree to publish. This study did not require an ethical board approval because it did not contain human or animal trials.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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