The Long Non-Coding RNA SNHG16 Promotes NPC Cell Progression by Competitively Binding miR-23b-3p

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Research

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Abstract

**Background:** This study aims at verifying the effect of non-coding RNA SNHG16 on promotes NPC cell progression via binding miR-23b-3p.

**Methods:** The expression of non-coding RNA SNHG16 was detected by qRT-PCR in cell lines including c666-1 and HONE-1. Si-MCM6 and si-SNHG16 are transfected to cells to verify their effects on cell proliferation and apoptosis. MTT is used to measure cell viability while flow cytometry assay and transwell assay were used for cell apoptosis, cell cycle and invasion respectively. The expression level of MCM6 was determined by western blot. Relationships between mRNA MCM6 and IncRNA SNHG16 were explored by qRT-PCR and nude mouse tumorigenicity assay.

**Results:** The MCM6 was overexpressed in NPC tissues and IncRNA SNHG16 showed the same trend. Those two factors were correlated with high cancer stage. The expression of MCM6 was decreased after si-SNHG16 and dual luciferase reporter system demonstrated their combine with miR-23b-3p. Further we explored the down-regulation of IncRNA SNHG16 could inhibit NPC cell proliferation, colony formation and also accelerate cell apoptosis rate. And this result could be altered by adding miR-23b-3p inhibitor.

**Conclusion:** The IncRNA SNHG16 is able to promote the NPC proliferation via binding miR-23b-3p, which has potential for future treatment.

Introduction

Nasopharyngeal carcinoma (NPC) is a highly chemo sensitive cancer, a malignancy derived from of the nasopharynx epithelial cells, is endemic in Southern China and Southeast Asia as well as association with Epstein-Barr virus (EBV).[1] Currently, concurrent radio chemotherapy is a mainstay of curative treatment for NPC.[2] Although excellent control has been achieved with the fast development of irradiation techniques and their combination with chemotherapy, local recurrence still mainly results in the failure of treatment, at a rate of approximately 8–10%.[3] The resistant to radiotherapy and chemotherapy remains the main cause of treatment failure of NPC.[4] Therefore, there is still an urgent need to develop effective therapeutic strategies to overcome chemoresistance and figure out its underlying molecular mechanism.

With the development of sequencing technologies, more than 90% of the human genome is actively transcribed, although only a minority of it is translated into proteins. Long non-coding RNA (lncRNA) is generally defined as RNA transcripts contains over 200 nucleotides without protein coding potential.[5] Increasing evidence has indicated that lncRNAs are implicated in a variety of pathophysiological processes, such as gene expression, cell proliferation, apoptosis, and tumorigenesis.[6] Importantly, lncRNA was found to function as either oncogene or anti-oncogene to participate in the pathogenesis and development of many kinds of diseases including cancers.[7] It was recently identified that another lncRNA, Small Nucleolar RNA Host Gene 16 (SNHG16), might act as a potential cancer biomarker or
functional cancer development modulator in various human cancers [8–10]. Yet, the function of SNHG16 in NPC still remains unclear and is necessary to explore.

MicroRNAs (miRNAs) belongs to small non-coding RNAs family, which can regulate the post-transcriptional level of gene expression through binding to target mRNAs, leading to target mRNA degradation or translation suppression.[11] As highly conserved, short non-coding RNAs contains 21–25 nucleotides, miRNAs negatively affect gene expression at the post-transcriptional level by binding to the 3’ untranslated region (3’-UTR) of target mRNA, leading to its degradation or translational repression.[12] Growing evidence implies that miRNAs have been related to the disease pathogenesis. Therefore, discerning the relationship between miRNAs and diseases has become an important goal in the biological context.[13] Furthermore, it has been well documented that lncRNAs function as molecular sponges for miRNA by negatively regulating its expression.[4] Nevertheless, the relationship of SNHG16 and miRNA on pathological process of NPC cells remains to be investigated.

Identifying the specific lncRNAs and miRNAs related to NPC was the target of this study. To attain this object, we used microarray-based gene expression profiling to characterize the differentially expressed mRNAs and lncRNAs in NPC cells compared with controls. We found that SNHG16 may mediate NPC progress through miR-23b-3p. Our present study provides a deeper understanding of the NPC pathogenesis and might indicate a new direction for the treatment of NPC.

Methods

Cells and tissues collection

Firstly, we collected 15 NPC tissues and 15 adjacent normal tissues from The Affiliated Hospital of Shaanxi University of Chinese Medicine. Then the samples were immediately encapsulated, put into liquid nitrogen and stored at -80 °C for subsequent analysis. Before the experiment, we received informed consents from each patient. The experimental protocol was approved by the Ethics Committee of West China Hospital, Sichuan University.

We used six cell lines including c666-1, HONE-1, NP69, CNE, HNE1 and HEK-293T. They were collected from the Cellbank of the Chinese Academy of science (Shanghai, China). Cells were grown routinely in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) with 10 % fetal bovine serum (Gibco, Carlsbad, CA, USA) and cultured in a 37 °C humidified atmosphere with 5% CO₂.

Bioinformatics analysis

The data of RNA expression microarray (GSE12452) from 41 samples including 31 NPC tissues and 10 non-NPC tissues was obtained from Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE12452). The differentially expressed mRNA and IncRNA were selected when |the logarithm of fold change value| > 1 and $P < 0.05$ based on R project (https://www.r-project.org/) analysis. Then GSEA v3.0 software
(http://software.broadinstitute.org/gsea/index.jsp) was used for gene set enrichment analysis (GSEA). All results are shown by “joyplot” and “dotplot” packages through R project. Based on above research we chose cell cycle pathway to find the deep relationship between differential mRNAs and IncRNAs. TargetScan (http://www.targetscan.org/vert_71) was used to predict binding sites for miRNAs and mRNAs, while miRcode (http://www.mircode.org/index.php) was used to predict binding sites for miRNAs and IncRNAs.

**Plasmid construction and cell transfection**

On the GenePharma (Shanghai, China), the transfected materials MiR-23b-3p inhibitor, si-MCM, si-SNHG16, sh-MCM and sh-SNHG16 were purchased. Twenty-four hours before transfection, c666-1 and HONE-1 cells in the exponential phase were digested by pancreatin and made into cell suspension. Si-MCM6 and si-SNHG16 were used for cell experiment and the *in vitro* experiment. After trypsinization from flasks, cells were cultured in six-pore plates, incubated at 37 °C with 5 % CO\(_2\) for 18-24 h. Three hours before transfection, cells at about 80-90 % confluency were changed to the serum and antibiotic-free media. Then, cells were transfected using Lipofectamin 3000 reagent (Life Technologies, Gaithersburg, MD, USA) referring to manufacturer's instructions and incubated at the same conditions as above for 48 h. In addition to the *in vitro* experiment, based on the manual, concentrated lentiviral solutions of sh-SNHG16 and sh-MCM6 were mixed with two wells of nutrient solution contains c666-1 cells, respectively. Finally, the cells were digested by pancreatin and injected into mice after incubation for 48 h.

**RNA isolation and qRT-PCR**

The RNA in the two cell lines was extracted with TRIZol reagent (Invitrogen) following the instruction of manufacturer. The concentration and purity of extracted RNA were detected by a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA, USA). Then cDNA was synthesized using the Reverse Transcription System Kit (Applied Biosystems, Foster City, CA, USA). The detection of MCM6, GAPDH and miR-23b-3p expression was performed by RT-PCR using SYBR Green PCR Master Mix (Takara, Dalian, China) and TaqMan MicroRNA Assay Kit (Applied Biosystems) on Bio-Rad iQ5 Multicolor Real-Time qRT-PCR Detection system (Bio-Rad, Hercules, CA, USA). GAPDH was a reference gene for SNHG16 and MCM6, while U6 was the reference gene for miR-23b-3p. The primers for SNHG16, MCM6, GAPDH, miR-23b-3p and U6 (Table 1) were synthesized by Guangzhou Ribo-Bio (Guangzhou, China). All statistics were analyzed based on \(2^{-\Delta\Delta Ct}\) method.

**MTT assay**

Cell viability was analyzed via 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay (Sigma, St, Louis, MO, USA). Cells (3\times10^3 per well) were inoculated into 96-well plates, and followed by transfection with si-SNHG16, si-MCM6, si-SNHG16+ miR-23b-3p inhibitor, si-MCM6+miR-23b-3p inhibitor or respective negative control. After incubation for 24 h, 48 h, 72h, and 96 h at 37 °C, 20 μl of 0.5 mg/ml MTT was subsequently added to the wells (200 μl/well) and incubated for another 4 h. Then the culture
medium was discarded, and the precipitate was dissolved in 150 μl of DMSO (Sigma). Optical density value was measured at 570 nm using a Beckman microplate reader (Beckman, Brea, CA, USA).

Invasion assays

Invasion assays were applied using cell culture inserts with 8 μm pore transparent polyethylene terephthalate filters (Becton Dickinson, Bedford, MA, USA) coated with Matrigel. A total of 3×10⁴ cells disperse in 200 μl culture medium without serum were added to each insert, and 500 μl culture medium with 10 % FBS was added to the bottom chamber. Then the cells on the upper filter were removed at the end of incubation for 24 h at 37 °C, and those cells invaded the lower membrane were fixed in methanol and dyed with crystal violet. Five optical fields for each filter with triplicate inserts were randomly selected to calculate the number of invaded cells.

Western blot

Western blotting was performed as described in previous study. The BCA Kit (Sigma-Aldrich) was used to quantify the protein concentration. Then samples were separated through electrophoresis on 10-12% sodium dodecyl sulfate polyacrylamide gels, and the separated proteins were moved to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA), which was blocked with 5 % bovine serum albumin in Tris-buffered saline (TBS) buffer for 30 min at room temperature later and incubated by first antibody (MCM6 rabbit anti-human, 1:1000, Abcam, Cambridge, MA, USA; GAPDH rabbit anti-human, 1:2500, Abcam) at 4˚C overnight. After that, secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000, Abcam) were used, and immunoreactivity was visualized by the ECL western blotting detection system (GE Healthcare, Amersham, UK). Densitometric analysis of immunodetected bands was performed using Image Analysis software (Biorad).

Luciferase reporter assays

293T cells (l×10⁵) were transferred to 24-well plates in each well and incubated before transfection. Then the cells were co-transfected using miR-23b-3p, the wild type or mutant SNHG16 and MCM6. After 48 h of transfection, the cells were washed by PBS before incubated with shake for 15 min at room temperature. Then cell lysis buffer was collected at 4 °C and centrifuged for 2 min. The supernatant was collected and refrigerated at -80 °C refrigerator. Cell lysis buffer was added to 96-well plate with 10 μl per well. During the detection, 30 μl luciferase reagents II (Promega, Madison, WI, USA) were added. Then 30 μl stop buffer was added to terminate the activities. The luciferase activities were detected by the Dual-Glo luciferase reporter assay kit (Promega) for 48 h post transfection.

Flow Cytometric Analysis of Apoptosis

A total of 2×10⁵ cells of the transfection group and NC group were collected after post transfection, washed before resuspended with pre-cooled PBS following the instruction of Annexin V apoptosis detection kit (Life Technologies). Then the cells were incubated for 15 min before washed and
resuspended in 500 μl of binding buffer. Ten microliter of Annexin-V–FITC and propidium iodide respectively were added and the mixture was analyzed using the FACS Calibur. The data was analyzed by FACS Diva software.

Flow cytometry analysis of cell cycle

Cells in different groups were collected and washed in cold phosphate-buffered saline (PBS), fixed in 70% ethanol, and stored at 4 °C. DNA was treated with RNase A solution (500 units/ml) at 37 °C for 15 min and stained by PI (50 μg/ml) in 1.12% sodium citrate at room temperature before analysis. Flow cytometry determination of DNA content was done by FACS Calibur. The fractions of the cells in different phases were analyzed using Multicycle (Phoenix Flow System, San Diego, CA) cell cycle analysis software.

Tumor xenograft in nude mice

All experimental procedures were acknowledged by the Animal Care and Use Committee of West China Hospital, Sichuan University, followed the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Twenty male BALB/C nude mice at the age of four weeks (n = 5 per group were purchased from Shanghai SLAC Laboratory Animal Co., Ltd, China) for xenograft tumor growth assay. Two hundred microliter of osteosarcoma cells (1x10^5) transfected with NC, sh-MCM6 and sh-SNHG16 were injected into the tibial medullary cavity of nude mice. Tumor size was measured and calculated regularly every week (7 days). After 35 days, the tumor tissues were excised and fixed, followed by dehydration, paraffin-embedding, and cutting into sections.

Immunohistochemistry

Immunohistochemical staining was done on formalin-fixed and paraffin embedded tissue sections from tumors tissue. The tissue was placed in the incubator at 65 °C for 2 hours before removing paraffin by xylene. After gradient elute the tissue by absolute ethyl alcohol, 95 % ethyl alcohol, 85 % ethyl alcohol, washed the tissue by redistilled water. In the third step, target retrieval was achieved with BOND Novocastra Epitope Retrieval Solution 1 (Leica Biosystems) at 100 °C for 20 min. Monoclonal Anti-ki67 antibody (2 µg/ml, Abcam) were used as primary antibody. After that, secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000, Abcam) were used. In this way, ki67 was stained in the tissue and the tissue was photographed by optical microscope.

Statistical analysis

Statistical analysis was finished using GraphPad Prism 6.0 software. The data was showed by mean value±standard deviation (SD) in each group. Student’s t-test was applied to compare the differences between two groups, while the ANOVA was used to analyze differences among multi-samples. P< 0.05 was considered as statistically significance in the data.
Results

**Difference analysis identified miR-23b-3p as a potential target for SNHG16 in NPC**

To investigate the differential expressed genes, bioinformatic methods were applied to analyze the data from GEO. As shown in the heat map, the results described part of IncRNAs and mRNAs that were up-regulated and down-regulated in NPC tissues, compared with that in normal tissues ($P < 0.05$, $|\log_2(FC)| > 1$, Fig. 1A, 1B). SNHG16 and MCM6 were among the top 10 up-regulated IncRNAs and mRNAs respectively. Then KEGG analysis was used by GSEA to screen the relevant signaling pathways in NPC tissue. And then, we found that the cell cycle pathway was enriched in the 30 activated signaling pathway by analysis of R package joyplot and dotplot (Fig. 1C, 1D).

Gene ontology (GO) analysis was performed by GOplot through R project (Fig. 2). The results showed that cell cycle related cell functions were enriched. Biological process that focuses on cell cycle also gained a high mark of Z-score with a low $P$ value, hence the cell cycle was high enrichment and reliable. Therefore, we chose cell cycle pathway for further investigation. To further detect the relationship between genes and cell cycle, a network between mRNA and IncRNA on cell cycle was performed by Cytoscape based on previous analysis (Fig. 3A). LncRNA SNHG16 and mRNA MCM6 were chosen to find the potential links and from the Venn diagram, 2 common miRNAs were found including miR-23b-3p and miR-33b-3p (Fig. 3B). Since previous study has reported that miR-23b-3p is a tumor suppressor in hepatocellular carcinoma [14] and there is few studies focus on the effect of miR-23b-3p in NPC, miR-23b-3p was selected for following study. Overall, we made the hypothesis that SNHG16 help develop NPC cell progression by competitively binding miR-23b-3p.

**LncRNA SNHG16 and mRNA MCM6 were overexpressed in NPC tissues and cell lines**

We next investigated how SNHG16 and MCM6 expressed in the NPC tissues and cell lines. MCM6 and LncRNA SNHG16 were up-regulated while miR-23b-3p was down-regulated in tumor tissues by qRT-PCR validation ($P < 0.001$, Fig. 4A, 4B, 4C). To set up appropriate cell model, a series of cell lines including human normal nasopharyngeal cell line (NP69) and carcinoma cell lines (HONE-1, c666-1, CNE, HNE) were used to detect the expression of MCM6, SNHG16 and miR-23b-3p by qRT-PCR. We found that the SNHG16 and MCM6 expressed remarkably higher in c666-1, HNE-1 and HONE-1 than that in normal cell lines (all $P < 0.05$), while the increase of SNHG16 and MCM6 level in CHE cell was not significant ($P > 0.05$). On the contrary, the expression of miR-23b-3p in the three cell lines above was notably declined ($P < 0.05$). But in the CHE cells, the miR-23b-3p expression showed slightly decrease ($P > 0.05$, Fig. 4E, 4F, 4G). Therefore, c666-1 and HONE-1 which had higher expression of SNHG16 and MCM6 than others were chosen for the lateral study.

**Silence of SNHG16 inhibited the expression of MCM6 through up-regulation of miR-23b-3p**
To detect the function of SNHG16 and MCM6 in NPC cells, c666-1 and HONE-1 cells were transfected with si-SNHG16, si-MCM6 before detected by qRT-PCR, evaluating the transfection efficiency of si-SNHG16 and si-MCM6. Our results markedly showed that the expression level of SNHG16 and MCM6 decreased sharply in both cell lines transfected with si-SNHG16 and si-MCM6 (all \( P < 0.01 \)), while the expression of control group was not affected obviously (\( P > 0.05 \), Fig. 5). And as shown in Fig. 6 the putative binding site of miR-23b-3p and SNHG16, miR-23b-3p and MCM6 were predicted by TargetScan and miRcode. Then the luciferase reporter assay indicated the interaction between miR-23b-3p and SNHG16, miR-23b-3p and MCM6 since miR-23b-3p could combine with both of the SNHG16 and MCM6 in wild type instead of in the mutant type (\( P < 0.01 \)). Based on this conclusion, the expression level of MCM6, miR-23b-3p in c666-1 and HONE-1 cell lines were detected in groups of si-SNHG16, miR-23b-3p mimics and si-SNHG16 with miR-23b-3p mimics respectively. The miR-23b-3p was up-regulated notably when SNHG16 was silenced (\( P < 0.01 \)). Rather, when the SNHG16 was silenced or the miR-23b-3p was increased, the MCM6 expression was down-regulated (\( P < 0.01 \)), implying that miR-23b-3p could combine with MCM6, and SNHG16 could promote MCM6 expression level by combing with miR-23b-3p (all \( P < 0.05 \), Fig. 7A, 7B). The down-regulation of SNHG16 might promote miR-23b-3p to decline the expression of MCM6. To further understand the protein expression level of MCM6, western blot was performed and similar results were obtained compared with MCM6 mRNA expression (Fig. 7C). Overall, silence of SNHG16 expression limited the expression of MCM6 through up-regulation of miR-23b-3p.

Silence Of Snhg16 Suppressed Npc Cell Progression

To understand the functions of SNHG16-miR-23b-3p-MCM6 axis in NPC, MTT assay and transwell assay were performed to detect cell proliferation and the ability of invasion separately. The results demonstrated that SNHG16 or MCM6 knockdown respectively significantly inhibited the cell viability (\( P < 0.001 \), Fig. 8A, 8B) and invasion ability (\( P < 0.001 \), Fig. 8C, 8D) in both cell lines when compared with control group. But the situation could be rescued when miR-23b-3p inhibitor was added even though SNHG16 and MCM6 were silenced before. The cell propagation ability was enhanced in si-SNHG16 + miR-23b-3p inhibitor group and si-MCM6 + miR-23b-3p inhibitor group compared with si-SNHG16 group and si-MCM6 group (all \( P < 0.05 \), Fig. 8).

Furthermore, the flow cytometry assay on cell apoptosis analysis in HONE-1 and c666-1 cells showed the apoptosis influenced by the same operations as above. When SNHG16 or MCM6 was silenced, apoptosis could be activated most significantly in comparison to the control (all, \( P < 0.01 \), Fig. 9A, 9B). When the miR-23b-3p inhibitor was added, apoptosis showed a diversity with the silent groups. Following this, to determine whether the IncRNA SNHG16 and MCM6 attributed to cell cycle arrest, we performed cell cycle analysis by flow cytometry. As shown in Fig. 10A and 10B, silence of SNHG16 and MCM6 resulted in G0/G1 phase accumulation in HONE-1 cells (\( P < 0.05 \)), whereas adding the miR-23b-3p inhibitor led to lower accumulation level of G0/G1 phase (\( P < 0.05 \)). Similar results were obtained in c666-1 cells (Fig. 10C, 10D). SNHG16 or MCM6 silence induced a significant accumulation of cells in the G0/G1
phase with concomitant decrease of cells at the G2/M and S phases. All these results showed that the decrease of SNHG16 could inhibit NPC cell progression through cell cycle pathway.

**Silenced Snhg16 Or Mcm6 Inhibited Tumor Growth**

Tumor cell transplantation experiment was performed to determine the potential of SNHG16 on NPC in alleviating the symptoms of the disease. C666-1 cells after transfection by lentivirus were injected to nude mice and the tumor was excised after 35 days (Fig. 11A). Tumor volume was significantly smaller in the sh-SNHG16 and sh-MCM6 groups compared to the control ($P<0.001$, Fig. 11B). The tumor weight followed the same pattern that was lower in the sh-SNHG16 and sh-MCM6 groups than that in others groups (all $P<0.01$, Fig. 11C). Moreover, we detected the content of SNHG16, MCM6 and miR-23b-3p in tumors. Just as we predicated, the expression of MCM6 was well suppressed by silence of SNHG16 ($P<0.01$, Fig. 11E), while the SNHG16 expression was not affected by MCM6 level ($P>0.05$, Fig. 11D). The miR-23b-3p showed a higher expression level than the control ($P<0.05$, Fig. 11F). To make the result of in vivo experiment more intuitively, we made paraffin section to detect the tumor cell activity by ki67 score through IHC. Compare to the control, IHC revealed that the ki67 expression was decreased in sh-SNHG16 and sh-MCM6 groups ($P<0.05$, Fig. 11G, 11H). Animal experiment further confirmed our hypothesis that rising SNHG16 could promote the NPC cell progression by competitively binding miR-23b-3p.

**Discussion**

In this study, lncRNA SNHG16 expression level is increased in NPC cells and its up-regulation can promote NPC cell proliferation and invasion. Additionally, our study verified that SNHG16 can post-transcriptionally affect the expression of MCM6 and further promote the progression of NPC by binding with miR-23b-3p.

IncRNAs play vital roles in cancer development. To date, only a small number of IncRNAs have been characterized at functional level.[15] Previous studies have proved that IncRNA differentially expressed patterns can be represented by the microarray probers and Inc-BCL2L11-3 can be a distinguishing IncRNA identified in the recurrent NPC.[16] The IncRNA NEAT1 regulates epithelial to mesenchymal transition (EMT) and radio resistance in NPC through miR-204/ZEB1 has been reported.[17] Song et al. find that SNHG16 which is overexpressed in osteoarthritis chondrocytes is known to inhibit cell survival ability and is aberrantly expressed in several cancers and stimulates proliferation by regulating cyclin-dependent kinase 6.[18] Zhao et al. find that increase of SNHG16 in neonatal hypoxic brain injury identifies a novel mechanism of IncRNA-mediated neural injury.[19] Furthermore, the different expression of SNHG16 may suggest the differential role of SNHG16 in carcinogenesis of different tissues.[20] For example, SNHG16 is overexpressed in esophageal cancer cells. Nevertheless, few studies focus on SNHG16 in NPC. We identify that SNHG16 may act as an oncogene in NPC first based on the article research.

MiRNAs play key roles in many complex diseases, including various cancers. For instance, miR-139-5p induced timeless down-modulation and inhibited cell proliferation and apoptosis.[21] Nowadays
mounting evidence has proved that miR-23b-3p not only functions as stated above, but also works as a biomarker for diagnosis for various cancers. For example, its expression is notably decreased in hepatocellular carcinoma tissues compared with their paired adjacent tissues.[22] Besides, overexpression of miR-23b-3p reverses cancer cell resistance to plenty of chemotherapy drugs in vitro, and sensitizes tumors to chemotherapy in vivo.[23] Francesco et al. also find a significant down-regulation of miR-23b-3p in Duchenne muscular dystrophy patients.[24] All previous studies prove that miR-23b-3p is important in cancer procession and consistent with our results that miR-23b-3p may suppress the NPC development.

A novel computational approach suitable to explore the potential role of IncRNAs as miRNA sponges for preserving homeostasis and preventing disease has already been proposed.[25] In previous study, Paci et al. have identified a sponge interaction network between long non-coding RNAs and messenger RNAs in human breast cancer.[26] In present research, we predicted the possible miRNAs for SNHG16 using bioinformatics analysis and found miR-23b-3p sharing the complementary binding sites to mediate the expression of MCM6. Among others proliferation markers, the mini-chromosome maintenance (MCM) proteins as the key proteins in the initiation of DNA synthesis and DNA replication, are considered to be linked with histological grades in various neoplastic development.[27] A recent report indicates that MCM is associated with histological grade and survival ability of cells in endometrioid endometrial adenocarcinoma, which may be a new marker in early diagnosis.[28] In our study, we verify that SNHG16 accelerates NPC progression by acting as a sponge of miR-23b-3p and activating MCM6 indirectly, which may provide a new insight for NPC prediction or prognosis.

However, several limitations exist in our study. For instance, despite the relative large sample size, the total number of samples in this study is still small. This study is based on only two cell lines and more experiments should be applied. In addition, due to the difficulty to investigate the downstream metabolic network of MCM6, more researches should be executed in future.

In summary, this study provides a comprehensive report of the relationship between SNHG16, miR-23b-3p and MCM6 axis. Highly expressed SNHG16 promotes NPC cell growth by competitively binding with miR-23b-3p. Further studies regarding the mechanism of SNHG16 in NPC may provide clues as to whether they can serve as potential therapeutic targets. Taken together, our study has demonstrated the importance of SNHG16 expression in NPC, which may be a novel predictive indicator for NPC patients.

**Abbreviations**
### Abbreviation Table

| Abbreviation | Full name                        |
|--------------|----------------------------------|
| SNHG16       | Small Nucleolar RNA Host Gene 16 |
| NPC          | Nasopharyngeal carcinoma         |
| IncRNA       | Long non-coding RNA              |
| EBV          | Epstein-Barr virus; miRNAs       |
| miRNAs       | MicroRNAs                        |
| 3′-UTR       | 3’ untranslated region           |
| SD           | standard deviation               |
| GO           | Gene ontology                    |

### Declarations

**Ethical approval:** All procedures performed in studies involving animals were in accordance with the ethical standards of West China Hospital, Sichuan University.

**Informed Consent:** All samples were collected with the informed consent of the patients and the study was approved by West China Hospital, Sichuan University.

**Conflict of Interest:** The authors confirm that there are no conflicts of interest.

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**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

**Authors’ contributions:** Shixi Liu: critical revision of the manuscript; Jiaojiao Peng: substantial contribution to the conception and design of the work, manuscript drafting; Feng Liu and Hong Zheng: acquisition, analysis, and interpretation of the data; Qi Wu: revising the manuscript critically, final approval of the version to be published. All authors have read and approved the final article.

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Tables

Table 1. Primer sequences for qRT-PCR
| Primer       | Sequence (5'-3')                                      |
|-------------|-------------------------------------------------------|
| miR-23b-3p F | 5'- ATCACATTGCCAGGGATTACCAC-3’                        |
| miR-23b-3p R | 5'-GCGAATACCTCGGACCCTGC-3’                            |
| U6 F        | 5'-TCGCTTCCGAGCACA-3’                                 |
| U6 R        | 5'-GAATACCTCGGACCCTGC-3’                              |
| MCM6 F      | 5'-TGATAAGATGGACGTCGCGG-3’                             |
| MCM6 R      | 5'-GGCGTTTCAGAGTGCCTTCA-3’                             |
| SNHG16 F    | 5'-GCGTCGTGACGTCATCAAAA-3’                             |
| SNHG16 R    | 5'-AAAAACCGCAACATTCGCAA-3’                             |
| GADPH F     | 5'-GAAAGCCTGCCGTTGACTAA-3                             |
| GADPH R     | 5'-GCCCCATACGACCAAATCAGAG-3                           |

*F: forward primer; R: reverse primer.

**Figures**
Figure 1

Heatmaps and signaling pathways of discrepant mRNA and lncRNA. (A) The heatmap of top 10 up-regulation and down-regulation differential genes in mRNA by R project. (B) The heatmap of top 10 up-regulation and down-regulation differential genes in lncRNA by R project. (C) The joyplot diagram refers 30 up-regulated metabolism pathway by R project. (D) The dot plots diagram refers to the same results of joyplot with the enrich degree by R project.
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Figure 2

The results of GO analysis. (A) The GOplot indicates the GO terms targeted by enrichment genes and log2 (FC) values. (B) The top 5 Enrichment results of biological process (BP), cell component (CC) and molecular function (MF) in the GOplot.
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The relationship between lncRNA and mRNA. (A) Relationship between discrepant mRNA (roundness) and lncRNA (rhombus) (cutoff >0.7, P<0.05) was verified by Cytoscape. (B) Venn diagram indicates the common miRNA between extremely differential expressed mRNA and lncRNA, showing two coincident miRNAs.
MCM6 and SNHG16 were up-regulated in nasopharyngeal carcinoma tissues and cells. (A, B, C) High expression of MCM6 and SNHG16 in nasopharyngeal carcinoma tissue respectively, while low expression of miR-23b-3p shows opposite result through qRT-PCR (***P<0.001 compare with normal tissues). (E, F, G) The expression of MCM6, SNHG16 and miR-23b-3p in cell lines NP69, HONE-1, c666-1, CNE, HNE cell lines through qRT-PCR (*P<0.05, **P<0.01 compare with NP69 cell).
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Figure 5

Cell transfection results in three groups determined by qRT-PCR. (A, B) The SNHG16 expression level was down-regulated in two cell lines (c666-1 and HONE-1) after silencing SNHG16 (**P<0.01 compare with control). (C, D) The MCM6 expression level was down-regulated in two cell lines (c666-1 and HONE-1) after silencing MCM6 (**P<0.01, ***P<0.001 compare with control).
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Figure 6

The wild type miR-23b-3p could combine with MCM6 in cells. (A) The putative binding site of miR-23b-3p and SNHG16 is shown, followed by the luciferase activity which was measured in c666-1 co-transfected with wild type (SNHG16-WT) or mutant (SNHG16-MUT) using the luciferase reporter assay (**P<0.01 compare with control). (B) The putative binding site of miR-23b-3p and MCM6, as well as the Luciferase activity which was measured in c666-1 co-transfected with wild type (MCM6-WT) or mutant (MCM6-MUT) (**P<0.01 compare with control).
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Figure 7

SNHG16 could influence the expression of MCM6 and miR-23b-3p in cells. (A, B) The expression level of MCM6 and miR-23b-3p were measured by qRT-PCR in the cells transfected with si-SNHG16, miR-23b-3p mimics, si-SNHG16 with miR-23b-3p inhibitor, blank or control (***P<0.001 compare with control, # P<0.05, ## P<0.01 compare with si-SNHG16). (C) Western blot results of MCM6 in the five groups as above when compared with the control in c666-1 and HONE-1 cell lines (***P<0.01 compare with control, #P<0.05 compare with si-SNHG16).
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Figure 7
Figure 8

SNHG16 promoted nasopharyngeal carcinoma cell proliferation and invasion. (A, B) The cell viability of four groups compared with the control group in HONE-1 and c666-1 cells through MTT assay (***P<0.001 compare with control, ##P<0.01, ###P<0.001 compare with si-MCM6 and si-SNHG16). (C, D) Silence of SNHG16 or MCM6 inhibited the invasion of c666-1 and HONE-1 cells, but stimulated the cell migration
after adding miR-23b-3p inhibitor in Transwell assay (***P<0.001 compare with control, #P<0.05, ##P<0.01 compare with si-SNHG16, &&P<0.01 compare with si-MCM6).

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Figure 9

SNHG16 suppressed nasopharyngeal carcinoma cell apoptosis. (A, B) Apoptosis was measured by flow cytometry assay in treated c666-1 and HONE-1 cells (**P<0.01, ***P<0.001 compare with control, ##P<0.01 compare with si-SNHG16, &&P<0.01 compare with si-MCM6).
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Figure 10

The inhibition effect of SNHG16 and MCM6 on cell cycle in cells. (A) The cell number in various phases of cell cycle was detected by Flow cytometry in HONE-1 cell. (B) The statistical cell numbers in three phases in HONE-1 cell including G2/M, S and G0/G1 phases (*P<0.05 compare with control, #P<0.05 compare with si-SNHG16, &P<0.05 compare with si-MCM6). (C) The cell number in various phases of cell cycle detected by Flow cytometry in c666-1 cell. (D) The statistical cell numbers in three phases in c666-1 cell including G2/M, S and G0/G1 phases (*P<0.05 compare with control, #P<0.05 compare with si-SNHG16, &P<0.05 compare with si-MCM6).
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Figure 11

Effects of silence SNHG16 or MCM6 on tumor growth in vivo. (A) Tumors were collected from nude mice injected with nasopharyngeal carcinoma cells transfected with sh-SNHG16, sh-MCM6, blank or control. (B) The tumor volume changes in 35 days was measured every 7 days. (**P<0.001 compare with control). (C) The tumor weight was measured 35 days after tumor transplantation (***P<0.001 compare with control). (D, E, F) Expression levels of SNHG16, MCM6 and miR-23b-3p were measured by qRT-PCR in nude mice tissue (*P<0.05 compare with control). (G) The tumor tissue was made to paraffin section and ki67, the brown particles, were stained by immunohistochemistry. (H) Ki67 positive cell numbers in the tumor tissues (*P<0.05 compare with control).
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