Downregulated long non-coding RNA SNHG7 restricts proliferation and boosts apoptosis of nasopharyngeal carcinoma cells by elevating microRNA-140-5p to suppress GLI3 expression

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ABSTRACT

Long non-coding RNAs (lncRNAs) have been proposed to correlate with various carcinomas, yet the role of lncRNA SNHG7 in nasopharyngeal carcinoma (NPC) is hardly studied. This study intents to examine the molecular mechanism of SNHG7 on NPC cells. The NPC tissues and nasopharyngeal tissues of mild inflammation of nasopharyngeal mucosa were obtained. SNHG7, miR-140-5p, and GLI3 mRNA and protein expression in tissues and in the CNE1, HONE1, C666-1, CNE2, and normal NP69 cell lines was detected. IC50 and the protein expression of related drug-resistant genes of CNE2 and CNE2/DDP cells were determined. Proliferative ability, cell colony formation rate, cell cycle, and apoptosis of CNE2 and CNE2/DDP cells were also detected. SNHG7, miR-140-5p, and GLI3 mRNA and protein expression in CNE2 and CNE2/DDP cells in each group was detected. SNHG7's cell localization, the binding sites of SNHG7 and miR-140-5p along with miR-140-5p and GLI3 were detected. Overexpressed SNHG7 and GLI3, and underexpressed miR-140-5p were found in NPC tissues and cells. SNHG7 silencing and miR-140-5p elevation declined the drug resistance of drug-resistant NPC cells and their parent cells, restrained NPC cell colony formation ability and proliferation, and boosted cell apoptosis. SNHG7 specially bound to miR-140-5p and SNHG7 silencing elevated miR-140-5p expression. GLI3 was a direct target gene of miR-140-5p and miR-140-5p elevation diminished GLI3 expression. MiR-140-5p inhibition reversed the impacts of SNHG7 silencing on NPC cells. In summary, our study reveals that downregulated SNHG7 restricts GLI3 expression by upregulating miR-140-5p, which further suppresses cell proliferation, and promotes apoptosis of NPC.

Introduction

Derived from epithelial cells on the surface of the nasopharynx, nasopharyngeal carcinoma (NPC) is classified into three categories in light of the criteria of World Health Organization (type I, keratinizing squamous cell tumor; type II, nonkeratinizing differentiated tumor; type III nonkeratinizing undifferentiated tumor) [1]. Risk factors for NPC are chiefly comprised of Epstein-Barr virus infection, human papillomavirus, genetic susceptibility as well as dietary and social practices [2]. NPC frequently arises in southern China, especially in Hainan, Guangdong, Fujian, Jiangxi, and Guangxi provinces [3]. Lately, correlations of aberrantly long non-coding RNAs (lncRNAs) with NPC have been substantially reported [4], which inspired this study to be focused on the exploration of new biomarkers and treatment of NPC.

LncRNAs refer to RNA transcripts with over 200 nucleotides and nearly without protein-coding ability, which function as molecular biomarkers in human cancers [5,6]. There is a report suggesting that in lung cancer, lncRNA SNHG7 is dysregulated and serves as a promoter of cell progression [7]. Also, the promotion of cell proliferation and inhibition of apoptosis by SNHG7 have also been discovered in gastric cancer [8]. Evidence has shown that microRNA-140-5p (miR-140-5p) can be targeted by lncRNA SNHG16 [9]. MiRNAs, which refers to short noncoding RNAs with about 22 nucleotides in length, have been proposed to be dysregulated in...
many cancers and function in their development [10,11]. A previous study has revealed that in hepatocellular carcinoma (HCC), miR-140-5p is under-expressed and it directly targets Pin1 (a kind of unique isomerase) to stop multiple cancer-driving pathways, thus depressing HCC development [12]. Similar research by Hu Y et al. has demonstrated that in glioma, miR-140-5p modulates VEGFA/MMP2 signaling for cell proliferation and invasion restriction [13]. GLI-Kruppel family member 3 (GLI3), a kind of the zinc finger transcription factor, is a regulator of Sonic hedgehog signaling, and it has been found to be targeted by miR-506 in cervical cancer to depress tumor progression [14,15]. There is also a report revealing that in oral squamous cell carcinoma, silencing of GLI3 can decline stemness and simultaneously circumscribe cell progression [16]. Nevertheless, the impacts of IncRNA SNHG7 in NPC have hardly been reported. Therefore, this study intends to explore the mechanism of SNHG7/miR-140-5p/GLI3 axis in NPC.

Materials and methods

Ethics statement

This study was reviewed and approved by the Ethics Committee of Affiliated Otolaryngological Hospital, the First Affiliated Hospital of Zhengzhou University and was supervised by the Ethics Committee of Affiliated Otolaryngological Hospital, the First Affiliated Hospital of Zhengzhou University. Written informed consents were obtained from all patients before the study.

Subjects

Patients with NPC who underwent nasopharyngeal biopsy from September 2017 to September 2018 were included in the study. The following ones were included: patients according to diagnostic criteria for NPC; patients pathologically diagnosed as NPC; patients first diagnosed as NPC and not received any surgical treatment; patients with complete and detailed medical information; and the following ones were excluded: patients with other tumors; patients with severe heart, liver, and kidney dysfunction; patients with other systemic immune diseases; patients not cooperating with the treatment.

A total of 55 qualified NPC patients (41 males and 14 females) aged 24 to 57 y with an average age of \((32.48 \pm 10.35)\) y were selected for the experiment. All the patients were staged according to the 1992 Fuzhou Conference [17]: 7 in stage II, 31 in stage III, and 17 in stage IVa. They were diagnosed as poorly differentiated squamous cell carcinoma or non-keratinized undifferentiated carcinoma by histopathology at the first visit, and were set as the cancer tissue group. At the same time, 30 nasopharyngeal tissues from patients with mild inflammation of nasopharyngeal mucosa in the corresponding period of the Affiliated Otolaryngological Hospital, the First Affiliated Hospital of Zhengzhou University were collected as the normal tissue group.

Cell selection and culture

Human NPC cells (CNE1, HONE1, C666-1, and CNE2) were all purchased from Shanghai Huiying Biological Technology Co., Ltd. (Shanghai, China). The normal nasopharyngeal epithelial cell line NP69 was purchased from Jianglin Biotechnology Co., Ltd. (Shanghai, China). All cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) for incubation \((37^\circ C, 5% CO_2)\). The medium was changed every 2 d, and passage was started when the cell density reached 80%-90%. After 2–3 stable passages, the cells in logarithmic growth phase were taken for the detection of SNHG7, miR-140-5p, and GLI3 mRNA expression by reverse transcription quantitative polymerase chain reaction (RT-qPCR), and GLI3 protein expression level by western blot analysis. Follow-up experiments were performed with CNE2 cells with the greatest expression difference from NP69 cells.

Human NPC cell-resistance modeling

Drug-resistant cells were induced by a combination of high-dose pulse and increasing doses. Human NPC CNE2 cells in the logarithmic growth phase were inoculated into a 250 mL culture flask. When the cell confluence was 70%-80%, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed that the half maximal inhibitory concentration (IC50) of cisplatin (DDP) on sensitive cells was 0.25 \(\mu\)mol/L. The cells were then cultured in medium
containing 3.3 μmol/L DDP for 24 h, followed by 5-10-d culture with DDP of IC50. Then, IC50 was determined after stable growth and three passages of the cells. Next, the cells were cultured with the medium containing 3.3 μmol/L DDP for 24 h, and the medium with gradually increasing DDP IC50 was replaced. After the cells stably grew and serially passaged 3 times in the medium containing 3.3 μmol/L DDP, the final IC50 of DDP was 7 μmol/L and named CNE2/DDP.

Cell grouping and transfection
CNE2 and CNE2/DDP cells in the logarithmic growth phase (2 × 10⁵ cells/well) were inoculated in 6-well cell culture plates, and transfected when the cell confluence reached 80% based on the instructions of lipofectamine 2000 kit (11,668–027, Invitrogen, Carlsbad, California, USA). Two hundred and fifty μL serum-free RPMI 1640 medium was used to dilute each transfection sequence (all purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China) with a final concentration of 50 nM) before 5-min incubation at room temperature. Then, 5 μL lipofectamine 2000 was also diluted by 250 μL serum-free RPMI 1640 medium for 5-min incubation at room temperature. The above two mixtures were incubated for 20 min and added into the cell culture wells. Then, the cells were cultured for 6 h (37°C, 5% CO₂, saturated humidity), and the medium containing the transfection solution was replaced with RPMI 1640 medium containing 10% FBS for follow-up experiments. All cells were divided into seven groups: blank group (CNE2 and CNE2/DDP cell lines without any treatment), sh-negative control (NC) group (CNE2 and CNE2/DDP cell lines transfected with SNHG7 interference NC plasmid), sh-SNHG7 group (CNE2 and CNE2/DDP cell lines transfected with SNHG7 interference plasmid), mimic-NC group (CNE2 and CNE2/DDP cells transfected with miR-140-5p mimic NC), miR-140-5p mimic group (CNE2 and CNE2/DDP cells transfected with miR-140-5p mimics), sh-SNHG7 + inhibitor NC group (CNE2 and CNE2/DDP cell lines transfected with SNHG7 interference plasmid and miR-140-5p inhibitor NC), sh-SNHG7 + miR-140-5p inhibitor group (CNE2 and CNE2/DDP cell lines transfected with SNHG7 interference plasmid and miR-140-5p inhibitor).

MTT assay
CNE2 and CNE2/DDP cells were passaged before transfection and cultured in drug-free medium for 3 to 5 d until in logarithmic growth phase, followed by the cell density adjustment to 1 × 10⁵ cells/mL. Then, the cells were added to a 96-well plate at 100 μL per well. Next, 100 μL medium with 10-fold drug (DDP, 5-fluorouracil [5-FU]) concentration gradient was added, respectively, with a total volume of 200 μL per well (all 7 concentration gradients, 1 blank control), and 3 parallel wells for each drug concentration. After 48-72-h culture, 15 μL of 10 mg/mL MTT solution was put in each well for 4-h continuous culture. After removing the medium, 200 μL dimethyl sulfoxide (DMSO) was added to each well, and dissolved and crystallized by slight shaking. The optical density (OD) value of each well was detected on a microplate reader with 490 nm as the detection wavelength. Data analysis was performed by MTT analysis software for IC50 calculation, followed by cell resistance index (RI) calculation. CNE2 and CNE2/DDP cells after transfection of each group were treated, and MTT detection and RI calculation were performed under the functions of 7.86 μmol/L DDP and 347.86 μmol/L 5-FU.

MTT assay for cell proliferation detection
Cells in the logarithmic growth phase in each group (200 μL) were inoculated in a 96-well cell culture plate at 1 × 10⁴ cells/mL with 6 wells repeated. When the cells were attached after 24-h incubation, the medium was replaced by 200 μL RPMI 1640 with 10% FBS and 20 μL MTT solution (5 mg/mL) was then added, followed by a 4-h incubation at 37°C. With the supernatant abandoned, 150 μL DMSO was added to every well and shook at low speed for 10 min, followed by OD measurement by a microplate reader at 490-nm wavelength. The experiment was repeated 3 times.

5-ethynyl-2ʹ-deoxyuridine (EdU) assay
CNE2 and CNE2/DDP cell suspension (100 μL) containing 2000 cells were inoculated in 96-well plates with 3 replicate wells per group, followed by 48-h incubation (37°C, 5% CO₂). With the medium removed, the serum-free RPMI 1640 containing
EdU (1:1000) was added to the incubator for a 2-h culture. The culture plate was taken out, then treated based on the EdU kit (Nanjing Xinfan Biotechnology Co., Ltd., Jiangsu, China), and finally placed under an inverted fluorescence microscope for fluorescent photo collection through a random selection of three fields. The EdU-positive rate (%) = the number of EdU-positive cells/the total number of cells × 100%.

**Colony formation assay**

CNE2 and CNE2/DDP cells were inoculated on 6-well plates at 400 cells/well. Then, the cells were incubated in a 37°C incubator with 5% CO₂ for 14 d, followed by 15-min 4% paraformaldehyde fixation and 20-min 0.4% crystal violet staining. After washing and air-drying, the number of cell colonies was counted through a random selection of 5 fields under a fluorescence microscope (>50 colonies were considered as an effective colony). Cell colony formation rate = the number of cell colonies/the number of inoculated cells × 100%.

**Flow cytometry**

The cell cycle of a portion of transfected cells was detected. All cells were supplemented with 100 μL propidium iodide (PI)-Rnase A for 15-min incubation without light, and DNA content in each phase of cell cycle was analyzed by flow cytometry. The cells were detached and centrifuged, followed by 2 phosphate buffered saline (PBS) rinses. Next, the cells were resuspended in 75% pre-cooled ethanol before overnight fixation at −20°C. After supernatant removal and 2 PBS washes, the cells in each sample were resuspended with 450 μL PBS, and supplemented with 100 μL Rnase A for 30-min water bath at 37°C, followed by 30-min PI (400 μL) staining at 4°C without light. Finally, cell cycle distribution was measured and analyzed by flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA). The experiment was repeated three times independently.

The transfected cells of each group were rinsed with PBS three times and supplemented with 100 μL pre-cooled 1× binding buffer for cell resuspension. Then, 5 μL Annexin and 5 μL PI were added in sequence, mixed, and kept for 15 min, followed by apoptosis detection by flow cytometry. The results were judged as follows: AnnexinV were used as the lateral axis and PI as the vertical axis; the negative normal cells were in the upper right quadrant; the early apoptotic cells were in the upper left quadrant; the late apoptotic cells or the necrotic cells were in the lower left quadrant; the early apoptotic cells were in the lower right quadrant.

**RT-qPCR**

Based on the Trizol (Takara, Otsu, Shiga, Japan) method, the total RNA of the collected tissues and cells were extracted, and the concentration and purity of RNA were then determined. In light of the instructions of the reverse transcription kit (K1621, Fermentas, Maryland, NY, USA), the RNA was reversely transcribed into cDNA which was then preserved in a refrigerator at −20°C. The primer sequences (Table 1) of miR-140-5p, SNHG7, and GLI3 were designed and entrusted to Shanghai Genechem Co., Ltd. (Shanghai, China) for synthesis. The expression of each gene was determined by an RT-qPCR kit (Takara, Otsu, Shiga, Japan), followed by RT-qPCR machine (ABI 7500, ABI, Foster City, CA, USA) detection. U6 was used as the internal reference of miR-140-5p, and glyceraldehyde phosphate dehydrogenase (GAPDH) as that of SNHG7 and GLI3. The relative expression of each target gene was calculated by 2^ΔΔCt method. Each experiment was repeated 3 times.

**Western blot analysis**

The protein concentration was measured based on the instructions of bicinchoninic acid kit (Boster Biological Technology Co., Ltd., Wuhan, China). The extracted proteins were added to the loading buffer and loaded onto a 10% SDS-PAGE gel. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) for 1 h at room temperature, and then incubated overnight at 4°C with the primary antibodies. After washing, the membrane was incubated with the secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 h at room temperature. The protein bands were visualized using an enhanced chemiluminescence (ECL) kit. The results were quantified using ImageJ software. The expression levels of the target proteins were normalized to GAPDH, which was used as an internal control.

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**Table 1. Primer sequence.**

| Gene  | Primer sequence (5’–3’) |
|-------|------------------------|
| SNHG7 | Forward: 5’-CAACTGGTCCGAAACCCCATCT-3’<br>Reverse: 5’-CGGGTCCAAGGCGATTCTCCT-3’ |
| miR-140-5p | Forward: 5’-GAGTGTCAAGGGTTTACCT-3’<br>Reverse: 5’-GAGGGGTCAGGAGGTATTC-3’ |
| GLI3  | Forward: 5’-AGGGTTAAATGGTGATCAAGATGG-3’<br>Reverse: 5’-CCCACGGTTTGGTCATAGAA-3’ |
| U6    | Forward: 5’-CTGCAGTTCCCGAGCA-3’<br>Reverse: 5’-AAGGGTTTCAAGGAATTGG-3’ |
| GAPDH | Forward: 5’-GCCACGCTATTGTCTAGAAA-3’<br>Reverse: 5’-CATCACGCCACGGTTTCC-3’ |

miR-140-5p, microRNA-140-5p; GLI3, GLI-Kruppel family member 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
buffer before 10-min boiling at 95°C, and each well was loaded with 30 μg samples. Then, the proteins were separated by 10% polyacrylamide gel (Boster Biological Technology Co., Ltd., Wuhan, China) through electrophoresis with the electrophoresis voltage changed from 80 v to 120 v. Next, the proteins were transferred to polyvinylidene difluoride membrane through 45–70 min wet transfer with a voltage of 100 mv, followed by 1-h 5% bovine serum albumin (BSA) sealing. Then, primary antibody Ki-67 (1:1000), GLI3 (1:300) and P-glycoprotein (P-gp) (1:300) (Abcam, Cambridge, UK) were added together with CyclinD1 (1:1000), B-cell lymphoma-associated X (Bax, 1:1000), B-cell lymphoma-2 (Bcl-2, 1:1000), multidrug resistance-associated protein 1 (MRP1, 1:200) (Santa Cruz Biotechnology, Santa Cruz, California, USA), GAPDH (1:2000, Jackson ImmunoResearch, Grove, PA, USA) and glutathione-S-transferase-π (GST-π, 1:2000, Millipore, Darmstadt, Germany) for 24- to 48-h incubation at 4°C, and then placed in horseradish peroxidase-labeled secondary antibody (1:500, Jackson ImmunoResearch, Grove, PA, USA) for 1-h incubation at room temperature. Images were obtained by Odyssey (a two-color infrared fluorescence scanning imaging system), and the gray value of the bands was measured by Quantity One (an image analysis software). The ratios of the value of target bands to that of the internal reference bands in each group were obtained and compared.

**RNA-fluorescence in situ hybridization (FISH) assay**

Prediction of the subcellular localization of SNHG7 was carried out with the bioinformatics website (http://lncatlas.crg.eu/), which was then verified by FISH assay. The assay was performed based on the instructions of Ribo™ IncRNA FISH Probe Mix (Red) (Guangzhou RiboBio Co., Ltd., Guangzhou, China). The coverslip was put in a 24-well culture plate, followed by cell inoculation at 6 × 10^4 cells/well to achieve about 80% cell confluence. After taking the coverslip out, the cells were washed by PBS before fixation by 1 mL 4% paraformaldehyde and treatment with proteinase K, glycine and acetamidine reagent. Subsequently, 250 μL pre-hybrid solution was added for 1-h incubation at 42°C. Then, the pre-hybrid solution was replaced with 250 μL SNHG7 hybridization solution (300 ng/mL) with probe for overnight hybridization at 42°C. After 3 phosphate-buffered saline/Tween 20 (PBST) washes, 4',6-diamidino-2-phenylindole (DAPI, ab104139, 1:100, Abcam, Shanghai, China) diluted with PBST was added for 5-min nucleus staining in a 24-well culture plate, followed by 3 PBST washes (3 min each time). At last, the cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan) and photographed after anti-fluorescence quencher sealing.

**Dual luciferase reporter gene assay**

The binding site of lncRNA SNHG7 and miR-140-5p were predicted and analyzed at the bioinformatics website (https://cm.jefferson.edu/rna22/ Precomputed/), which was then verified by dual luciferase reporter gene assay. The synthetic SNHG7 3’-untranslated regions (3’-UTR) gene fragment was introduced into the pMIR-reporter (Beijing Huayueyang Biotechnology Co., Ltd., Beijing, China) through endonuclease sites (Bamh1 and EcoR1). The complementary sequence mutation site of the seed sequence was designed on the wild type (WT) of SNHG7, and the target fragment was inserted into the pMIR-reporter plasmid by T4 DNA ligase after restriction endonuclease digestion. The correctly sequenced luciferase reporter plasmids (WT and mutant type [MUT]) were independently co-transfected with mimic NC and miR-140-5p mimic into CNE2 cells (Shanghai North Connaught Biotechnology Co., Ltd., Shanghai, China). After 48-h transfection, cells were lysed, and luciferase activity was measured with a luciferase assay kit (BioVision, San Francisco, CA, USA) and Glomax20/20 luminometer (Promega, Madison, Wisconsin, USA). The experiment was repeated three times.

The targeting relationship of miR-140-5p and GLI3 and the binding site of miR-140-5p and GLI3 3’UTR were predicted with a bioinformatics software (http:// www.targetscan.org/vert_72/). The GLI3 3’UTR promoter sequence containing the miR-140-5p binding
site was synthesized, and a GLI3 3’UTR WT plasmid (GLI3-WT) was constructed. Based on this plasmid, a binding site was mutated for GLI3 3’UTR MUT plasmid (GLI3-MUT) establishment in light of the procedures of point mutation kit (Takara, Otsu, Shiga, Japan). CNE2 cells in the logarithmic growth phase were inoculated in 96-well plates, and transfected with the mixtures of GLI3-WT and GLI3-MUT plasmids with mimic NC and miR-140-5p mimic plasmids, respectively, by Lipofectamine 2000 at a cell density of approximately 70%. Forty-eight hours later, cells were lysed, and luciferase activity was detected with a luciferase assay kit. Each experiment was repeated three times.

RNA-pull down

The cells were transfected with biotin-labeled miR-140-5p WT plasmid and biotin-labeled miR-140-5p MUT plasmid (50 nM each), respectively. Forty-eight hours later, the cells were washed with PBS and incubated with specific cell lysate (Ambion, Austin, Texas, USA) for 10 min, after which 50 mL cell lysate sample was collected. Then, residual lysate was incubated with M-280 streptavidin magnetic beads (Sigma, St. Louis, MO, USA) pre-coated with RNase-free BSA and yeast tRNA (Sigma, St. Louis, MO, USA) at 4°C for 3 h, followed by 2 cold lysate washes, 3 low salt buffer wash, and a high salt buffer wash. An antagonistic miR-140-5p probe was set up as an NC. SNHG7 expression was detected by RT-qPCR after total RNA extraction with Trizol.

Statistical analysis

All data were statistically analyzed with SPSS 21.0 (SPSS, IBM Corp., Armonk, NY, USA) statistical software. Measurement data were expressed as mean ± standard deviation and those subject to normal distribution between two groups were compared with independent sample t-test. Comparison among the group was analyzed by one-way analysis of variance (ANOVA), after which Tukey’s multiple comparison test was used for pairwise comparison. The difference was considered statistically significant at P < 0.05.

Results

There are overexpressed SNHG7 and GLI3, and underexpressed miR-140-5p in NPC tissues

SNHG7 and miR-140-5p levels along with GLI3 mRNA level in normal tissues (nasopharyngeal tissues of mild inflammation of nasopharyngeal mucosa) and cancer tissues were detected by RT-qPCR. The results showed ascended SNHG7 and GLI3 levels, and decreased miR-140-5p level in the cancer tissues (all P < 0.05; Figure 1(a–c)). GLI3 protein level detection by Western blot analysis also suggested elevated GLI3 protein expression in cancer tissues (P < 0.05) (Figure 1(d)).

There are overexpressed SNHG7 and GLI3, and underexpressed miR-140-5p in NPC cells

SNHG7 and miR-140-5p levels, as well as GLI3 mRNA level in the NP69, CNE1, HONE1, C666-1, and CNE2 cells, were detected by RT-qPCR. Elevated SNHG7 and GLI3 levels, and reduced miR-140-5p level were found in the CNE1, HONE1, C666-1, and CNE2 cells versus the NP69 cells (all P < 0.05), and the highest SNHG7 and GLI3 levels and the lowest miR-140-5p level were discovered in the CNE2 cells, which showed the most difference from the levels in the NP69 cells (Figure 2(a–c)). Western blot analysis revealed that GLI3 protein level in the CNE1, HONE1, C666-1, and CNE2 cells ascended versus the NP69 cells (all P < 0.05), and the highest GLI3 level was found in the CNE2 cells, which was the most different from the level in the NP69 cells (Figure 2(d)). Based on the above findings, CNE2 cells were selected for subsequent experiments.

SNHG7 silencing and miR-140-5p elevation decline the drug resistance of drug-resistant NPC cells and their parent cells

Calculation of the drug resistance of drug-resistant cells and their parent cells to different drugs showed that CNE2/DDP had an RI of 21.82 for DDP (RI > 15, Table 2), which met the criterion for high drug resistance, indicating successful drug resistance modeling for human NPC cells.
Figure 1. There are overexpressed SNHG7 and GLI3, and underexpressed miR-140-5p in NPC tissues. (a), SNHG7 level in nasopharyngeal carcinoma tissues and nasopharyngeal tissues of mild inflammation of nasopharyngeal mucosa; (b), Expression of miR-140-5p in nasopharyngeal carcinoma tissues and nasopharyngeal tissues of mild inflammation of nasopharyngeal mucosa; (c), GLI3 mRNA expression in nasopharyngeal carcinoma tissues and nasopharyngeal tissues of mild inflammation of nasopharyngeal mucosa; (d), GLI3 protein expression in nasopharyngeal carcinoma tissues and nasopharyngeal tissues of mild inflammation of nasopharyngeal mucosa; the data in the figure were measurement data expressed as mean ± standard deviation; *, P < 0.05 vs the normal tissue group.

Figure 2. There are overexpressed SNHG7 and GLI3, and underexpressed miR-140-5p in NPC cells. (a), relative SNHG7 expression in the NP69, CNE1, HONE1, C666-1, and CNE2 cells; (b), relative miR-140-5p expression in the NP69, CNE1, HONE1, C666-1, and CNE2 cells; (c), relative GLI3 mRNA expression in the NP69, CNE1, HONE1, C666-1, and CNE2 cells; (d), relative GLI3 protein expression in the NP69, CNE1, HONE1, C666-1, and CNE2 cells; the data in the figure were measurement data expressed as mean ± standard deviation; *, P < 0.05 vs the NS69 cells.
Moreover, it was indicated that the inhibitory impacts of different drugs with different concentrations on drug-resistant cells and their parent cells were concentration-dependent, and that CNE2/DDP cells were most resistant to 5-FU (Figure 3(a,b)).

The mRNA and protein expression of relevant drug resistance genes (P-gp, MRP1, and GST-π) in CNE2 and CNE2/DDP cells were detected by Western blot analysis. The results (Figure 3(c)) showed that P-gp, MRP1, and GST-π expression in each drug-resistant cell line grew to a varying degree versus the parental strain (all \( P < 0.05 \)).

The RI of transfected CNE2/DDP cells under the functions of DDP and 5-FU in each group was calculated. The results showed that versus the blank group, the change in cell RI of the sh-NC group was not conspicuous (\( P > 0.05 \)); the same parameter of cells transfected with miR-140-5p mimic diminished versus those transfected with miR-140-5p mimic NC (\( P < 0.05 \)); the cell proliferation rate in the sh-SNHG7 + miR-140-5p inhibitor group increased versus the sh-SNHG7 + inhibitor NC group (\( P < 0.05 \)) (Figure 4(a,b)).

SNHG7 silencing and miR-140-5p elevation restrain colony formation ability and proliferation of NPC cells

Through the determination of the colony formation ability of CNE2 and CNE2/DDP cells, it was suggested that the sh-NC group had no conspicuous change in cell colony formation ability versus the blank group (\( P > 0.05 \)); the same ability of cells transfected with SNHG7 interference plasmid dropped versus those transected with SNHG7 interference NC plasmid (\( P < 0.05 \)); the same ability of cells transfected with miR-140-5p mimic fell versus those transfected with miR-140-5p mimic NC (\( P < 0.05 \)); the same parameter of the sh-SNHG7 + miR-140-5p inhibitor group rose (\( P < 0.05 \)) (Figure 3(d,e)).

Table 2. IC50 and RI of DDP and 5-FU on CNE2 and CNE2/DDP cells.

| Drugs | IC50 (μmol/L) | CNE2 | CNE2/DDP | RI |
|-------|---------------|------|----------|----|
| DDP   | 0.37 ± 0.10   | 7.86 ± 0.71 | 21.82 |
| 5-FU  | 6.42 ± 0.22   | 347.86 ± 37.61 | 53.45 |

DDP, cisplatin; 5-FU, 5-fluourouracil; IC50, half maximal inhibitory concentration; RI, resistance index.

SNHG7 silencing and miR-140-5p elevation boost cell apoptosis of NPC

Flow cytometry revealed that the proportion of CNE2 and CNE2/DDP cells in G0/G1, S, and G2/M phases in the sh-NC group was nearly identical with that in the blank group (\( P > 0.05 \)).

Table 2. IC50 and RI of DDP and 5-FU on CNE2 and CNE2/DDP cells.

Versus the sh-NC group, the proportion of cells grew in the G0/G1 phase in the sh-SNHG7 group, and dropped in S and G2/M phases (all \( P < 0.05 \)).

In the sh-SNHG7 + miR-140-5p inhibitor group, the proportion of cells fell in G0/G1 phases, and rose in S and G2/M phases versus the sh-SNHG7 + inhibitor NC group (all \( P < 0.05 \)) (Figure 5(a–c)).

Apoptosis detection by flow cytometry illustrated that the apoptosis rate of the sh-NC group was nearly the same as that of the blank group (\( P > 0.05 \)). The apoptosis rate of cells in the sh-SNHG7 group elevated
versus the sh-NC group \((P < 0.05)\). The same parameter in the miR-140-5p mimic group also increased relative to the mimic NC group \((P < 0.05)\), and declined in the sh-SNHG7 + miR-140-5p inhibitor group versus the sh-SNHG7 + inhibitor NC group \((P < 0.05)\) (Figure 5(d,e)).

The levels of Bax and Bcl-2 in the CNE2 and CNE2/DDP cells were detected by Western blot analysis. The

Figure 3. SNHG7 silencing and miR-140-5p elevation decline the drug resistance of drug-resistant NPC cells and their parent cells. (a), survival rate of CNE2 and CNE2/DDP cells under different concentrations of DDP; (b), survival rate of CNE2 and CNE2/DDP cells under different concentrations of 5-FU; (c), Western blot analysis of protein expression of related drug-resistance genes in CNE2 and CNE2/DDP; (d), RI of CNE2/DDP cells in each group under the function of DDP; (e), RI of CNE2/DDP cells in each group under the function of 5-FU; the data in the figure were measurement data expressed as mean ± standard deviation *, \(P < 0.05\) vs the parental strain CNE2 cells; a, \(P < 0.05\) vs the sh-NC group; b, \(P < 0.05\) vs the mimic-NC group; c, \(P < 0.05\) vs the sh-SNHG7 + inhibitor NC group.
results illustrated that Bax and Bcl-2 levels in cells transfected with SNHG7 interference NC plasmid were almost the same as those without any treatment ($P > 0.05$). The level of pro-apoptotic protein Bax ascended, and anti-apoptotic protein Bcl-2 reduced in the sh-SNHG7 group versus the sh-NC group (both...
Bax level rose while Bcl-2 level dropped in the miR-140-5p mimic group versus the mimic NC group (both \( P < 0.05 \)). In the sh-SNHG7 + miR-140-5p inhibitor group, Bax level descended and Bcl-2 level elevated versus the sh-SNHG7 + inhibitor NC group (both \( P < 0.05 \)) (figure 5(f,g)).
SNHG7 specially binds to miR-140-5p and SNHG7 silencing elevates miR-140-5p expression

Detection of SNHG7 and miR-140-5p expression in CNE2 and CNE2/DDP cells was performed by RT-qPCR. The results indicated that SNHG7 and miR-140-5p expression in the sh-NC group did not differ from that in the blank group \((P > 0.05)\). SNHG7 expression reduced while miR-140-5p level grew in the sh-SNHG7 group versus the sh-NC group \((P < 0.05)\). No conspicuous changes in SNHG7 expression were seen \((P > 0.05)\) and miR-140-5p expression elevated in the miR-140-5p mimic group versus the mimic-NC group \((P < 0.05)\). SNHG7 expression did not change conspicuously \((P > 0.05)\) and miR-140-5p expression diminished in the sh-SNHG7 + miR-140-5p inhibitor group versus the sh-SNHG7 + inhibitor NC group \((P < 0.05)\) (Figure 6(a,b)).

To examine the mechanism of SNHG7, we initially analyzed the online analysis website \(\text{http://lnca.tlas.crg.eu/}\) and found that SNHG7 was mainly distributed in the cytoplasm (Figure 6(c)), which was further verified by RNA-FISH assay (Figure 6(d)), indicating that SNHG7 may function in the cytoplasm. Furthermore, online analysis software predicted that there was a specific binding site between SNHG7 gene sequence and the miR-140-5p sequence (Figure 6(e)), and further verification by the dual luciferase reporter gene assay revealed that versus the mimic-NC group, the luciferase activity in the WT-miR-140-5p mimic/sh-SNHG7 group fell substantially \((P < 0.05)\), while no marked changes were found in that in the MUT-miR-140-5p mimic/sh-SNHG7 group \((P > 0.05)\), indicating that miR-140-5p may specifically bind to SNHG7 (figure 6(f)). RNA-pull down assay revealed that the enrichment of SNHG7 in the bio-miR-140-5p-WT group grew \((P < 0.05)\), and little difference was seen in that of the bio-miR-140-5p-MUT group versus the bio-probe NC group \((P > 0.05)\) (Figure 6(g)).

GLI3 is a direct target gene of miR-140-5p and miR-140-5p elevation diminishes GLI3 expression

GLI3 expression in CNE2 and CNE2/DDP cells were detected by RT-qPCR and Western blot analysis. The results showed that GLI3 expression in the sh-NC group were not palpable versus the blank group \((P > 0.05)\), and diminished in the sh-SNHG7 group versus the sh-NC group \((P < 0.05)\). Moreover, the same parameter in the miR-140-5p mimic group dropped versus the mimic NC group \((P < 0.05)\), and ascended in the sh-SNHG7 + miR-140-5p inhibitor group versus the sh-SNHG7 + inhibitor NC group \((P < 0.05)\) (Figure 7(a,b)).

The bioinformatics software (\(\text{http://www.targetscan.org} \)) predicted a targeted site between miR-140-5p and GLI3 (Figure 7(c)). Luciferase activity assay indicated that after CNE2 cells were co-transfected with GLI3-WT and miR-140-5p mimic, the relative luciferase activity reduced substantially \((P < 0.05)\), whereas co-transfection with GLI3-MUT and miR-140-5p mimic exerted no impact \((P > 0.05)\) (Figure 7(d)). These findings indicate that GLI3 is a direct target gene of miR-140-5p.

Discussion

NPC is a mysterious malignancy that infrequently arising in most regions of the world [18]. NPC is a big health problem in southern China, and the chief cause of treatment failure is ascribed to the local recurrence and distant metastasis [19]. LncRNAs have long been proposed to be implicated in tumorigenesis and cell growth [20]. However, few studies have probed into the action of lncRNA SNHG7 on NPC development, so in our study, a series of assays were performed to examine the role of SNHG7/miR-140-5p/GLI3 axis in NPC. Collectively, our study revealed that depleted lncRNA SNHG7 restricts GLI3 expression by upregulating miR-140-5p, which further suppresses cell proliferation and boosts apoptosis of NPC.

To begin with, we found elevated SNHG7 and GLI3, and underexpressed miR-140-5p in NPC tissues and cells. In accord with our result, an early study has demonstrated that SNHG7 expression was enhanced in non-small cell lung cancer [21]. In a study by Zhang W et al., miR-140-5p expression has been discovered to be much low in tissues and cell lines of colorectal cancer (CRC) [22]. Report has shown that GLI3 expression in liver fibrosis was elevated [23]. Moreover, we discovered that SNHG7 specially bound to miR-140-5p, and SNHG7 silencing enhanced miR-140-5p expression. It is indicated in esophagus cancer that miR-140-5p is targeted by...
SNHG16, and a decrease of SNHG16 expression leads to the enhancement of mature miR-140-5p [9]. In addition, we demonstrated that GLI3 was a direct target gene of miR-140-5p and miR-140-5p elevation diminished GLI3 expression. Wen SY et al. have confirmed that GLI3 is miR-506’ target gene, and that the

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**Figure 6.** SNHG7 specially binds to miR-140-5p and SNHG7 silencing elevates miR-140-5p expression. (a), SNHG7 expression of CNE2 cells and CNE2/DDP cells in each group; (b), miR-140-5p expression of CNE2 cells and CNE2/DDP cells in each group; (c), prediction of subcellular localization of SNHG7 by online analysis website; (d), confirmation of subcellular localization of SNHG7 by FISH assay; (e), prediction of the binding site between miR-140-5p and SNHG7 by bioinformatics website; (f), verification of the regulatory relationship between SNHG7 and miR-140-5p by dual luciferase reporter gene assay; (g), verification of the enrichment level of SNHG7 and miR-140-5p by RNA-pull down assay; the data in the figure were all measurement data expressed as mean ± standard deviation; a, P < 0.05 vs the sh-NC group; b, P < 0.05 vs the mimic-NC group; c, P < 0.05 vs the sh-SNHG7 + inhibitor NC group.
growth of miR-506 expression can result in the restriction of GLI3 expression [15]. A similar study by Yao C has also indicated that in human Sertoli cells, GLI3 was miR-133b’s direct target and the upregulation of miR-133b can cause an increase of GLI3 expression [24].

Furthermore, subsequent assays revealed that SNHG7 silencing and miR-140-5p elevation declined the drug resistance of drug-resistant NPC cells and their parent cells, restrained NPC cell colony formation ability and proliferation, and boosted cell apoptosis. In line with our study, previous research has shown that in neuroblastoma (NB), SNHG7 silencing negatively works on the development of NB cells by modulating miR-653-5p/STAT2 axis [25]. In breast cancer, SNHG7 is found to be enhanced, and if SNHG7 expression is repressed, tumor cell progression will also be circumscribed [26]. There is evidence showing that lower SNHG7 level has something to do with lower taxol resistance in hypopharyngeal cancer, and patients with low SNHG7 level possesses higher overall survival (OS) than those with high SNHG7 level [27]. It has been indicated that in cervical cancer, reduced SNHG20 expression and elevated miR-140-5p restrained cancer cell proliferation and invasion [28]. In a recent study, linc00515 downregulation has been indicated to constrain the chemoresistance of multiple myeloma cells by elevating miR-140-5p and diminishing ATG14 expression [29]. Besides, we discovered that miR-140-5p suppression reversed the impacts of SNHG7 silencing on NPC cells. Close associations of miR-140-5p downregulation with promoted CRC stage and worse OS have been proposed in an early study [22]. Also, the contributory impacts of miR-140-5p silencing on the reduction of OS of patients with gastric cancer have been illustrated lately [30]. All the above findings are in full support of our results.

To sum up, our study revealed that lncRNA SNHG7 knockdown restricts cell proliferation and promotes apoptosis of NPC by elevating miR-140-5p for GLI3 downregulation, and miR-140-5p inhibition can reverse the impacts of SNHG7 knockdown on NPC cells. Our study...
suggests lncRNA SNHG7 is a new biomarker of NPC, which can be a new target for NPC treatment. Nevertheless, more studies have to be done for better elaboration of the role of SNHG7/miR-140-5p/GLI3 axis in NPC.

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Authors’ contributions

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Disclosure statement

No potential conflict of interest was reported by the authors.

Ethical statement

This study was reviewed and approved by the Ethics Committee of Affiliated Otolaryngological Hospital of Zhengzhou University and was supervised by the Ethics Committee of Affiliated Otolaryngological Hospital, the First Affiliated Hospital of Zhengzhou University. Written informed consents were obtained from all patients and their family before the study.

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