

Long non-coding RNA ZNF674-1 acts as a cancer suppressor in nasopharyngeal carcinoma

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Abstract. Nasopharyngeal carcinoma (NPC) is the most frequently occurring carcinoma of the head and neck. The complexity of NPC makes it difficult for it to be diagnosed and treated at an early stage. Certain long non-coding RNAs (lncRNAs) are closely associated with the carcinogenesis of NPC. In the present study, the expression of lncRNA ZNF674-1 in NPC tissues and an NPC cell line was analyzed and was revealed to be downregulated compared with normal tissues and cells. When the expression of lncRNA ZNF674-1 was reduced in NPC cells, the proliferation, migration and invasion of these cells was promoted, whereas the apoptosis of these cells was decreased. On the contrary, when overexpressed, the expression of lncRNA ZNF674-1 inhibited the proliferation, invasion and migration of cells, but promoted cell apoptosis. The results of the present study reveal that the lncRNA ZNF67-1 may restrain the carcinogenesis of NPC, and may also serve as a potential biomarker for the early diagnosis and treatment of NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is a squamous cell malignancy that originates from the nasopharyngeal epithelium (1,2). In 2001, the incidence rates of NPC in China at 25-30 per 100,000 were the highest globally (3), as are the mortality rates for NPC in China (4). NPC has no typical symptoms in the early stage of the disease and is characterized by gross invasion; thus, the majority of patients with NPC are diagnosed at an advanced disease stage (5). Thus, there is a requirement for further research into the molecular mechanisms involved in the carcinogenesis of NPC, which may aid the identification of an independent biomarker for the early detection and treatment of NPC (1,6).

In the process of the transcription and translation of genetic information, 98.5% of all RNAs do not possess the ability to be translated into a protein (7,8). Long non-coding RNAs (lncRNAs) are one such type of these RNAs, and are >200 nucleotides in length (9). lncRNAs were considered to be transcriptional noise for a number of years (10); however, previous studies have revealed that abnormally expressed lncRNAs influence the progression of human diseases, particularly cancer, via gene silencing, chromatin remodeling, splicing regulation and cell cycle regulation (10,11). Furthermore, the lncRNAs HOX transcript antisense RNA, H19 and metastasis associated lung adenocarcinoma transcript 1 (MALAT1) were closely associated with human tumorigenesis (12-16).

lncRNA ZNF674-1 (OTTHUMG00000021416) was dysfunctionally expressed in primary NPC tissues, which indicates that it may serve a role in the tumorigenesis of NPC (17). In the present study, the expression of lncRNA ZNF674-1 in the NPC 6-10B cell line and 24 primary NPC tissues was assessed. Next, the expression of lncRNA ZNF674-1 was overexpressed and knocked down *in vitro* in order to identify its role in the proliferation, invasion, migration and apoptosis of NPC cells.

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Materials and methods

Cell lines and tissues. A total of 24 primary NPC specimens (mean age, 43 years; age range, 25-64 years; 18 men, 6 women) and 24 normal nasopharyngeal epithelial specimens were obtained from Peking University Shenzhen Hospital (Shenzhen, China) between May 2013 and September 2015. The patients were not age- and sex-matched. Fresh clinical specimens were obtained by nasal endoscope and stored immediately in liquid nitrogen following collection from the Ear-Nose-Throat Department. Ethical approval was obtained from the Research Ethics Board of Peking University Shenzhen Hospital. Each of the patients with NPC had no history of radiotherapy, chemotherapy or surgery prior to the experiment. Written informed consent was obtained from all patients prior to the study. Clinicopathological information for the patients is presented in Table I. The clinical staging of all patients were determined using the 2010 Cancer Staging Standard published by the America Joint Committee (18).

Cell culture. An NPC 6-10B cell line and the human normal nasopharyngeal NP460 cell line were obtained from Peking University Shenzhen Hospital and Southern Medical University (Guangzhou, China). The two cell lines were maintained in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 50 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.), 50 U/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator at 37°C with 5% CO₂.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from clinical tissues and cell lines using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Next, the purity of RNA was examined using the SmartSpec Plus Spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA). For RT-qPCR, the Reverse Transcription kit (Takara Biotechnology, Co., Ltd., Dalian, China) was used to reverse transcribe the RNA to cDNA according to the manufacturer's protocol. The Roche Lightcycler 480 Real-Time PCR (Roche Applied Science, Penzberg, Germany) system was used to detect the expression of lncRNA ZNF674-1 in clinical tissues and cell lines using the Synergy Brands Green kit (Takara Biotechnology, Co., Ltd.). The thermocycling conditions for RT-qPCR were: 94°C for 3 min, 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min for 40 cycles and finally 72°C for 10 min. All samples were tested in triplicate. The expression of lncRNA ZNF674-1 were normalized using GAPDH, which was set as an internal reference. The primer sequences for lncRNA ZNF674-1 were as follows: Forward, 5'-AGCACTTGGCCCTAAAGAGA-3' and reverse, 5'-AACATACTGGCCCAAACAGA-3'. The primer sequences for GAPDH were as follows: Forward, 5'-TCCAAATCAAGTGGGGCGGA-3' and reverse, 5'-TGATGACCCTTTTGGCTCCCC-3'. The 2^{-ΔΔC_q} method was used to calculate the relative expression of lncRNA ZNF674-1 in clinical tissues and cell lines (19).

Knockdown and overexpression of lncRNA ZNF674-1. One non-targeting control (si-NC) and four small interfering RNAs (siRNAs) against lncRNA ZNF674-1 (si-ZNF674-1) were used. All siRNAs were synthesized by GenePharma (Shanghai GenePharma Co., Ltd., Shanghai, China). The four si-ZNF674-1 were: sense, 5'-CCCUGAUCUUGAUGGCCAUTT-3' and antisense, 5'-AUGGCCAUCAAGAUUAGGTT-3'; sense, 5'-CCUGAUUAUCUGAAGUAACATT-3' and antisense, 5'-UGUUACUUCAGAUUAUCAGGTT-3'; sense, 5'-GGCGGAUUCAUUACAGUUATT-3' and antisense, 5'-UCA AUGAGAGCAGGUAUGTT-3'; sense, 5'-CUACUCUAUAAGGCAUCUATT-3' and antisense, 5'-UAGAUGCCUUAUAGAGUAGTT-3'.

A total of 3x10⁶ 6-10B cells were transfected with si-NC (100 nM) and si-ZNF674-1 (100 nM) using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following seeding in a six-well plate overnight. After 48 h of transfection, the interfering efficiency of the four si-ZNF674-1 was examined. Consequently, si-ZNF674-1, which exhibited the highest knockdown efficiency of lncRNA ZNF674-1 expression (>78.8%) of the four si-ZNF674-1, was selected for further experimentation. The sequences of the si-ZNF674-1 selected were as follows: Sense, 5'-CCCUGAUCUUGAUGGCCAUTT-3'; antisense, 5'-AUGGCCAUCAAGAUUAGGGTT-3'; si-NC sense, 5'-GAGGCGUGGAGUCUUGUUUTT-3'; antisense, 5'-AAACAAGACUCCACGCCUUTT-3'.

ADV4 was used as a template to synthesize the adenovirus (ADV4-lncRNA-ZNF674-1), which was produced by Shanghai GenePharma Co., Ltd. (Shanghai, China), and contained the target sequence to overexpress the lncRNA ZNF674-1. A total of 3x10⁶ 6-10B cells were seeded in a 6-well plate for 24 h and then 10 μl ADV4-lncRNA-ZNF674-1 (9x10⁹ PFU/ml) or ADV4-NC (9x10⁹ PFU/ml) was added into the medium. Then, 48 h later, the overexpression efficiency of ADV4-lncRNA-ZNF674-1 was examined.

Cell proliferation assays. Cell proliferation was assessed using the Cell Counting Kit-8 (CCK8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. A total of 3,000 6-10B cells/well were plated into a 96-well plate with 10 replicates. Next, half of the wells were transfected with si-ZNF674-1 or infected with ADV4-lncRNA-ZNF674-1, and the other half were transfected with si-NC or infected with ADV4-NC. Cellular proliferation was assessed at 450 nm every 24 h post-transfection.

Cell scratch assay. 6-10B cells (2x10⁶ cells/well) were seeded in a 6-well plate. Cells were transfected with si-ZNF674-1 or infected with ADV4-lncRNA-ZNF674-1 or the respective controls when they grew to 90-100% confluence. Next, a sterile 200-μl pipette tip was used to scratch a line through the well at 6 h post-transfection. Finally, serum-free RPMI-1640 medium was used to replace the serum-containing medium. Images of migration distance were captured and assessed using an inverted microscope (magnification, x100; Olympus Corporation, Tokyo, Japan) at 0 and 24 h after scratch assay.

Transwell assay. 6-10B cells were harvested at 24 h after transfection with the aforementioned siRNAs or lentiviruses. For the migration assays, a total of 1x10⁴ cells in 100-μl serum-free

Table I. Clinicopathological characteristics of patients.

Characteristics	Patients, n
Sex	
Male	18
Female	6
Degree of differentiation	
Undifferentiated	15
Differentiated	7
Histology	
Squamous	24
Other	0
Lymph node metastasis	
Positive	17
Negative	7
Distal metastasis	
Positive	0
Negative	24
Clinical TNM stage	
I-II	4
III-IV	20

TNM, Tumor-Node-Metastasis.

RPMI-1640 medium were seeded into the upper chamber (8- μ m pore size; EMD Millipore, Billerica, MA, USA). For the invasion assays, a total of 1×10^4 cells in 100- μ l serum-free RPMI-1640 medium were seeded in the upper chamber, which was coated with Matrigel (1:5; 50 μ l/well; BD Biosciences, Franklin Lakes, NJ, USA). The lower chambers were filled with 500 μ l RPMI-1640 medium which contained 10% FBS. Subsequent to culturing for 24 h, the cells that migrated or invaded the membrane were fixed using 4% paraformaldehyde for 25 min at room temperature and stained using 0.1% crystal violet for 25 min at room temperature in the dark. Finally, the stained cells were imaged using an inverted confocal microscope (magnification, x100; Olympus Corporation) and the total stained cells in 5 fields of view were counted by using an inverted confocal microscope (magnification, x100; Olympus Corporation).

Cell apoptosis assay. 6-10B cells were plated into a 6-well plate and transfected with aforementioned siRNAs or lentiviruses. A total of 48 h after transfection, the cells were collected and washed three times with pre-chilled phosphate buffered saline. Next, the cells were double stained using an Annexin V-fluorescein isothiocyanate/propidium iodide detection kit (Invitrogen; Thermo Fisher Scientific, Inc.) for 15 min at 4°C, according to the manufacturer's protocol. Finally, a flow cytometer (EPICS XI-4; Beckman Coulter, Inc., Brea, CA, USA) was used to detect the frequency of apoptotic cells using the FCS Express 3.0 software (De Novo Software, Glendale, CA, USA).

Statistical analysis. All experiments were performed at least three times and the data are presented as the mean \pm standard

deviation. All statistical analyses were performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). The differences in expression between the NPC and matched normal clinical specimens and between the two cell lines were analyzed using an unpaired Student's t-test. The cell proliferation, cell scratch assay, Transwell assay and cell apoptosis assay data were analyzed by one-way analysis of variance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Expression of lncRNA ZNF674-1 was downregulated in human NPC clinical tissues and NPC cell line. The expression of lncRNA ZNF674-1 was examined in 24 non-cancer nasopharyngeal epithelial specimens and 24 NPC specimens. The results revealed that lncRNA ZNF674-1 was significantly downregulated in NPC specimens compared with non-cancer nasopharyngeal epithelial specimens ($P < 0.001$; Fig. 1A). As presented in Fig. 1B, the expression of lncRNA ZNF674-1 in the NPC 6-10B cell line was significantly lower compared with that in the normal NP460 cell line ($P < 0.01$).

Knockdown and overexpression of lncRNA ZNF674-1 in 6-10B. To investigate the role lncRNA ZNF674-1 serves in the progression of NPC, a siRNA transient transfection to knock down lncRNA ZNF674-1 in 6-10B cells was established. si-ZNF674-1 significantly decreased the expression of lncRNA ZNF674-1 by 78.8% in 6-10B cells compared with si-NC ($P < 0.01$; Fig. 2A). Next, an adenovirus resulting in the stable overexpression of lncRNA ZNF674-1 in 6-10B cells was synthesized. The ADV4-lncRNA-ZNF674-1 adenovirus could overexpress lncRNA ZNF674-1 at a level 1,370 times higher than that in ADV4-NC transfected cells in 6-10B cells, a difference which was identified to be significant ($P < 0.001$; Fig. 2B).

lncRNA ZNF674-1 restrained the proliferation of NPC cells. A CCK8 assay was used to investigate the effect of lncRNA ZNF674-1 in NPC cell proliferation. The results revealed that the cells infected with ADV4-lncRNA-ZNF674-1 exhibited significantly decreased cell proliferation compared with cells infected with ADV4-NC ($P < 0.05$; Fig. 3A), while the 6-10B cells transfected with si-ZNF674-1 exhibited significantly increased NPC cell proliferation compared with cells transfected with si-NC ($P < 0.05$; Fig. 3B).

lncRNA ZNF674-1 inhibited the migration and invasion of NPC cells. The migratory capacity of NPC cells was investigated through scratch assays and Transwell migration assays. Scratch assays revealed that the migratory ability of 6-10B cells following infection with ADV4-lncRNA-ZNF674-1 were significantly reduced compared with cells infected with the negative control ($P < 0.001$; Fig. 4A), whereas cell migration was increased following transfection with si-ZNF674-1 compared with cells transfected with si-NC ($P < 0.01$; Fig. 4B). A similar result was obtained in the Transwell migration assays. The migratory ability was significantly lower in cells infected with ADV4-lncRNA-ZNF674-1 than in those infected with ADV4-NC ($P < 0.001$; Fig. 5A), meanwhile, the

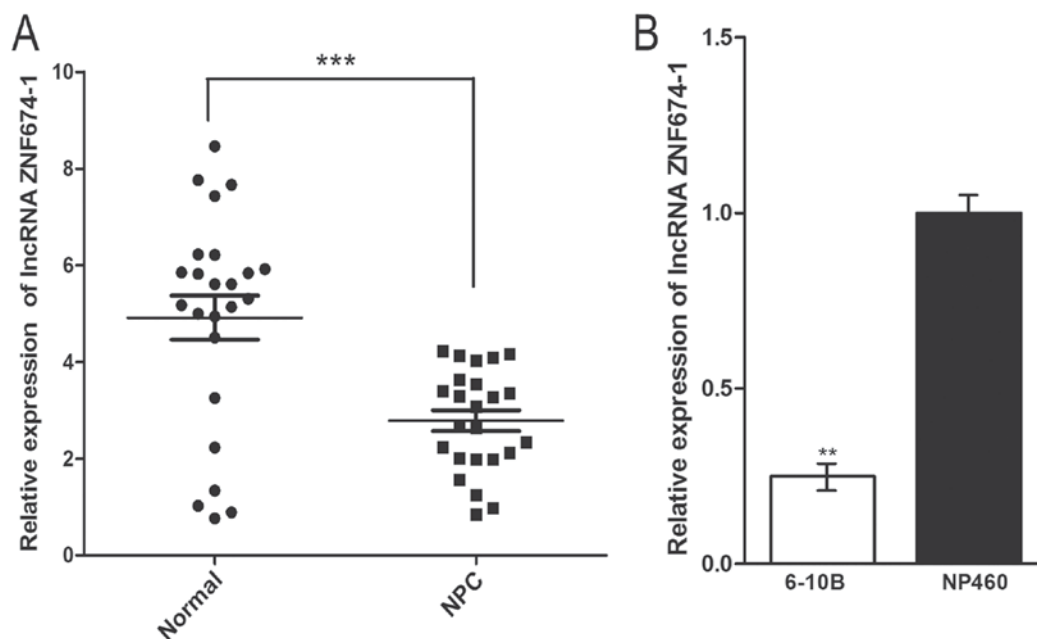


Figure 1. Relative lncRNA ZNF674-1 expression in NPC specimens and NPC-derived cells were assessed using reverse transcription-quantitative polymerase chain reaction. (A) lncRNA ZNF674-1 was downregulated in 24 NPC specimens compared with 24 normal nasopharyngeal epithelial specimens. *** $P < 0.001$. (B) lncRNA ZNF674-1 was expressed at a lower level in the NPC 6-10B cell line compared with that in NP460. ** $P < 0.01$ vs. NP460 cells. The data were analyzed using the $2^{-\Delta\Delta C_q}$ method and presented as the mean \pm standard deviation. NPC, nasopharyngeal carcinoma; lncRNA ZNF674-1, long non-coding RNA zinc finger protein 674-1.

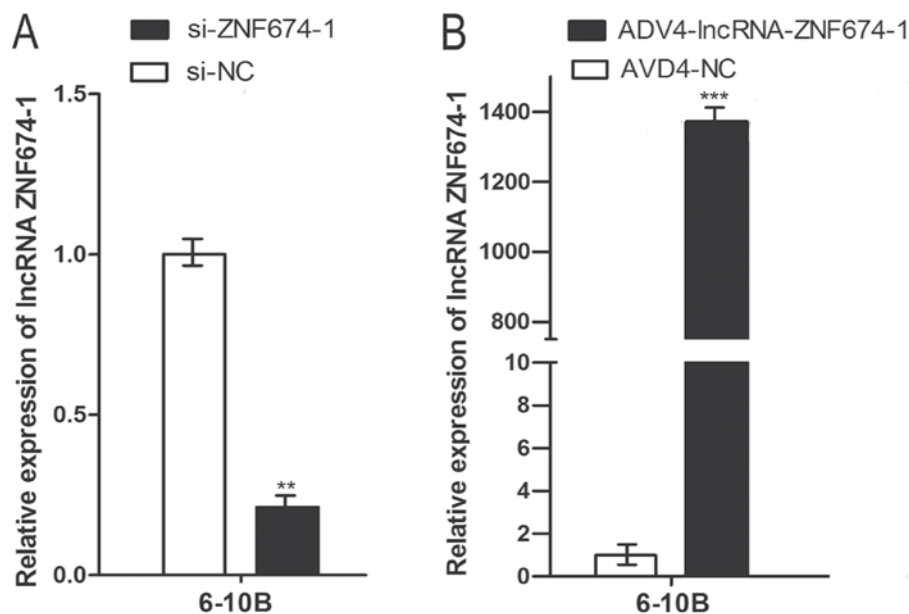


Figure 2. Knockdown and overexpression of lncRNA ZNF674-1 in 6-10B cells transfected with siRNA or adenoviruses. (A) Relative expression levels of lncRNA ZNF674-1 in 6-10B cells transfected with si-ZNF674-1 or si-NC. ** $P < 0.01$ vs. si-NC. (B) Relative expression levels of lncRNA ZNF674-1 in 6-10B cells infected with ADV4-lncRNA-ZNF674-1 or ADV4-NC. *** $P < 0.001$ vs. ADV4-NC. si-ZNF674-1, small interfering RNA targeting lncRNA ZNF674-1; lncRNA, long non-coding RNA; ADV4-lncRNA-ZNF674-1, adenovirus containing exogenous lncRNA ZNF674-1; NC, negative control.

migratory ability was significantly higher in cells transfected with si-ZNF674-1 than in those transfected with si-NC ($P < 0.01$; Fig. 5B). Transwell invasion assays revealed that cells infected with ADV4-lncRNA-ZNF674-1 had a significantly lower invasion ability than did ADV4-NC infected cells ($P < 0.01$; Fig. 6A). However, cells transfected with si-ZNF674-1 had a significantly higher invasive capability than cells transfected with si-NC ($P < 0.05$; Fig. 6B).

lncRNA ZNF674-1 impaired the apoptosis of NPC cells. Flow cytometry was used to analyze the role of lncRNA ZNF674-1 in the apoptosis of NPC cells. As presented in Fig. 7A, the apoptosis rate of the cells infected with ADV4-lncRNA-ZNF674-1 was significantly increased compared with the negative control ($P < 0.01$). Comparatively, the apoptosis rate of the cells transfected with si-ZNF674-1 was decreased compared with the cells transfected with si-NC (Fig. 7B).

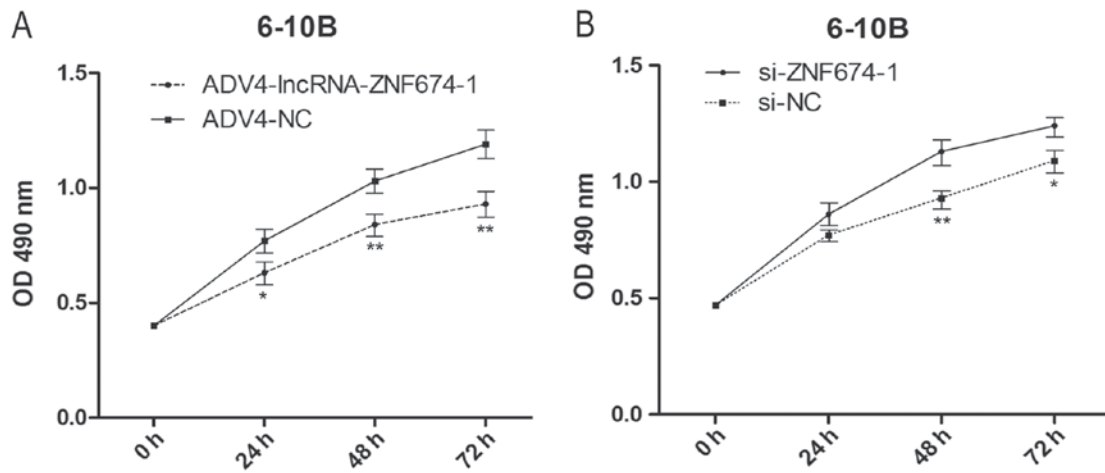


Figure 3. lncRNA ZNF674-1 inhibited the proliferation of nasopharyngeal carcinoma cells. (A) Proliferation of 6-10B cells infected with ADV4-lncRNA-ZNF674-1 or ADV4-NC. * $P < 0.05$ and ** $P < 0.01$ vs. ADV4-NC cells. (B) Proliferation of 6-10B cells transfected with si-ZNF674-1 or si-NC. * $P < 0.05$ and ** $P < 0.01$ vs. si-NC cells. Measured using a Cell Counting Kit-8 at different time intervals. lncRNA, long non-coding RNA; NC, negative control; si-ZNF674-1, small interfering RNA targeting lncRNA ZNF674-1; ADV4-lncRNA-ZNF674-1, adenovirus containing exogenous lncRNA ZNF674-1.

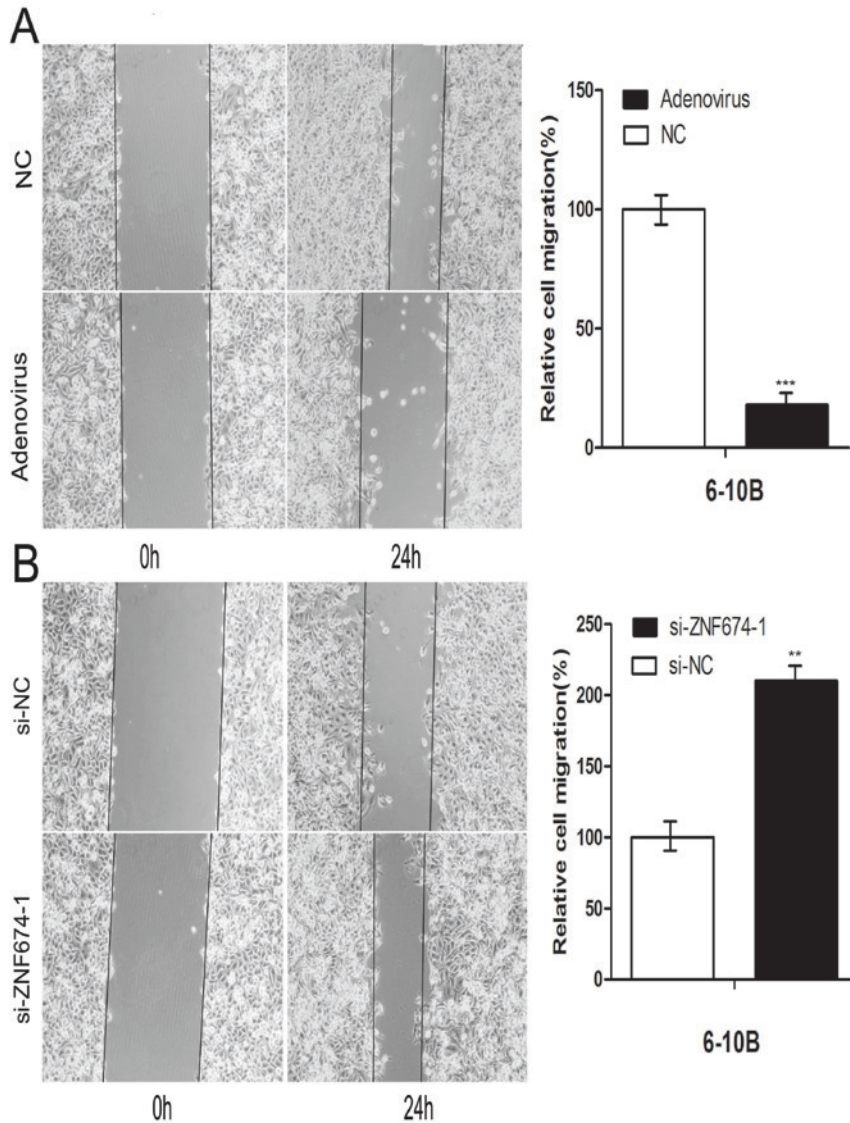


Figure 4. lncRNA ZNF674-1 restrained the migration of nasopharyngeal carcinoma cells. Cell scratch assay was used to measure the migration of 6-10B cells (A) following infection with ADV4-lncRNA-ZNF674-1 or ADV4-NC or (B) transfection with si-ZNF674-1 or si-NC. * $P < 0.05$ and ** $P < 0.01$. Magnification, $\times 100$. NC, negative control; si-ZNF674-1, small interfering RNA targeting lncRNA ZNF674-1; ADV4-lncRNA-ZNF674-1, adenovirus containing exogenous lncRNA ZNF674-1.

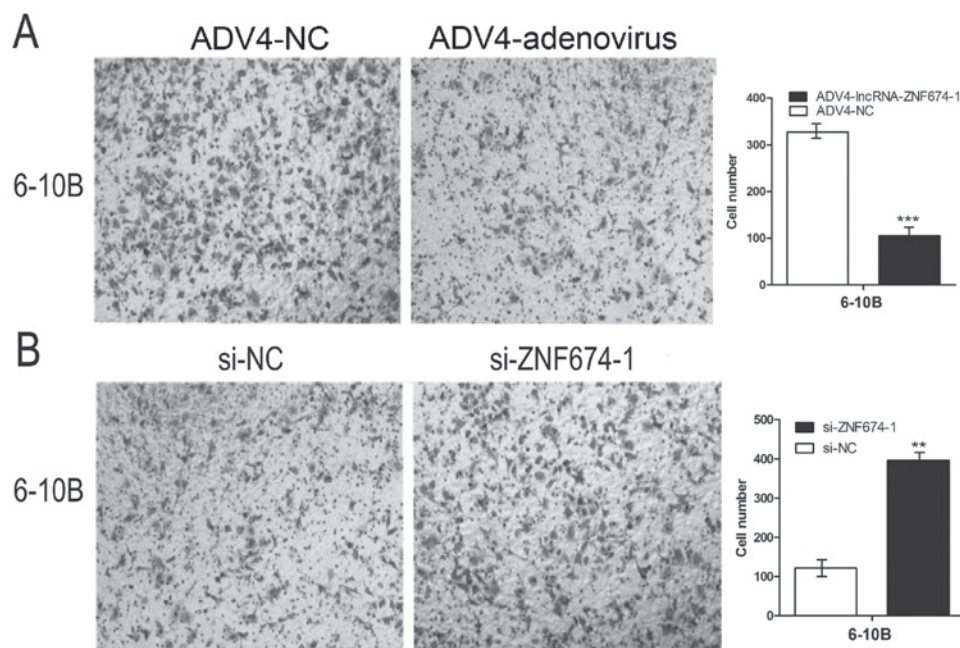


Figure 5. lncRNA ZNF674-1 suppressed the migratory ability of nasopharyngeal carcinoma cells. (A) Transwell migration assays revealed the migration of 6-10B cells following infection with ADV4-lncRNA-ZNF674-1 or ADV4-NC. *** $P < 0.001$ vs. ADV4-NC. (B) Transwell migration assays revealed the migration of 6-10B cells following transfection with si-ZNF674-1 or si-NC. ** $P < 0.01$ vs. si-NC. All data were presented as the mean \pm standard deviation. Magnification, $\times 100$. lncRNA, long non-coding RNA; si-ZNF674-1, small interfering RNA targeting lncRNA ZNF674-1; ADV4-lncRNA-ZNF674-1, adenovirus containing exogenous lncRNA ZNF674-1; NC, negative control.

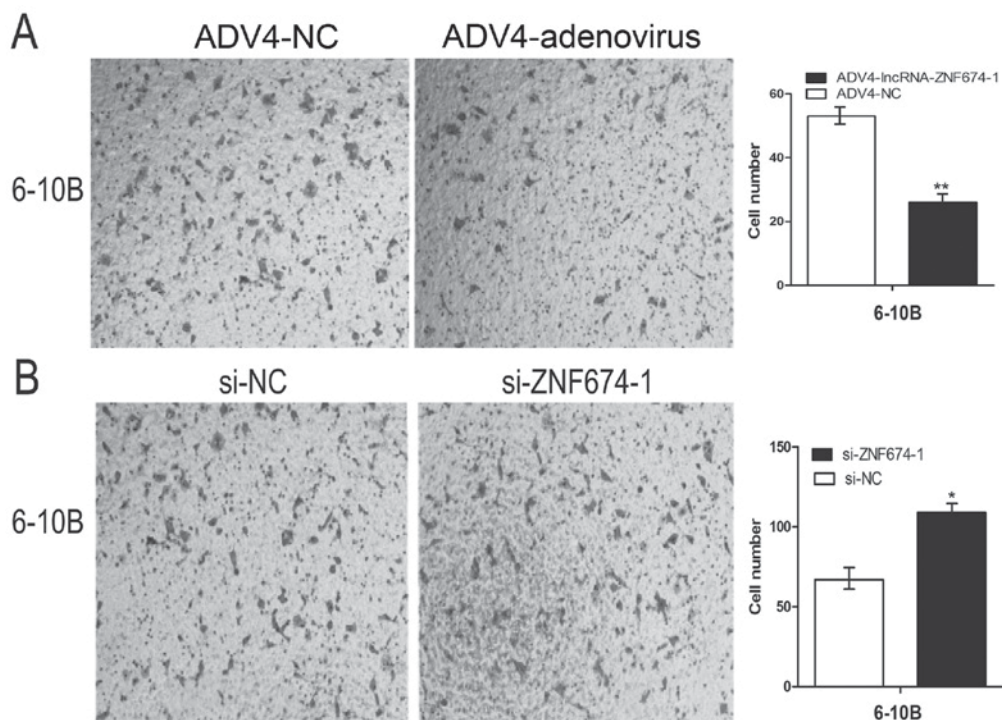


Figure 6. Transwell invasion assays demonstrated that lncRNA ZNF674-1 suppressed the invasive ability of nasopharyngeal carcinoma cells. (A) Invasion ability of cells infected with ADV4-lncRNA-ZNF674-1 or ADV4-NC. ** $P < 0.01$ vs. ADV4-NC. (B) Invasion ability of cells transfected with si-ZNF674-1 or si-NC. * $P < 0.05$ vs. si-NC. All data were presented as the mean \pm standard deviation. Magnification, $\times 100$. lncRNA, long non-coding RNA; si-ZNF674-1, small interfering RNA targeting lncRNA ZNF674-1; ADV4-lncRNA-ZNF674-1, adenovirus containing exogenous lncRNA ZNF674-1; NC, negative control.

Discussion

An epidemiological study has revealed that numerous factors may affect cell gene expression, which results in the

occurrence of NPC (20). Among all of these factors, the environment, genes and Epstein-Barr virus infection status are the most notable (21-24). The pathological process of NPC remains unclear, so when studying the pattern of NPC

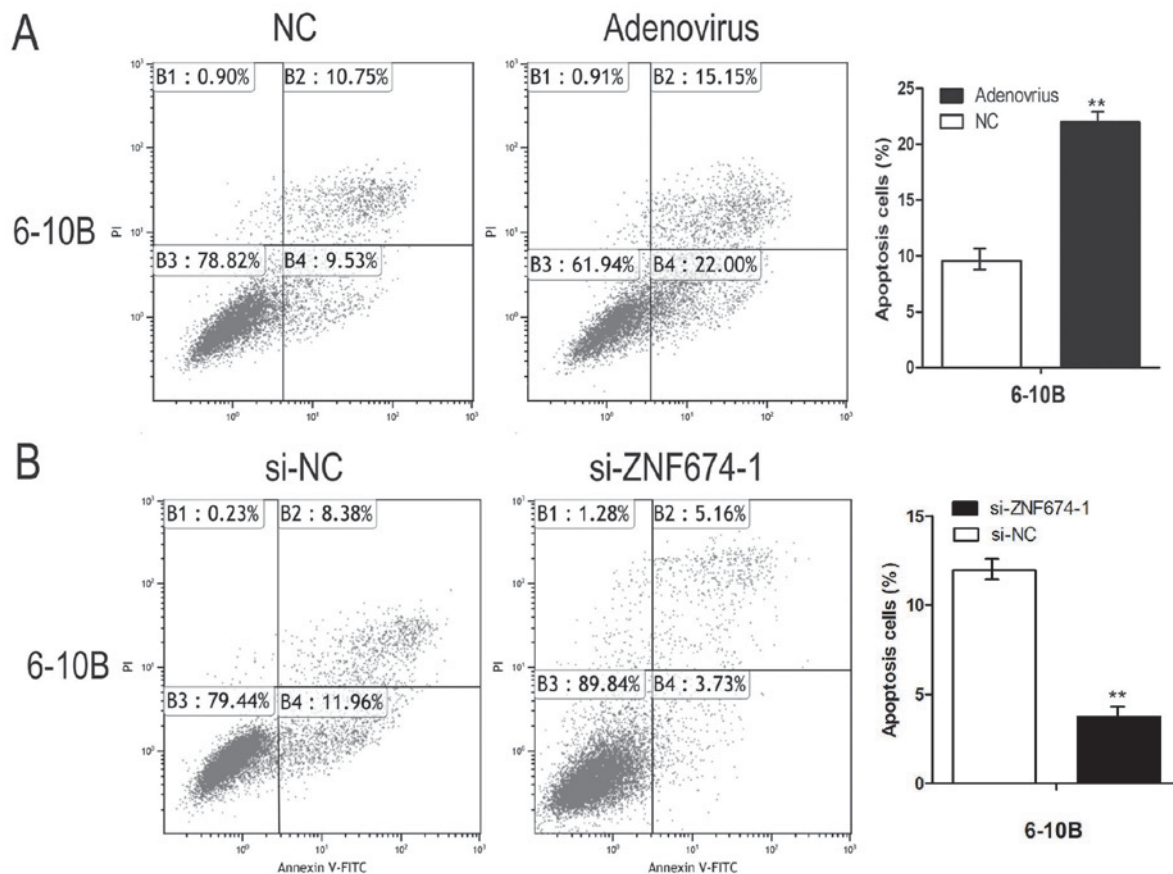


Figure 7. lncRNA ZNF674-1 decreased the apoptotic rate of the NPC cells. (A) Flow cytometry was used to analyze the apoptotic rates of NPC cells following infection with ADV4-lncRNA-ZNF674-1 and ADV4-NC. ** $P<0.01$ vs. NC. (B) Flow cytometry was used to analyze the apoptotic rates of NPC cells transfected with si-ZNF674-1 and si-NC. ** $P<0.01$ vs. si-NC. The data were presented as the mean \pm standard deviation. lncRNA, long non-coding RNA; NPC, nasopharyngeal carcinoma; NC, negative control; si-ZNF674-1, small interfering RNA targeting lncRNA ZNF674-1; ADV4-lncRNA-ZNF674-1, adenovirus containing exogenous lncRNA ZNF674-1.

occurrence, the identification of novel biomarkers could be performed. By combining these biomarkers with the presence of clinicopathological features, the detection of NPC could occur earlier, allowing for the earlier treatment of NPC (25,26). Previously, multiple studies have reported that aberrantly expressed lncRNAs were closely associated with the tumorigenesis of malignancies (27-29). A number of these aberrantly expressed lncRNAs may function as independent biomarkers of the disease, reflecting the progression of the disease and predicting patient outcome (30,31). Growth arrest specific 5, the expression of which is decreased in hepatocellular carcinoma, may restrain cell proliferation and predict poor patient prognosis (32,33). MALAT1, originally identified in non-small-cell lung cancer, has been used as an indicator for the early detection and judgment of the progress of non-small-cell lung cancer (15,16). A previous study identified that lncRNA ZNF674-1 was aberrantly expressed in primary NPC tissues; however, the function of lncRNA ZNF674-1 in NPC is yet to be investigated (17). lncRNA ZNF674-1 is situated at chromosomal location Xp11, and is 626 bp (17) and it has been identified that Xp11.2 translocation is closely associated with renal cell carcinoma tumorigenesis (34,35). This indicates that lncRNA ZNF674-1 may be a cancer-associated gene involved the carcinogenesis of NPC. In the present study, the role of lncRNA ZNF674-1 in NPC was characterized, to

the best of our knowledge, for the first time. In the present study, the expression of lncRNA ZNF674-1 in 24 primary NPC specimens, 24 normal nasopharyngeal epithelial specimens, the normal nasopharyngeal epithelial NP460 cell line and the NPC 6-10B cell line was examined. It was revealed that lncRNA ZNF674-1 was significantly downregulated in NPC tissues and NPC cell lines compared with their respective controls ($P<0.01$). lncRNA ZNF674-1 was knocked down or overexpressed to investigate the role of lncRNA ZNF674-1 in the progression of NPC. lncRNA ZNF674-1 knockdown in 6-10B cells promoted NPC cell proliferation, migration, invasion and restrained cell apoptosis. On the contrary, overexpression of lncRNA ZNF674-1 inhibited cell growth, the invasive and migratory abilities of cells, and promoted cell apoptosis *in vitro*.

Taken together, the results of the present study indicated that lncRNA ZNF674-1 serves a negative function in NPC carcinogenesis, meaning that it may be a useful diagnostic biomarker for the early detection and therapy of NPC. However, the precise molecular mechanisms of lncRNA ZNF674-1 in the carcinogenesis of NPC requires further study.

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Availability of data and materials

All data generated or analysed for the present study are included in this published article.

Author's contributions

GHN conceived the experiments. ZL carried out the molecular genetic studies. HFD and HYH collected clinical specimens. LL and XFC performed the statistical analysis. WZ analysed the results and drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the Research Ethics Board of Peking University Shenzhen Hospital. Written informed consent was obtained from all patients prior to the study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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