

lncRNA C22orf32-1 contributes to the tumorigenesis of nasopharyngeal carcinoma

GUO-HUI NIE^{1*}, ZHAO LI^{2,3*}, HONG-FANG DUAN⁴, LIANG LUO^{2,3}, HONG-YI HU², WEI-QIANG YANG², LI-PING NIE⁵, RU-FEI ZHU², XIAO-FAN CHEN⁶ and WEI ZHANG⁶

¹Department of Otolaryngology, Shenzhen Second People's Hospital, Shenzhen, Guangdong 518035;

²Department of Otolaryngology, Peking University Shenzhen Hospital, Shenzhen, Guangdong 518036;

³Guangzhou Medical University, Guangzhou, Guangdong 510000; ⁴Department of Otolaryngology, Shenzhen Children's Hospital, Shenzhen, Guangdong 518038; ⁵Department of Clinical Laboratory, Peking University Shenzhen Hospital; ⁶Biomedical Research Institute, Shenzhen Peking University-

The Hong Kong University of Science and Technology Medical Center, Shenzhen, Guangdong 518036, P.R. China

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Abstract. The mechanism of nasopharyngeal carcinoma (NPC) remains unclear. The present study investigated the abnormal expression of long non-coding (lnc)RNAs in NPC tissues and one NPC cell line to identify the involvement of lncRNAs in the tumorigenesis of NPC. Using a quantitative reverse transcription polymerase chain reaction (RT-qPCR), the expression of lncRNA C22orf32-1 in NPC tissues and an NPC cell line was verified. The effects of lncRNA C22orf32-1 on NPC cells were investigated with a cell proliferation assay, cell scratch assay, Transwell assay and a cell apoptosis assay. The expression levels of lncRNA C22orf32-1 in NPC tissues and an NPC cell line were upregulated. lncRNA C22orf32-1 promoted the proliferation, migration and invasion of NPC cells, and reduced the apoptosis of NPC cells. The data demonstrated that lncRNA C22orf32-1 may facilitate the tumorigenesis of NPC, and may be used for the early diagnosis and treatment of NPC.

Introduction

Nasopharyngeal carcinoma (NPC) is the most common type of malignancy of the neck and head in China (1,2). Due to the anatomical location, the nasopharynx is difficult to access and the early symptoms of NPC are not distinctive. Therefore, NPC is usually diagnosed at the advanced stage (3,4). At present, the main treatment of NPC is radiotherapy. Radiotherapy may inhibit the development of NPC, but it may seriously influence the quality of life of the patient due to the side effects (5-7). The regulatory mechanisms of NPC remain unclear. Therefore, research on the molecular mechanisms and genetic alterations involved in the progression of NPC is required, as this may help to find novel therapeutic and diagnostic methods (8-11).

Long non-coding (lnc)RNAs are non-protein-coding transcripts with >200 nucleotides (12). As the non-coding RNAs do not serve as the template for protein synthesis, they are considered superfluous in transcription. Multiple previous studies have demonstrated that lncRNAs take part in the development of numerous diseases, particularly via gene silencing, cell cycle regulating, chromatin remodeling and splicing regulating (13-15). The lncRNAs are categorized into oncogenes and tumor suppressor genes according to their different functions in variety of types of malignancy (16,17). It was demonstrated that the dysfunctionally expressed lncRNAs were associated with the occurrence of tumors (18). Therefore, lncRNAs may be used for the early diagnosis of tumors and as novel targets for treatments (19,20). lncRNA metastasis associated lung adenocarcinoma transcript 1 has already been used as a biomarker for the early diagnosis of non-small cell lung cancer, due to its specificity compared with the other serum-specific antigens (21).

lncRNA C22orf32-1 (OTTHUMG00000150697) was identified through gene expression profile analysis (22). The expression of lncRNA C22orf32-1 was significantly upregulated in primary NPC tissues compared with the normal nasopharyngeal epithelial tissues, and therefore may act as an

Correspondence to: Dr Guo-Hui Nie, Department of Otolaryngology, Shenzhen Second People's Hospital, 3002 Sungang West Road, Shenzhen, Guangdong 518035, P.R. China
E-mail: nghui@21cn.com

Dr Wei Zhang, Biomedical Research Institute, Shenzhen Peking University-The Hong Kong University of Science and Technology Medical Center, 1120 Lotus Road, Shenzhen, Guangdong 518036, P.R. China
E-mail: zhangweispace@yeah.net

*Contributed equally

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independent biomarker for early diagnosis and provide a novel therapeutic target for NPC (13,22,23). In the present study, the expression levels of lncRNA C22orf32-1 in 24 primary NPC tissues and 24 normal nasopharyngeal epithelial tissues were examined with one NPC and one normal nasopharyngeal epithelial cell line. The effect of lncRNA C22orf32-1 knockdown on NPC cell proliferation, migration, invasion and apoptosis was explored.

Materials and methods

Tissue specimens. A total of 48 samples were collected from Peking University Shenzhen Hospital (Shenzhen, China) from May 2013 to February 2015. Of these, 24 samples were NPC tissues from patients with primary NPC and others were normal nasopharyngeal epithelial from volunteers without cancer. Fresh samples of normal nasopharyngeal epithelium and NPC tissues were obtained from the Department of Head and Neck (Peking University Shenzhen Hospital, Shenzhen, China), and stored immediately in liquid nitrogen. The present study was approved by the Research Ethics Board at Peking University Shenzhen Hospital (Shenzhen, China). No NPC patients received radiotherapy or chemotherapy prior to the surgery. The clinical staging of all patients were determined using the 2010 Cancer Staging Standard published by the America Joint Committee (24). Details of the patient clinical information are summarized in Table I.

Cell culture. The normal human nasopharyngeal NP460 and NPC 6-10B cell lines were obtained from the Southern Medical University (Guangzhou, China) and Peking University Shenzhen Hospital (Shenzhen, China). Cells were grown in a humidified incubator with 5% CO₂ at 37°C and maintained in medium containing 88% RPMI-1,640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 50 U/ml streptomycin and 50 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.).

RNA extraction, complimentary (c)DNA synthesis and quantitative reverse transcription polymerase chain reaction (RT-qPCR). The RNA was extracted from samples and cell lines using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the protocol of the manufacturer. The purity of RNA was examined in the A260/A280 ratio using the SmartSpec Plus Spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, USA). The cDNA was synthesized from RNA using a Reverse Transcription kit (Takara Biotechnology Co., Ltd., Dalian, China). The lncRNA C22orf32-1 expression levels in samples and cell lines were detected with the SYBR Green kit (Takara Biotechnology Co., Ltd.), and analyzed using the Roche Lightcycler 480 Real-Time PCR System (Roche Applied Science, Penzberg, Germany). The thermocycling conditions for RT-qPCR were: 94°C for 3 min, then 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. Glycerinaldehyde 3-phosphate dehydrogenase (GAPDH) was set as the internal control to normalize the relative expression level of lncRNA C22orf32-1. The primer sequences were: Forward, 5'-AGC ACTGGCCCTAAAGAGA -3'; reverse, 5'-AACATACTG

GCCCAAACAGC -3' for lncRNA C22orf32-1, and forward, 5'-GAGCACAGAGCCTCGCCTT-3'; reverse, 5'-TCATCA TCCATGGTGAGCTGGC-3' for GAPDH. The expression levels of lncRNA C22orf32-1 and GAPDH in specimens were calculated using the $\Delta\Delta Cq$ method [$\Delta\Delta Cq = (\text{mean}Cq_{\text{lncRNA C22orf32-1(NPC)}} - \text{mean}Cq_{\text{GAPDH (NPC)}}) - (\text{mean}Cq_{\text{lncRNA C22orf32-1(normal)}} - \text{mean}Cq_{\text{GAPDH(normal)}})$]. The relative RNA expression in cells was calculated using the $2^{-\Delta\Delta Cq}$ method (25).

Small interfering (si)RNA and transfection of cell lines. A total of four siRNAs against lncRNA C22orf32-1, si-C22orf32-1, and one negative control, si-NC, were designed and synthesized by GenePharma (GenePharma, Shanghai, China). All the four si-C22orf32-1 were: Sense, 5'-CCCAGAGUCACU UAGAAGATT-3' and antisense, 5'-AUGGCCAUCAAGAUU AGGGTT-3'; sense, 5'-GAGGCGUGGAGUCUUGUUUTT-3' and antisense, 5'-AAACAAGACUCCACGCCUCTT-3'; sense, 5'-GGCGGAUUCAUACAGUUATT-3' and antisense, 5'-UAACUGUAAUGAAUCCGCCTT-3'; sense, 5'-CUGGAA UACAAUGCUCUAUTT-3' and antisense, 5'-AUAGAGCAU UGUUUUCCAGTT-3'. A density of 1×10^5 NPC cells were seeded onto 6-well plates overnight and transfected with 100 nM si-C22orf32-1 or 100 nM si-NC using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The interfering efficiency was tested using RT-qPCR 48 h following transfection. The most effective siRNA against si-C22orf32-1, which had a silence efficiency of >57.3%, was then selected for further analysis. The primers used were: Si-C22orf32-1 sense: 5'-CCC UAAUCUUGAUGGCCAUTT-3' and antisense: 5'-AUGGCC AUCAAGAUUAGGGTT-3'; si-NC sense: 5'-GAGGCGUGG AGUCUUGUUUTT-3' and antisense: 5'-AAACAAGACUCC ACGCCUCTT-3'.

Cell proliferation assays. The cell proliferation assay was conducted with Cell Counting Kit-8 (CCK8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). A total of 3,000 6-10B cells/well were seeded onto 10 replicate wells of 96-well plates. A total of 5-wells were transfected with si-NC and the others were transfected with si-C22orf32-1. Cell proliferation was monitored every 24 h following transfection up to 72 h, according to the protocol of the manufacturer. All assays were performed at least three times.

Cell scratch assay. The cell scratch assay was used to detect the migration of the NPC cells. A total of 2×10^6 cells/well of the 6-10B cell line were plated onto 6-well plates. The cells were transfected with si-NC and si-C22orf32-1 at 90-100% confluence. Sterile 200 μ l pipette tips were used to scrape a clear line through the well 6 h following transfection. The serum-free RPMI-1640 medium was used instead of the previous medium. The migration distance was assessed and images were captured at 0 and 24 h following scraping using an inverted microscope (Olympus Corporation, Tokyo, Japan). All experiments were performed in triplicate.

Transwell and matrigel assays. 6-10B cells were harvested 24 h following transfection with si-C22orf32-1 or si-NC. For the migration assays, 1×10^4 cells diluted in 100 μ l serum-free RPMI-1640 medium were plated into the upper chamber with a pore size 8 μ m (BD Biosciences, Franklin Lakes, NJ,

USA). For the invasion assays, 1×10^4 cells diluted in $100 \mu\text{l}$ serum-free RPMI-1640 medium were plated into the upper chamber, which was coated with Matrigel (BD Biosciences, San Jose, CA, USA). A total of $500 \mu\text{l}$ medium containing 10% FBS was added to the lower chambers. Following culture for 24 h, the cells that had invaded and migrated to the membrane were fixed with paraformaldehyde for 25 min, then stained with 0.1% crystal violet for 25 min. The images were captured and cells were counted using an inverted microscope (Olympus Corporation) and 5 fields of view were selected to be assessed. All experiments were performed in triplicate.

Cell apoptosis assay. 6-10B cells were plated onto 6-well plates and cultured in a humidified chamber supplemented with 5% CO_2 at 37°C following transfection. The cells were collected 48 h subsequent to this using EDTA-free pancreatic enzymes (Gibco; Thermo Fisher Scientific, Inc.) and washed three times with pre-chilled PBS. Subsequent to resuspension in 1X binding buffer (Invitrogen; Thermo Fisher Scientific, Inc.), the cells were stained with a propidium iodide detection kit (Invitrogen; Thermo Fisher Scientific, Inc.) and Annexin V-fluorescein isothiocyanate (Invitrogen; Thermo Fisher Scientific) in the dark for 15 min. Flow cytometry (EPICS XI-4; Beckman Coulter, Inc., Brea, CA, USA) was used to quantify the rate of apoptosis. All experiments were performed at least three times.

Statistical analysis. All experiments were performed at least three times, therefore the data were calculated as the mean \pm standard deviation. All statistical analyses were performed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). An unpaired t-test was used to analyze the expression difference between NPC specimens and normal specimens, and the NPC and the normal nasopharyngeal epithelial cell lines. Student's *t* test was used to analyze the data from the proliferation, invasion, migration, apoptosis and scratch assays. $P < 0.05$ (two-tailed) was considered to indicate a statistically significant difference.

Results

The expression of lncRNA C22orf32-1 is up-regulated in NPC cell lines and human NPC clinical tissues. The expression levels of lncRNA C22orf32-1 were tested in 24 normal nasopharyngeal epithelial tissues and 24 NPC tissues. The results revealed that lncRNA C22orf32-1 was significantly upregulated in NPC tissues compared with normal nasopharyngeal epithelial tissues (Fig. 1A). The NPC 6-10B cell line and normal nasopharyngeal epithelial NP460 cell line were selected to verify the expression pattern of lncRNA C22orf32-1. The expression level of lncRNA C22orf32-1 in the 6-10B cell line was significantly higher compared with the NP460 cells (Fig. 1B).

Knockdown of lncRNA C22orf32-1 inhibited the proliferation of NPC cells. To investigate the potential involvement of lncRNA C22orf32-1 in NPC progression, four siRNAs were used to decrease the expression of lncRNA C22orf32-1. The most effective siRNA was si-C22orf32-1, and further analysis

Table I. Clinicopathological characteristics of patients.

Characteristics	Number of cases
Mean age, range (years)	43 (25-64)
Sex (male/female)	18/6
Degree of differentiation (undifferentiated/differentiated)	15/7
Histology (squamous/others)	24/0
Lymph node metastasis (+/-)	17/7
Distal metastasis (+/-)	0/24
Clinical TNM stage (I-II/III-IV)	4/20

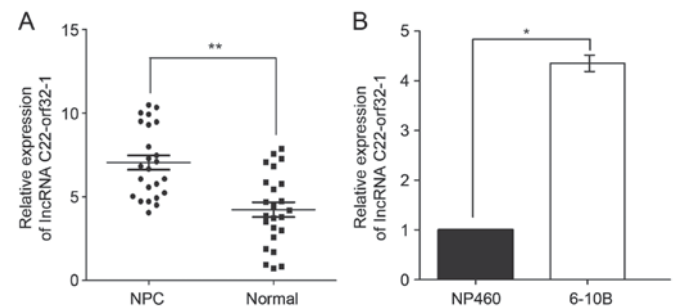


Figure 1. Relative lncRNA C22orf32-1 expression in NPC tissues and NPC cell lines assessed by quantitative reverse transcription polymerase chain reaction. (A) lncRNA C22orf32-1 was upregulated in 24 NPC tissues compared with 24 normal nasopharyngeal epithelial samples. (B) lncRNA C22orf32-1 presented higher expression in the NPC 6-10B cell line compared with normal nasopharyngeal epithelial NP460 cell line. The data were analyzed using $2^{-\Delta\Delta\text{Ct}}$ method and presented as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$, with comparisons indicated by lines. lncRNA, long non-coding RNA; NPC, nasopharyngeal carcinoma.

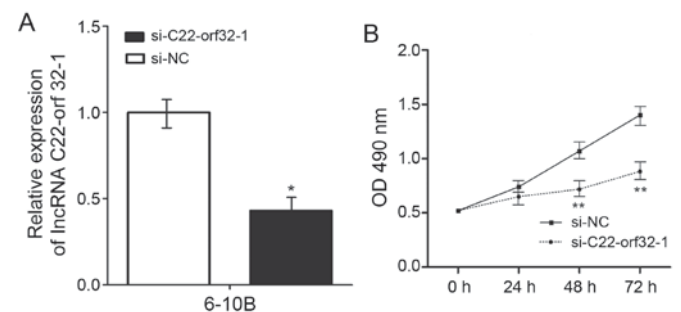


Figure 2. Knockdown of lncRNA C22orf32-1 in NPC cell lines. (A) Expression levels of lncRNA C22orf32-1 in 6-10B cells transfected with si-C22orf32-1 were significantly reduced compared with the cells transfected with si-NC. (B) Growth of 6-10B cells were detected by Cell Counting Kit-8 assay every 24 h from 0 h to 72 h. lncRNA, long non-coding RNA; si, small interfering RNA; NC, negative control; OD, optical density. * $P < 0.05$, ** $P < 0.01$.

revealed that it significantly decreased the expression levels of lncRNA C22orf32-1 in the 6-10B cell lines by $>57.3\%$ ($P < 0.05$; Fig. 2A). CCK-8 assays were used to examine the effect of lncRNA C22orf32-1 on the proliferation of NPC cells. Knockdown of lncRNA C22orf32-1 significantly suppressed the growth of the 6-10B cell line 48 and 72 h following transfection compared with the negative control (Fig. 2B).

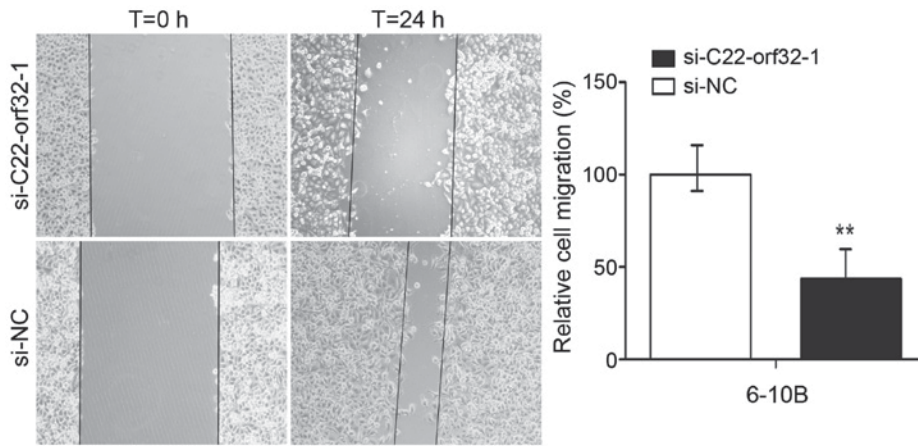


Figure 3. Long non-coding RNA C22orf32-1 promoted the migration of nasopharyngeal carcinoma cells. Cell scratch assays were used to detect the migration of the 6-10B cell line following transfection with si-C22orf32-1 and si-NC. The spacing measured from the cell transfected si-C22orf32-1 was obviously wider than the cell transfected si-NC. Magnification, x100. si, small interfering RNA; NC, negative control; T, time. **P<0.01.

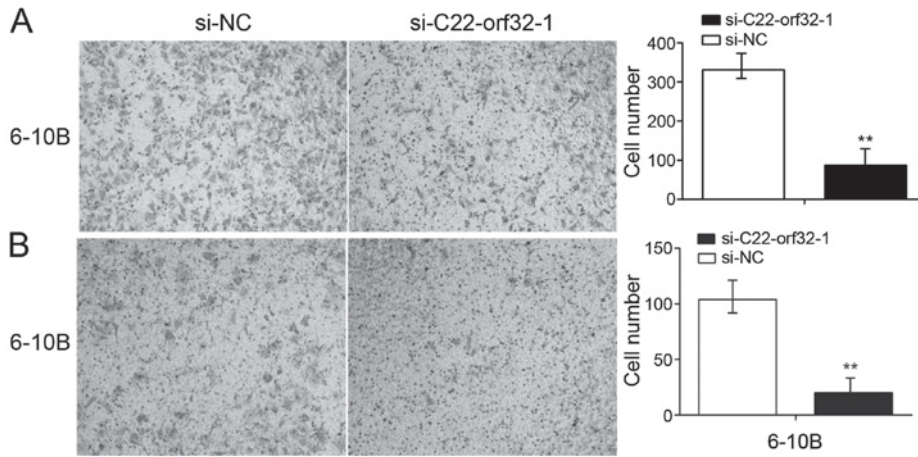


Figure 4. Inhibition of long non-coding RNA C22orf32-1 suppresses the invasion and migratory capacity of NPC cells. (A) Transwell migration assays and (B) Matrigel invasion assays demonstrated the migration and invasion activities of the 6-10B cell line subsequent to transfection with si-C22orf32-1 and si-NC. si, small interfering RNA; NC, negative control. *P<0.01, ***P<0.001.

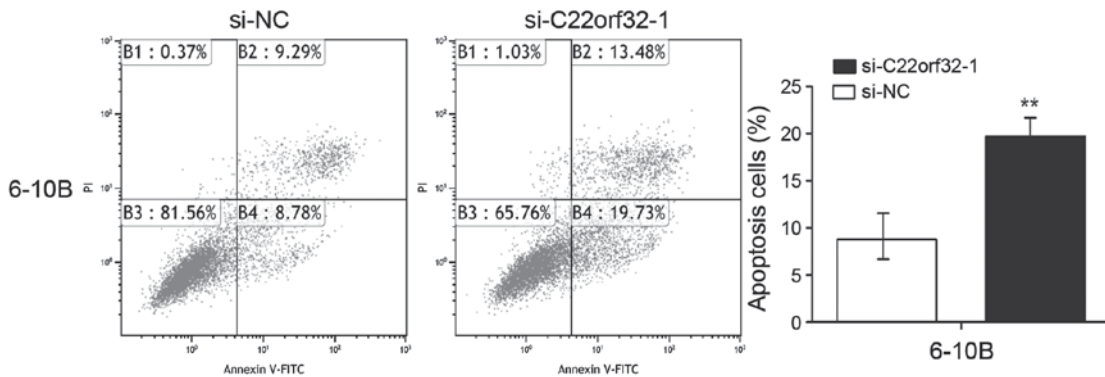


Figure 5. Long non-coding RNA C22orf32-1 decreased apoptosis of the NPC cells. Flow cytometry was used to analyze the apoptosis rates of the NPC 6-10B cell line following transfection with si-C22orf32-1 and si-NC. PI, propidium iodide; si, small interfering RNA; NC, negative control; NPC, nasopharyngeal carcinoma; FITC, fluorescein isothiocyanate. **P<0.01, ***P<0.001.

lncRNA C22orf32-1 promoted NPC cell migration and invasion. The scratch assays and Transwell migration assays were used to detect the effect of lncRNA C22orf32-1 on the migratory ability of NPC cells. In the scratch assays, it was

revealed that the migration abilities of the cells transfected with si-C22orf32-1 were significantly reduced compared with the cells transfected with si-NC. The migration rates were reduced to 58% in 6-10B cells (P<0.001; Fig. 3). Similarly,

the migration rates of the 6-10B cell lines following transfection with si-C22orf32-1 were decreased by 64.3% ($P < 0.001$; Fig. 4A) compared with the negative control. The Matrigel invasion assays were performed to investigate whether lncRNA C22orf32-1 was involved in the invasion of NPC cells. The results revealed that lncRNA C22orf32-1 knockdown cells exhibited impeded invasion abilities in the 6-10B cell line, and were reduced by 76.2% ($P < 0.001$; Fig. 4B).

Knockdown of lncRNA C22orf32-1 promoted NPC cell apoptosis. To investigate whether lncRNA C22orf32-1 was involved in the progression of NPC cell apoptosis, flow cytometry was used to analysis the rates of apoptosis in the 6-10B cells. The flow cytometry assay results indicated that the levels of apoptosis in the cells transfected with si-C22orf32-1 were significantly increased compared with the cells transfected with si-NC, with 8.78 vs. 19.73% ($P < 0.001$; Fig. 5).

Discussion

Epidemiological studies have demonstrated that NPC is a specific regional genetic disease with multiple factors. Exposure to the Epstein-Barr virus, the environment and genetics are the three main factors that influence the development of NPC (23,26-28). Like multiple other human malignancies, the occurrence of NPC is due to the activation of oncogenes and/or the inactivation of tumor suppressor genes that disrupt the homeostasis of cellular gene expression.

lncRNA is considered an important factor that influences the development of human tumors (29,30). Previous studies have demonstrated that abnormal expression of lncRNAs was able to change the biological functions of tumor cells by affecting various cellular processes. According to their alternative expression pattern, certain lncRNAs may reflect the progress of disease, and may be regarded as independent predictors for cancer diagnosis (14,16,31,32). The overexpression of Hox transcript antisense RNA in breast cancer promoted metastasis (33,34) and homeobox A transcript at the distal tip is considered an indicator for determining the progress of pancreatic cancer (35). Prostate cancer associated transcript 1 identified in hepatocellular carcinoma has been associated with the prognosis in patients with hepatocellular carcinoma (36). Yet, the specific functions of lncRNA in the occurrence of tumors remains unclear.

As demonstrated in a previous study, the expression of lncRNA C22orf32-1 was upregulated in primary NPC tissues (22). However, the mechanism of lncRNA C22orf32-1 in the development of NPC remains unknown (22). In the present study, the expression of lncRNA C22orf32-1 was examined in 24 primary NPC tissues and 24 normal nasopharyngeal epithelial tissues, and in one NPC and one normal nasopharyngeal epithelial cell line. It was revealed that the expression levels of lncRNA C22orf32-1 were higher in the NPC tissues and the NPC cell line. lncRNA C22orf32-1 is located in the human chromosome 22 with a length of 545 bp. Genetic diversity is a feature of chromosome 22, and as such this chromosome is associated with the occurrence of human disease. At present, at least 27 types of disease are

associated with chromosome 22, particularly malignancies including acute lymphoid leukemia, chronic myelogenous leukemia and malignant rhabdoid tumors (37-39). The high expression level and the specific location of lncRNA C22orf32-1 indicate that it may be associated with NPC. The effect of lncRNA C22orf32-1 knockdown on NPC cells was investigated, and it was demonstrated that the overexpression of lncRNA C22orf32-1 in the NPC cell line promoted cell proliferation, migration, invasion, and suppressed cell apoptosis. Conversely, subsequent to lncRNA C22orf32-1 knockdown, the capacities for proliferation, migration and invasion were decreased, and apoptosis was increased. This indicated that lncRNA C22orf32-1 is a cancer-associated gene involved in NPC, which may be a useful candidate biomarker for the early detection and treatment of NPC. Additional studies are required to determine the potential mechanisms of lncRNA C22orf32-1 in the development of NPC.

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