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LINC01551 promotes metastasis of nasopharyngeal carcinoma through targeting microRNA-132-5p

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Abstract. – OBJECTIVE: Previous studies have shown that long intergenic non-coding RNA01551 (LINC01551) is a cancer-promoting gene. However, the role of LINC01551 in nasopharyngeal carcinoma (NPC) has not been reported. Therefore, the aim of this study was to investigate the expression characteristics of LINC01551 in NPC, and to further explore its mechanism in promoting the metastasis.

PATIENTS AND METHODS: Quantitative real time-polymerase chain reaction (qRT-PCR) was used to detect the expression levels of LINC01551 in tumor tissue samples and paracancerous normal ones of 36 patients with NPC; meanwhile, the expression of LINC01551 in NPC cell lines was also verified using the qRT-PCR assay. In addition, the LINC01551 knockdown model was constructed in NPC cell lines (CNE2 and 6-10B) using lentivirus, and the influence of LINC01551 on the function of NPC cells was analyzed by cell counting kit-8 (CCK-8) and transwell invasion assays. Finally, the interaction between LINC01551 and microRNA-132-5p was examined by Luciferase reporter gene assay, while the potential mechanism was further explored by cell reverse experiments.

RESULTS: The results of qRT-PCR indicated that the expression level of LINC01551 in tumor tissue specimens of these patients was remarkably higher than that in adjacent tissues, and the difference was statistically significant. Meanwhile, LINC01551 expression was also found remarkably higher in cell lines than that in normal ones. In addition, compared with blank or control group, the proliferation, invasion and metastasis ability of NPC cells in LINC01551 knockdown group (si-LINC01551) was significantly reduced. Subsequently, the result of Luciferase reporting assay demonstrated that overexpression of microRNA-132-5p attenuated the Luciferase activity of the wild-type LINC01551 vector without attenuating that of the mutant vector, further demonstrating that LINC01551 can be combined with miR-132-5p. Additionally, the result of cell reverse experiment revealed that knockdown of microRNA-132-5p could reverse the effect of LINC01551 silencing on proliferation rate and metastasis of NPC cells, thus further demonstrating the mutual regulation between LINC01551 and microRNA-132-5p.

CONCLUSIONS: The above studies indicated that LINC01551 was remarkably up-regulated in NPC tissues, as well as in cell lines. In addition, studies have shown that LINC01551 may promote the metastatic ability by regulating microRNA-132-5p.

Key Words:
LINC01551, MicroRNA-132-5p, Nasopharyngeal carcinoma, Metastasis.

Introduction

Nasopharyngeal carcinoma (NPC) is a malignant tumor derived from nasopharyngeal epithelial cells[1-3]. There are approximately 80,000 new cases and 50,000 deaths each year in the world[4]. NPC is a disease attributed to different causes, and its pathogenesis includes genetics, environment EBV virus infection and other factors[5]. It has significant differences in ethnicity and geographical distribution, with a high incidence in Southeast Asia and China[2,6]. As for the treatment, this tumor is sensitive to radiotherapy, which has a significant effect on NPC[7,8]. However, the main reason for the failure of nasopharyngeal cancer treatment is the distant metastasis of tumor cells[9,10]. Therefore, an in-depth understanding of the underlying mechanisms of NPC progression and the identification of new biomarkers may provide new opportunities of tumor[11,12].

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Long-chain non-coding RNAs (lncRNAs) are a large class of RNAs with a transcription length greater than 200 nt[13]. LncRNA can be located on sense strand (covering the gene encoding the protein), antisense strand, bidirectional promoter (transcription within 1 kb of the antisense strand promoter of the protein), introns (transcription from introns of genes encoding proteins), intergenic (intergenic transcription of two encoded proteins) or enhancers (encoding of enhancer regions of genes encoding proteins). According to the functions, lncRNAs can be classified as five categories, which are, signals, molecular baits, guides, molecular scaffolds, and sponges[14]. Many lncRNAs have been reported to play pivotal roles in a wide range of biological processes, such as imprinting regulation, cell differentiation, development, and tumor formation, by regulating gene transcription or post-transcriptional regulation[15-17]. In addition, lncRNAs have been found to be down-regulated in a variety of tumors and can be used as tumor suppressor genes or oncogenes[18,19].

He et al[20] have found that the abnormal expression of lncRNA is closely relevant to the pathogenesis and progression of NPC. Nie et al[21] have demonstrated that high expression of lncRNA HOTAIR can affect the proliferation and migration ability of NPC cells and is positively correlated with the progression of NPC. In addition, low expression of lncRNA AFAP1 is found to be able to affect the migration and invasion of tumor cells[22]. Therefore, a deeper understanding of the function of lncRNA can provide a new perspective and idea for the molecular basis research of the development of NPC and provide new methods and means for the diagnosis and treatment of this tumor.

LINC01551 is a newly discovered lncRNA, and its mechanism of action in NPC is still unclear[23]. Therefore, in this study, we analyzed the expression of LINC01551 in 36 pairs of tumor tissue samples and adjacent ones of these patients and explored whether LINC01551 could regulate the function of these tumor cells by modulating microRNA-132-5p.

Patients and Methods

Patients and NPC Samples

A total of 24 pairs of NPC tissue specimens and their corresponding adjacent ones were selected from surgically treated cases and then stored at –80°C. All specimens of were diagnosed as squamous cell carcinoma, and no radiotherapy or chemotherapy was performed before diagnosis. The clinical staging of NPC patients was based on the AUCC/UICC NPC staging criteria, and all patients had signed informed consent. This investigation was approved by the Ethics Committee of the Affiliated Hospital of Jiangnan University (The Fourth People's Hospital of Wuxi).

Cell Lines and Reagents

Human NPC cell lines (HNE1, SUNE2, HONE1, CNE2, and 6-10B) and human nasopharyngeal immortalized epithelial cell line (NP460) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), and Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies (Gaithersburg, MD, USA). All cell lines were cultured with high-glucose DMEM containing 10% fetal calf serum (FCS), penicillin (100 U/mL), and streptomycin (100 μg/mL) in a 37°C, 5% CO_2 incubator.

Transfection

The control sequence (NC) and the lentivirus containing the LINC01551 knockdown sequence (si-LINC01551) were purchased from Shanghai Jima Company (Shanghai, China). Cells were plated in 6-well plates and grown to a cell density of 40%, and then transfection was performed according to the manufacturer's instructions. After 48 h, cells were collected for quantitative Real Time Polymerase Chain Reaction (qRT-PCR) analysis and cell function experiments.

Cell Counting Kit-8 (CCK-8) Assay

The cells were collected and plated into 96-well plates (2000 cells/well) at 48 h after transfection. Next, the cells were cultured for 24 h, 48 h, 72 h, and 96 h separately, and then added with CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent for incubation for another 2 h. Finally, the optical density (OD) value of each well was measured at the absorption wavelength of 490 nm using a microplate reader.

Transwell Invasion Assay

The cells transfected for 48 hours were trypsinized and resuspended in serum-free medium. After cell counting, the cell density was adjusted to 2.0×10^5/mL by dilution, and the transwell chambers containing Matrigel were placed in a 24-well plate. Later, 200 μL of the cell suspen-

sion was added to the upper chamber, and then 500 μL of the medium containing 10% FBS was added to the lower chamber, followed by incubated in an incubator at 37°C for 48 h. After that, the chambers were taken out, fixed with 4% paraformaldehyde for 30 min, and stained with crystal violet for 15 min. Then, the chambers were washed with phosphate-buffered saline (PBS), and the inner surface of the basement membrane of the chambers was carefully cleaned to remove cells in the inner layer. Finally, the perforated cells stained in the outer layer of the basement membrane of the chambers were observed under the microscope, and then counted in 5 fields of view selected randomly.

Cell Wound Healing Assay

After transfection for 48 h, the cells were digested, centrifuged, and resuspended in serum-free medium to adjust the density to 5 x 10^5 cells/mL. The density of the plated cells was determined according to the size of the cells (the majority of the number of cells plated was set to 50000 cells/well) until the confluency of the cells reached 90% or more the next day. After the stroke, cells were rinsed gently with PBS for 2-3 times and observed again at 24 h after incubation with low-concentration serum medium (such as 1% FBS).

QRT-PCR

Total RNA was extracted from tissue samples using the TRIzol method (Invitrogen, Carlsbad, CA, USA). Reverse transcription reaction was performed using the AMV reverse transcription kit, and 2 μg of total RNA was added to the 20 μL system for complementary deoxyribose nucleic acid (cDNA) synthesis. Primers were designed using Primer 5.0 software. The qRT-PCR reaction was performed using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) and StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used for qRT-PCR reaction: LINC01551: forward: 5'-GTCTTTTTACGCACGCAGCA-3', reverse: 5'-AGGAAAGGCTTGGAACGAGG-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward: 5'-TGTTCGTCATGGGTGTGAAC-3', reverse: 5'-ATGGCATGGACTGTGGTCAT-3'; microRNA-132-5p: forward: 5'-ACCGTGGCTTTCGATTGTTACT-3', reverse: 5'-ACCTGGCATACAATGTAGATTT-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse:5'-AACGCTTCACGAATTTGCGT-3'.

Data analysis was performed using ABI Step One software (Applied Biosystems, Foster City, CA, USA) and the relative expression levels of mRNA were calculated using the 2-ΔΔCt method.

Dual-Luciferase Reporter Assay

NPC cell lines including CNE2 and 6-10B were seeded in 24-well plates and co-transfected with microRNA-132-5p mimics/NC and pMIR Luciferase reporter plasmids. Prior to this, the plasmid was paired with the LINC01551 mutation binding site by insertion into wild-type LINC01551, and the mutation binding site was constructed into pMIR. The plasmid was then transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. 48 h later, the reporter Luciferase activity was normalized to control firefly Luciferase activity using a Dual-Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistically Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA). The difference in expression of LINC01551 in tumor tissues and adjacent tissues of NPC patients was analyzed by analysis of variance (ANOVA) followed by Post-Hoc Test (Least Significant Difference). The expression of LINC01551 in tumor tissues and paracancerous tissues and its relationship with various clinicopathological parameters were analyzed using the Chi-Square test. Data were presented as mean ± standard deviation, and $p<0.05$ suggested statistically significant difference.

Results

LINC01551 Was Up-Regulated in NPC Tissues

The expression of LINC01551 in 36 pairs of NPC tissue specimens and their adjacent paracancerous ones, as well as in NPC cell lines was detected by qRT-PCR. The results showed that the expression level of LINC01551 was remarkably increased in tumor tissues compared with that in adjacent ones, and the difference was statistically significant (Figure 1A). Compared with that in human nasopharyngeal immortalized epithelial cell line NP460, LINC01551 was found highly expressed in NPC cell lines (Figure 1B); among them, LINC01551 expression was the highest in CNE2 and 6-10B

cell lines, which were therefore selected for transfection and subsequent experiments. These results revealed that LINC01551 is up-regulated and may serve as an oncogene in NPC.

LINC01551 Expression Was Correlated With Distance Metastasis and Overall Survival in NPC Patients

According to the results of qRT-PCR, the above collected tissue samples were divided into high expression and low expression groups. Meanwhile, Chi-square test was used to analyze the interplay between the expression of LINC01551 and age, sex, pathological stage, and lymph node metastasis of patients. As shown in Table I, the expression of LINC01551 was not remarkably associated with age and gender but with pathological stage and lymph node metastasis. In addition, qRT-PCR analysis revealed that the expression of microRNA-132-5p is also closely related to pathological stage and lymph node metastasis.

Inhibition of LINC01551 Impeded NPC Cell Proliferation and Metastasis

Si- LINC01551 and NC were transfected into CNE2 and 6-10B cell lines, and qRT-PCR was used to verify the transfection efficiency (Figure 2A). Subsequently, the impact of LINC01551 on the tumor cell proliferation ability was examined by CCK-8 assay. As shown in Figure 2B, si-LINC01551 remarkably inhibited proliferation of CNE2 and 6-10B cell lines, and the inhibition rates of proliferation of 6-10B cell lines at 24 h, 48 h, 72 h, and 96 h were 20.3% (24 h, $p<0.05$), 9.7% (48 h, $p<0.01$), 13.5% (72 h, $p<0.01$), 8.5% (96 h, $p<0.01$), respectively; while those of CNE2 cell lines at 24 h, 48 h, 72 h, and 96 h were 5.7% (24 h), 14.5% (48 h, $p<0.05$), 20.1% (72 h, $p<0.01$), and 16.5% (96 h, $p<0.01$), respectively. The above results confirmed that LINC01551 can remarkably promote the proliferation of NPC cells.

Later, the impact of LINC01551 on the invasiveness of CNE2 and 6-10B cell lines was analyzed by the transwell invasion assay. As shown in Figure 2C, the invasive ability of NPC cells transfected with si-LINC01551 was remarkably decreased compared with negative control group, and the inhibition rates of 6-10B and CNE2 cells were 49.1% ($p<0.001$) and 33% ($p<0.001$), respectively. In addition, cell wound healing assay also demonstrated that knockdown of LINC01551 inhibited the crawling ability of cell lines (Figure 2D). In summary, the above experiments revealed that LINC01551 can conspicuously promote the invasiveness of NPC cells.

MicroRNA-132-5p Is a Direct Target of LINC01551

To further verify the targeting of microRNA-132-5p to LINC01551, the LINC01551 sequence was cloned into the Luciferase reporter plasmid pmirGLO, and the mutation vector pmirGLO-LINC01551-MUT was also constructed. Meanwhile, pmirGLO-LINC01551-WT or pmirGLO-LINC01551-MUT was co-transfected with

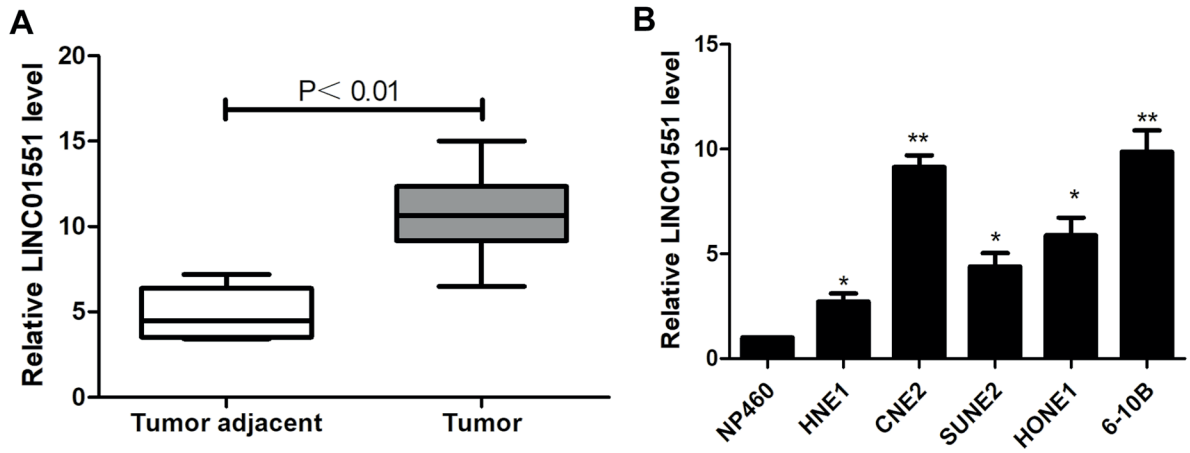

Figure 1. LINC01551 is highly expressed in nasopharyngeal carcinoma (NPC) tissues and cell lines. **A,** QRT-PCR detected the expression level of LINC01551 in tumor tissues and paracancerous tissues of patients; **B,** QRT-PCR verified the differential expression of LINC01551 in NPC cell lines and normal nasopharyngeal epithelial cells. Data are mean ± SD, *$p<0.05$, **$p<0.01$.

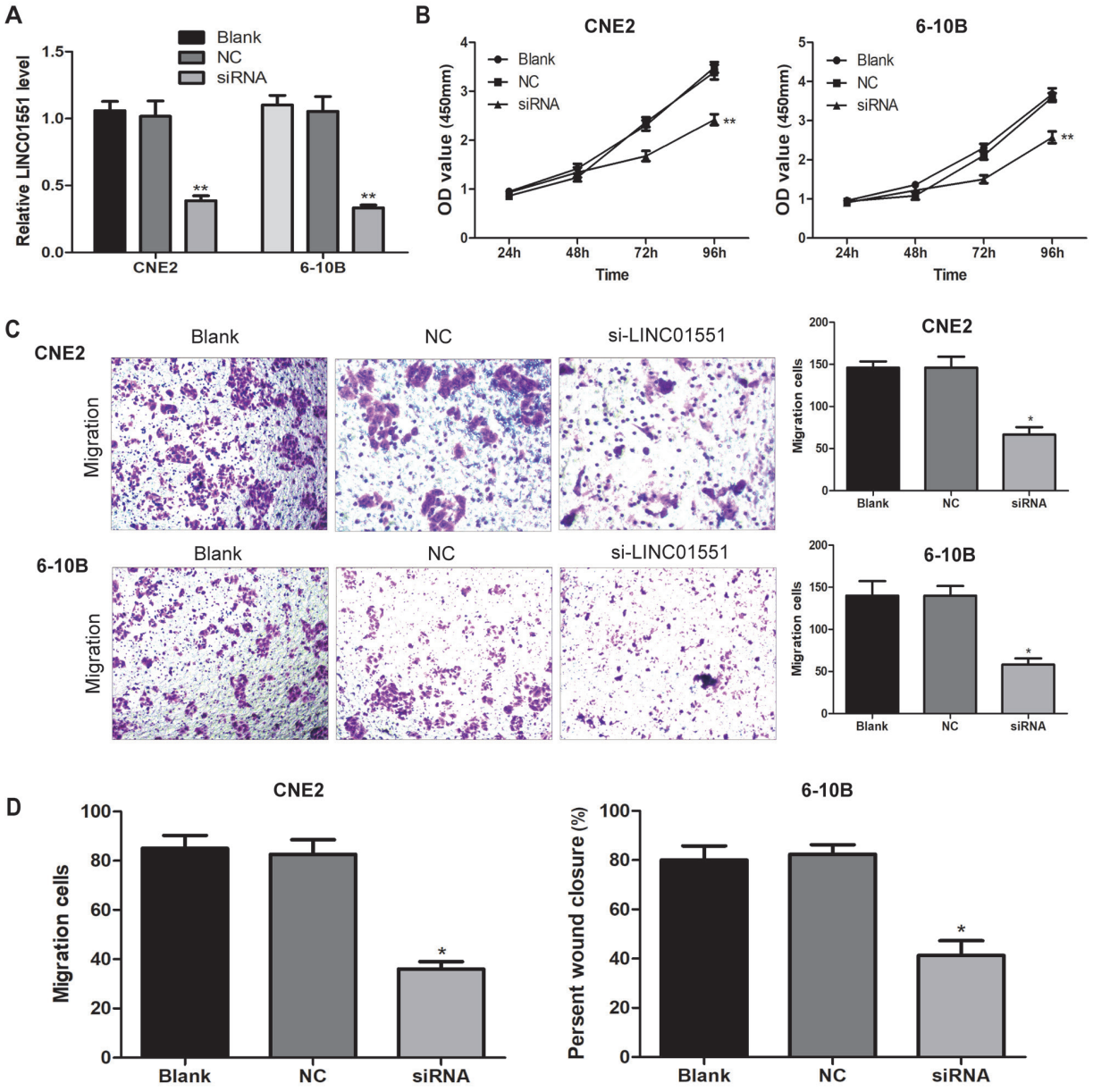

Figure 2. Knockdown of LINC01551 inhibits proliferation and invasion of NPC cells. **A,** QRT-PCR verified the interference efficiency of LINC01551 after transfection of the LINC01551 knockdown vector in cell lines 6-10B and CNE2; **B,** CCK-8 assay detected the effect of cell line 6-10B and CNE2 on the proliferation of NPC cells by lymphocyte cancer; **C,** Transwell invasion assay detected the ability of cell lines 6-10B and CNE2 to interfere with the invasion of NPC cells by LINC01551 (magnification: 40×); **D,** Cell wound healing assay detected the crawling ability of cell lines. Data are mean ± SD, *p<0.05, **p<0.01.

microRNA-132-5p in CNE2 and 6-10B cell lines for Luciferase reporter assay (Figure 3A). The results showed that overexpression of microRNA-132-5p remarkably attenuated the Luciferase activity of the wild-type LINC01551 vector (p<0.05) without attenuating the vector containing the mutant (p>0.05), further demonstrating that LINC01551 can be combined with miR-132-5p (Figure 3B). Also, qRT-PCR result confirmed that si-LINC01551 significantly enhanced microRNA-132-5p expression in CNE2 and 6-10B cell lines when compared to the control group (Figure 3C), suggesting that microRNA-132-5p may serve as a direct target of LINC01551

MicroRNA-132-5p Modulated LINC01551 Expression in NPC Cells

To further explore the ways in which LINC01551 promoted the malignant progression of NPC, bioinformatics analysis was applied to search for a possible relationship between microRNA-132-5p and LINC01551. In addition, in cell lines with LINC01551-silencing, we continued to knock down microRNA-132-5p to investigate the interaction between microRNA-132-5p and LINC01551, and then verified the transfection efficiency with qRT-PCR assay. It was found that the increased level of microRNA-132-5p induced by si-LINC01551 was significantly inhibited by microRNA-132-5p inhibitor (Figure 4A). The results of CCK-8 assay, transwell invasion test and cell wound healing assay showed that knocking down LINC01551 inhibited the activity and invasion ability of tumor cells, which, however, could be remarkably rescued by microRNA-132-5p inhibitor, suggesting that LINC01551 may promote metastasis by modulating microRNA-132-5p (Figure 4B-4D).

Discussion

The incidence rate of NPC ranks first among head and neck malignant tumors, which are often transformed from the phosphorous epithelium in the nasopharynx. The lower the differentiation degree is, the higher the malignancy degree will be. 95-98% of NPC are poorly differentiated squamous cell carcinoma[1-4]. As the nasopharynx has abundant lymphatic circulation, and the lymphatic circulation in the nasopharynx is bilateral, once NPC invades the lymphatic vessels, it is likely to cause cervical lymph node metastasis[9,10]. Therefore, cervical lymph node enlargement is often the reason for the first diagnosis of NPC patients. More than half of the patients with NPC have already had lymph node metastasis at the time of diagnosis. Therefore, the special anatomical structure and uncharacteristic clinical manifestations of NPC bring great difficulties to the early diagnosis[9]. Since most NPC is squamous-cell carcinoma, radiotherapy is the main treatment, which can be supplemented by chemotherapy, and surgical treatment can be performed when severe infiltration of surrounding tissues and obstruction of breathing and eating occur[5-8]. If specific biological molecules related to NPC can be found to guide the early diagnosis, improve the sensitivity of chemotherapy or conduct specific biological therapy will have important social and economic value for the diagnosis and treatment of this tumor[11,12].

The diverse sources of lncRNAs lead to different lengths and types of lncRNAs, and lncRNAs of different lengths and types are considered to play different functions[13-15]. LncRNA can play a role in post-transcription regulation after complementary pairing with target miRNA, thus causing related gene changes[16,17]. Other researches have shown that lncRNA is involved in these information transcription changes that can promote tumor development by influencing chromatin remodeling, shearing regulation of transcriptional regulatory factors, regulation of cell cycle and

Table I. Association of LINC01551 and miR-132-5p expression with clinicopathologic characteristics of NPC.

Parameter	No. of Case	LINC01551 expression		*p*-value	miR-132-5p expression		*p*-value
		Low (%)	High (%)		Low (%)	High (%)	
Age (years)				0.137			0.452
<60	13	9	4		4	9	
≥60	23	10	13		10	13	
Gender				0.317			0.494
Male	18	11	7		6	12	
Female	18	8	10		8	10	
T stage				0.047			0.036
T1-T2	23	15	8		6	17	
T3-T4	13	4	9		8	5	
Lymph node metastasis				0.015			0.018
No	26	17	9		7	19	
Yes	10	2	8		7	3	

influence on apoptosis[18]. The most important one is the series of functional changes such as cell proliferation, differentiation and apoptosis caused by the activation of a series of regulatory carcinogenic factors and/or the inactivation of tumor suppressor factors, which lead to the occurrence of tumor[19]. LncRNA, we widely involved in the occurrence and development of human cancer, and some lncRNAs even play a key role and can be used as special biomarkers to guide clinical diagnosis and treatment[11,19-22]. LINC01551, as a member of lncRNA, influences cell cycle, transcription, and translation of information through the action of related pathways or target genes, so as to be closely related to the occurrence and development of certain tumors[23].

In this report, we found that LINC01551 was remarkably increased in NPC tissues and cell

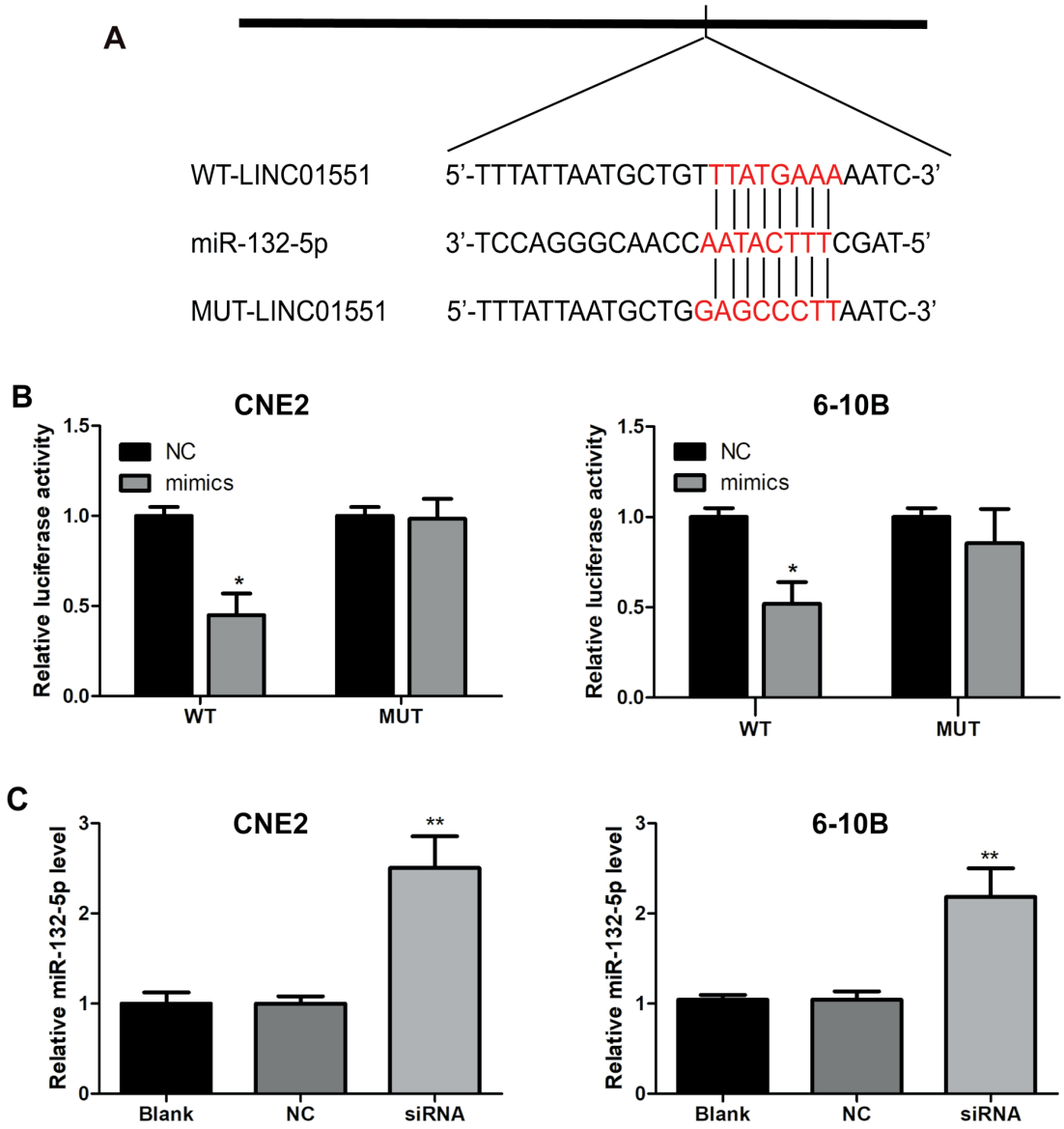

Figure 3. LINC01551 direct targeting of miR-132-5p. **A,** Predicted binding site of LINC01551 and miR-132-5p; **B,** Dual-Luciferase reporter gene assay verified the direct targeting of LINC01551 and miR-132-5p. Dual-Luciferase reporter gene assay showed that overexpression of miR-132-5p significantly attenuated the Luciferase activity of the wild-type LINC01551 vector ($p<0.001$), while the mutant-containing vector was not attenuated ($p>0.05$); **C,** QRT-PCR verified the interference efficiency of miR-132-5p after transfection of the LINC01551 knockdown vector in cell lines. Data are mean ± SD, *$p<0.05$, **$p<0.01$.

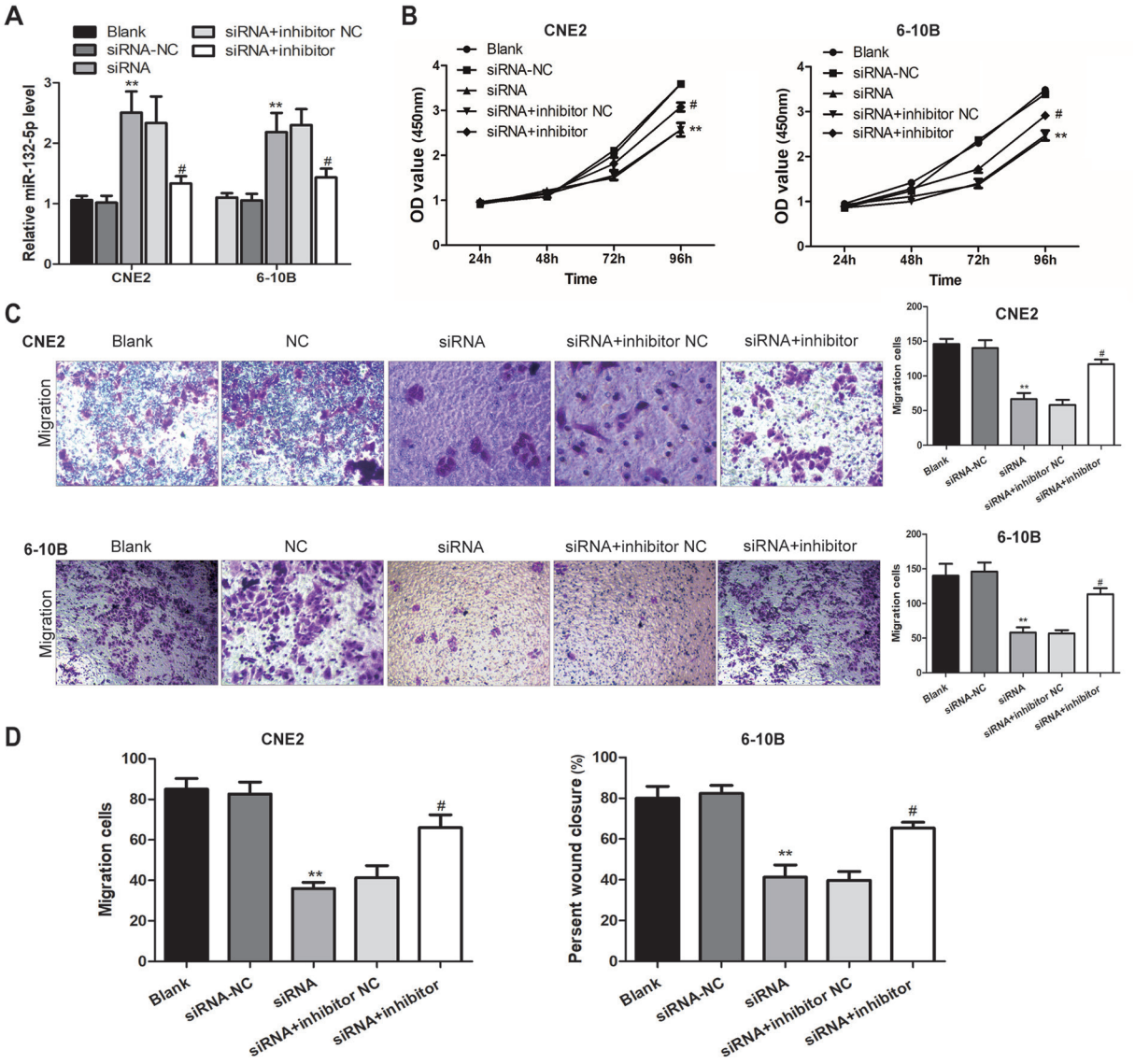

Figure 4. LINC01551 regulates the malignant progression of miR-132-5p in NPC. **A,** Expression level of miR-132-5p was detected by qRT-PCR after co-transfection of LINC01551 and miR-132-5p in 6-10B and CNE2 cell lines; **B,** CCK-8 assay detected the proliferation of cells after co-transfection of LINC01551 and miR-132-5p in 6-10B and CNE2 cell lines; **C,** Transwell migration assay was performed to detect the invasion ability of tumor cells after co-transfection of LINC01551 and miR-132-5p in 6-10B and CNE2 cell lines (magnification: 40×); **D,** Cell wound healing assay was performed to detect the crawling ability of carcinoma cells after co-transfection of LINC01551 and miR-132-5p in 6-10B and CNE2 cell lines. Data are mean ± SD, *#p<0.05, **p<0.01.

lines. To further study the regulatory mechanism of LINC01551 in the occurrence and development of NPC, the effect of LINC01551 silencing on the function of 6-10b and CNE2 cells was verified. Subsequently, CCK-8 assay, cell scratch test, and transwell invasion test were performed and it was revealed that LINC01551 silencing could significantly inhibit the proliferation and invasion ability of NPC cells, indicating that LINC01551 has an important effect on the function of NPC cells.

In recent years, several studies[14,15] have revealed a new mechanism of the body's post-transcriptional regulation of lncRNA: "sponge adsorption" mechanism of lncRNA in absorbing miRNA. Lncrna can reduce the effective concentration of miRNA by means of miRNA adsorption, while

protecting other target genes with important physiological functions from miRNA inhibition[15]. This mechanism has demonstrated the key significance of lncRNA in the rapid changes of miRNA abundance in tumor genesis, muscle development, embryonic stem cell differentiation and inflammation[16,17]. In this study, we found through bioinformatics analysis that LINC01551 contained a microRNA-132-5p binding site, and the direct binding of LINC01551 to downstream microRNA-132-5p was verified by Dual-Luciferase reporter gene assay. In addition, results revealed that there may be a feedback loop regulatory loop: LINC01551 could lead to the rapid decline of microRNA-132-5p concentration, which in turn promoted the malignant progression of NPC.

Conclusions

Taken together, the above studies indicated that LINC01551 is remarkably upregulated in NPC tissues and cell lines. In addition, LINC01551 may promote the metastatic ability *via* regulating microRNA-132-5p.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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