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Research Paper

High Expression of LINC01420 indicates an unfavorable prognosis and modulates cell migration and invasion in nasopharyngeal carcinoma

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Abstract

Recent studies demonstrated that long non-coding RNAs (lncRNAs) deregulated in many cancer tissues including nasopharyngeal carcinoma (NPC) and had critical roles in cancer progression and metastasis. In this study, we aimed to assess a lncRNA LINC01420 expression in NPC and explore its role in NPC pathogenesis. Our research revealed that the expression level of LINC01420 in NPC tissues were higher than nasopharyngeal epithelial (NPE) tissues. Moreover, NPC patients with high LINC01420 expression level showed poor overall survival. Knockdown LINC01420 inhibited NPC cell migration and invasion in vitro. In summary, LINC01420 may play a critical role in NPC progression and may serve as a potential prognostic biomarker in NPC patients.

Key words: lncRNAs, nasopharyngeal carcinoma

Introduction

Head and neck cancer includes carcinomas arising from the mucosal epithelia of the head and neck region as well as various cell types of salivary glands and the thyroid [1]. Nasopharyngeal carcinoma (NPC) is a kind of head and neck cancers, derived from the epithelial lining of the nasopharynx [2]. NPC is rare (annual incidence, < 1/100,000) in most parts of the world. However, it is endemic in regions such as southern China and Southeast Asia, where the annual incidence ranges from 3 to 30 per 100,000 persons. Epstein-Barr virus, environmental influences and heredity each play important roles in NPC development [3-10]. Undifferentiated carcinoma is the most common histological type of NPC and is associated with advanced local regional disease at diagnosis and a high incidence of distant metastasis. Distant relapse remains the major cause of treatment failure in NPC [11-17].

Long noncoding RNAs (lncRNAs) are non-protein-coding transcripts that are > 200 nucleotides in length and reside in the nucleus or cytoplasm [18]. Although the function and mechanism of most lncRNAs remain unknown, accumulated evidence suggests that lncRNAs play important roles in the transcriptional, epigenetic, and post-transcriptional regulation of gene expression. More and more evidence has shown that lncRNAs are capable of influencing various cellular processes such as proliferation, cell cycle progression, cell growth, apoptosis and metastasis [19-22].

To find functional lncRNAs in NPC, we analyzed 2 previously published online datasets to find dysregulated lncRNAs in head and neck cancer. One novel lncRNA, *LINC01420*, was significantly overexpressed in the two head and neck cancer datasets. Then we examined the expression of *LINC01420* in NPC tissues and demonstrated that *LINC01420* might play a critical role in NPC progression and prognosis as a potential prognostic biomarker.

Materials and methods

Tissue samples

Two sets of tissue samples were collected for this study: Set 1 contained 26 NPC and 10 non-tumor NPE biopsies to verify *LINC01420* expression with real-time PCR; Set 2 included 110 paraffin-embedded NPC tissue samples for *in situ* hybridization to confirm the expression of *LINC01420*. All tissue samples were collected from newly diagnosed NPC patients without any treatment at the Xiangya Hospital and the Affiliated Cancer Hospital of Central South University (Changsha, China). All the samples were handled according to the ethical and legal standards and approved by the Research Ethics Committee of the Xiangya Hospital and the Affiliated Cancer Hospital of Xiangya school of Medicine. All patients provided informed consent before surgery. After these specimens were confirmed by histopathological examination, these patients had received routine radiotherapy, and all of them were sensitive to radiotherapy [23].

RNA extraction and quantitative real-time PCR analyses (qRT-PCR)

Tissue RNA isolation and amplification were performed as our laboratory described previously [24-26]. Cell RNA was extracted using TRIzol reagent (Invitrogen, Carlbad, CA, USA). For qRT-PCR, RNA was reverse transcribed to cDNA by using a PrimeScript RT reagent Kit (Takara, Dalian, China). qRT-PCR was performed using a SYBR_Premix ExTaqII kit (Takara, Dalian, China) in the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) to determine the relative expression levels of target genes. The sequences of qRT-PCR primers: LINC01420: forward primer 5'-CACTCTACCCTCCG CACC-3' and reverse primer 5'-AGGAAGTGAAATC GTGCTGA-3'; β-actin: forward primer 5'-TCACCAA CTGGGACGACATG-3' and reverse primer 5'-GTCACCGGAGTCCATCACGAT-3'; β-actin was used as reference and normalization control.

Data mining and analysis

To find functional lncRNAs in NPC, we downloaded two head and neck GEO expression data from GEO database: GSE6791 and GSE30784 (Affymetrix Human Genome U133 Plus 2.0 platform). We used Significant Analysis of Microarray (SAM) software to analyze the expression of lncRNAs between the non-tumor NPE biopsies and NPC tissue samples in the two datasets. The cut off value for differentially expressed lncRNA was set at ≥1.2-fold change and the false discovery ratio (FDR) was < 0.05.

In situ hybridization (ISH)

In situ hybridization was performed to detect *LINC01420* expression in NPC specimens using three nucleotide probes from different *LINC01420* regions.

The sequences of LINC01420 ISH probes: Probe-1: 5'-ATTTAAAGAGGGTGGGATTTGGTCAG AAACTCAC-3'; Probe-2: 5'-CAGGACTTGGACCTTC AACACGAAAAATTCAGAAT-3'; Probe-3: 5'-CACT TGAGAAAACCACTGTAGGACAAGAACAACAT-3'. The probes were synthesized and labeled with DIG-dUTP at the 3′ and 5′ end (Invitrogen, Shanghai, China). *In situ* hybridization was performed as previously described [12]. All sections were independently scored by two pathologists who were blinded to the clinicopathological features and the clinical data.

Cell line and siRNAs

Human nasopharyngeal epithelium cell line NP69 and nasopharyngeal carcinoma HNE1, HK-1, HNE2 and 5-8F cell lines were maintained in our laboratory [27-33]. Cell were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS, Invitrogen, Shanghai, China) in a humidified incubator with 5% CO_2 at 37 ℃.

Three sequences of *LINC01420* targeting siRNAs were: 5'-CAUCUCAGGUCUCUUGGCUUUGCCA-3'; 5'-GCGUUGGGAUUAUCCGGAAGGAACU-3'; and 5'-CCUCUGAGAUUUAAGGCCAUGCCCU-3'. Sequences of non-target scramble controls were provided by Invitrogen.

For gene knockdown, cells were seed overnight and transfected with either a mixture of three siRNAs that targeting *LINC001420* or non-target scramble control siRNA (Invitrogen) using Lipofectamine RNAiMAX Reagent (Invitrogen) in OptiMEM medium (Invitrogen).

Cell migration and invasion assay

Cell migration was evaluated using a Transwell assay. A total of 2×10^4 cells in 200 μl of serum-free medium were added to the top chamber of the transwell (8 μm pore size, BD Biosciences, New Jersey, USA) at 24h after siRNA transfection. The bottom well contained growth medium with 20 % FBS. Cells were incubated at 37°C for 24 h. After 24 h, the cells that

had migrated to the lower face of the filters were fixed with 100% methanol and stained with 0.5% crystal violet and counted [24, 25].

Matrigel invasion assays were performed as described previously [34]. Briefly, filters coated with Matrigel in the upper compartment were loaded with 200μl serum-free medium containing 5 × 10^4 transfected cells, and the lower compartment filled with 20% FBS. After 24 h, migrated cells on the bottom surface were fixed with 100% methanol and counted after staining with 0.5% crystal violet.

Numbers of invaded cells were counted in six randomly selected fields under a microscope, and the average value was calculated. Each experiment was conducted in triplicate.

Statistical analysis

The expression levels of *LINC01420* in NPC and NPE tissues were analyzed by unpaired t test. The chi-square and t tests were performed to assess the relationship between *LINC01420* expression and clinicopathological features. Overall survival (OS) was calculated using the Kaplan-Meier method, and the results of the analysis were considered significant in a log-rank test if $p < 0.05$.

Results

LINC01420 is highly expressed in head and neck cancer

To find dysregulated lncRNAs in head and neck cancer, two online GEO datasets (GSE6791 and GSE30784) based on the Affymetrix HG_U133 Plus 2 arrays were reanalyzed. One novel lncRNA *LINC01420* was significantly highly expressed in head and neck cancer compared with non-tumor tissues according to the GSE6791 (Normal, n = 14; Tumor, n = 42, $p = 0.041$, Fig. 1A) and GSE30784 (Normal, n = 62; Tumor, n = 167, $p = 0.001$, Fig. 1B) datasets.

LINC01420 is overexpressed in NPC

NPC is one kind of head and neck cancer that arise from cells in nasopharynx. To assess the role of *LINC01420* in head and neck cancer, we examined the expression of *LINC0142* in 26 NPC and 10 non-tumor nasopharyngeal epithelium samples using qRT-PCR. The results showed that *LINC01420* was highly expressed in 26 NPC samples, compared with 10 non-tumor nasopharyngeal epithelium samples ($p = 0.002$, Fig. 2A). *LINC01420* was also overexpressed in four NPC cell lines (HNE1, HK1, HNE2 and 5-8F) compared with NP69, a normal human nasopharyngeal epithelium cell line (Fig. 2B). These results suggested that *LINC01420* was overexpressed in NPC.

Overexpression of *LINC01420* predicts a poor prognosis and could be regarded as an independent predictor for overall survival in NPC

We next assessed the expression of *LINC01420* in paraffin embedded NPC samples via *in situ* hybridization and found that *LINC01420* expression was highly expressed in NPC cancer nests compared with surrounded nasopharyngeal epithelium (Fig. 3A and 3B). The correlation between *LINC01420* expression and NPC clinicopathological features was also analyzed and the results demonstrated that the higher expression of *LINC01420* was significantly correlated with NPC distant metastasis ($p = 0.026$, Fig. 3C) and the male NPC patients had a higher positive rate of *LINC01420* than the female NPC patients ($p = 0.029$, Fig. 3D). However, *LINC01420* expression did not correlate with other clinicopathological characteristics, such as age, clinical stage and relapse (data not show).

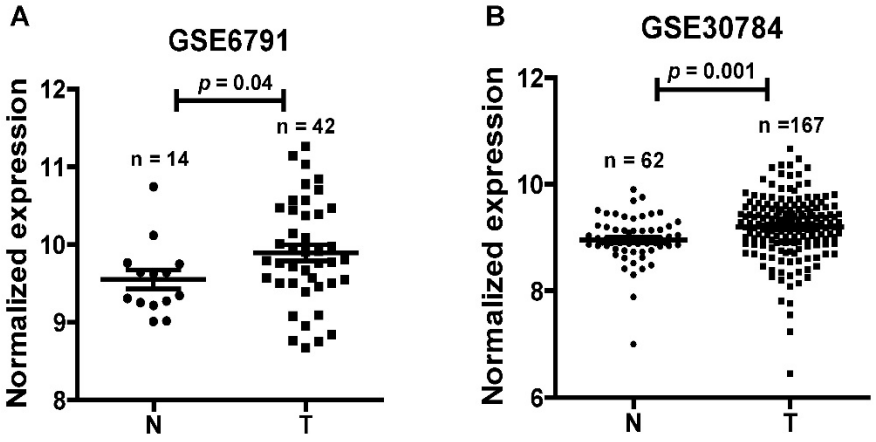

Figure 1. *LINC01420* is highly expressed in head and neck cancer. *LINC01420* significantly highly expressed in Gene Expression Profiling (GEP) datasets GSE6791 (Normal, n = 14; Tumor, n = 42, $p = 0.041$) and GSE30784 (Normal, n = 62; Tumor, n= 167, $p = 0.001$).

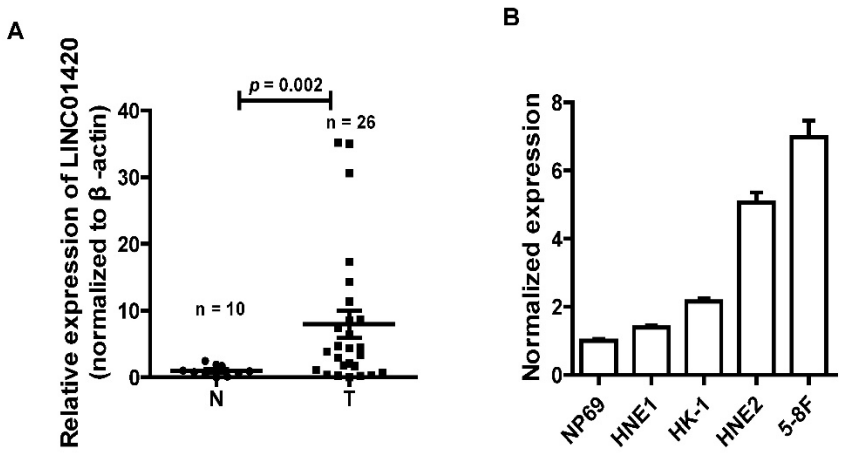

Figure 2. *LINC01420* is highly expressed in NPC tissues and cell lines. (A) *LINC01420* expression was higher in NPC samples (Tumor, n = 26) than that in non-cancerous tissues (Normal, n = 10). (B) *LINC01420* expression was significantly increased in NPC cell lines (HNE1, HK1, HNE2, and 5-8F) compared with NP69, a normal human nasopharyngeal epithelium cell line.

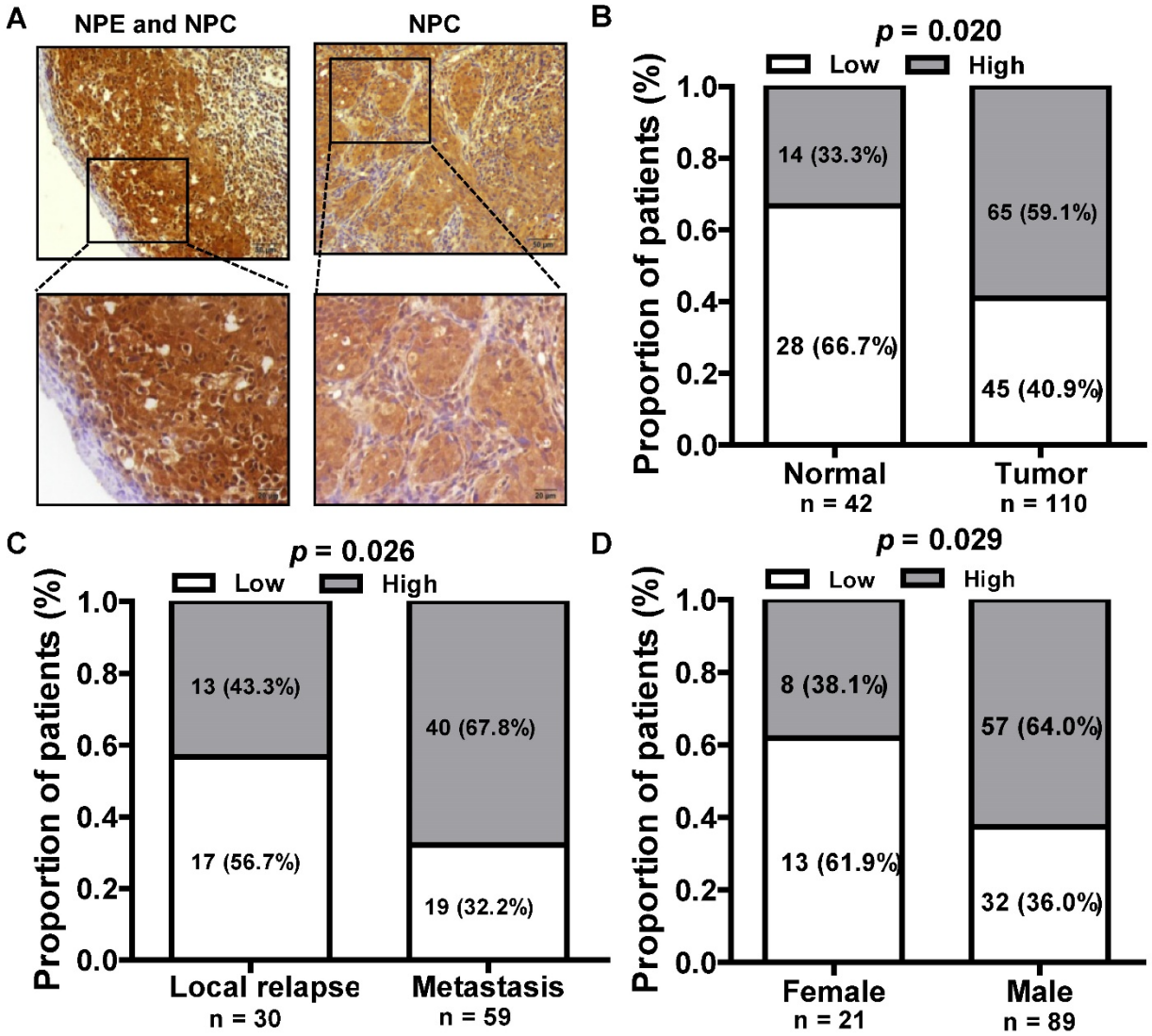

Figure 3. The relationship between *LINC01420* expression and clinicopathological features in NPC patients. (A) *LINC01420* expression measured by in *situ* hybridization in paraffin embedded NPC biopsies. (B) *LINC01420* expression was highly expressed in NPC cells (Tumor) compared to surrounded non-cancer NPE cells (Normal, p = 0.02). (C) High *LINC01420* expression was associated with distant metastasis (p = 0.026). (D) More male NPC patients have high *LINC01420* expression than female patients (p = 0.029).

To assess prognostic value of *LINC01420* expression in NPC patients, we examined the association between *LINC01420* expression and overall survival using Kaplan-Meier analysis with the log-rank test. The expression of *LINC01420* was negatively correlated with NPC patients' overall survival (p = 0.015, Fig. 4), NPC patients with high *LINC01420* expression displaying lower overall survival. Taken together, these data indicated that high *LINC01420* expression could be an independent risk factor for NPC patients.

Knockdown of *LINC01420* inhibited cell migration and invasion in NPC

We also explored the effect of *LINC01420* knockdown on NPC cell migration/invasion. First, the expression of *LINC01420* was examined and successfully decreased after knockdown by a mixture of three siRNAs in 5-8F cells (p < 0.001, Fig. 5A). Then, cell migration capacity was evaluated using a Transwell assay without Matrigel. The results showed that knockdown of *LINC01420* inhibited 5-8F cells migration capacity compared to the control group (p = 0.045, Fig. 5B). Matrigel invasion assays also demonstrated that 5-8F cells transfected with *LINC01420* siRNAs had lower invasive capability (p = 0.001, Fig. 5C).

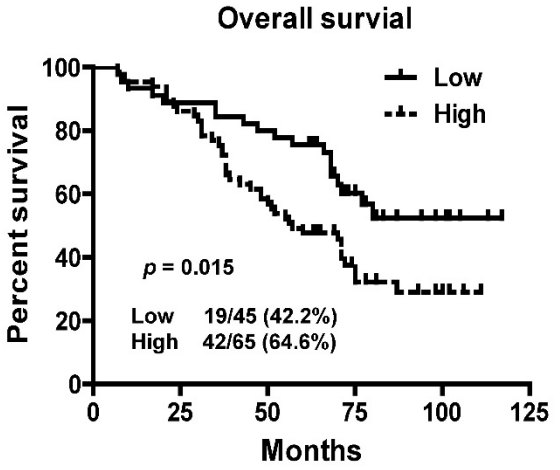

Figure 4. Kaplan-Meier survival curves of patients with NPC based on *LINC01420* expression. Patients with high *LINC001420* expression had a significantly unfavorable prognosis than those in low expression group (p = 0.015).

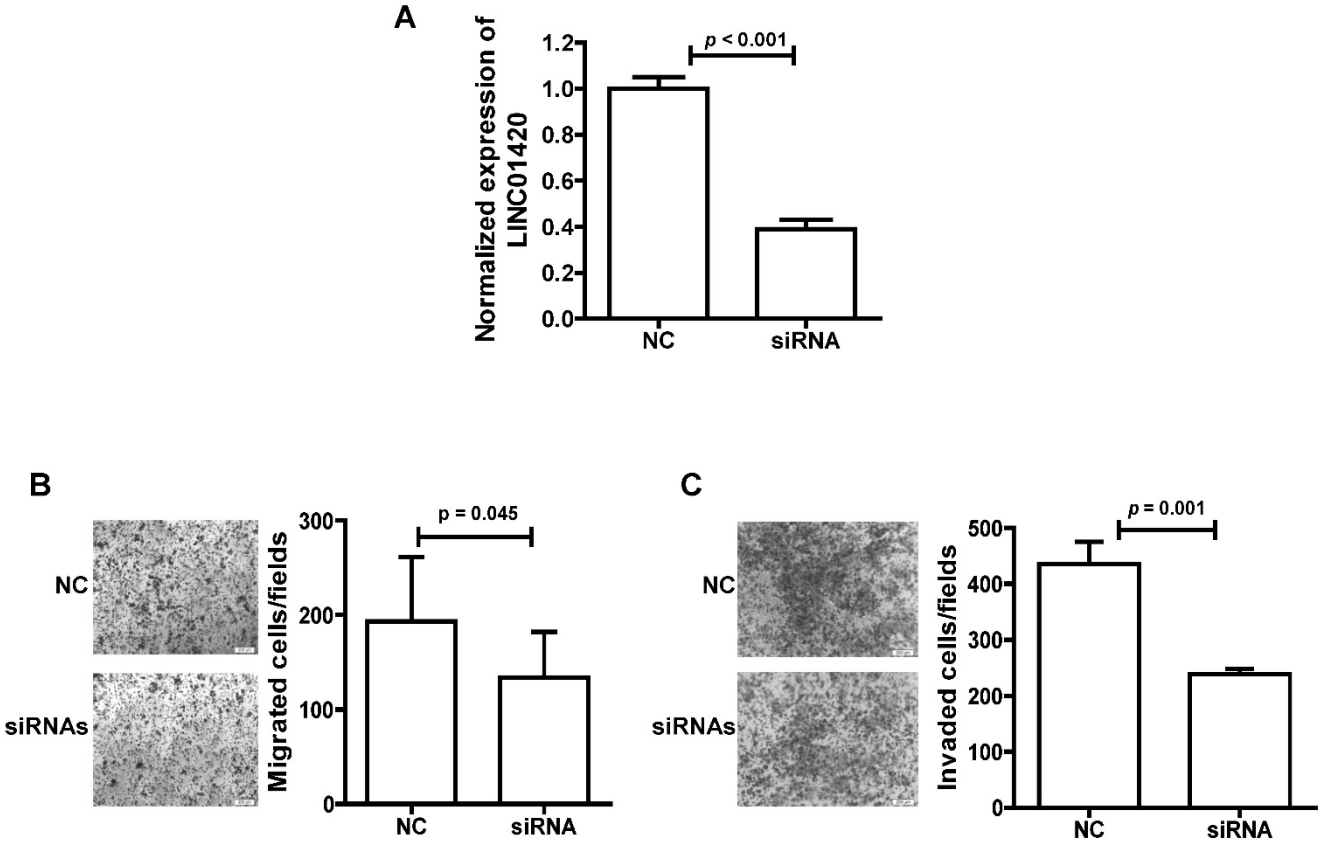

Figure 5. *LINC01420* Knockdown suppressed tumor cell migration and invasion *in vitro*. (A) siRNAs dramatically suppressed *LINC01420* expression in 5-8F cells (p < 0.001). (B and C) 5-8F cells were grown and transfected with *LINC01420* siRNAs, or scramble siRNA. Twenty-four hours after transfection, cells were subjected to a Transwell assay without or with Matrigel to measure migration or invasion capacity. *LINC01420* knockdown significantly inhibited 5-8F cell migration (p = 0.045) and invasion (p = 0.01).

Discussion

Cancer is still the major cause of morbidity and mortality in most areas in the world [35]. The incidence of NPC is high in endemic regions, with a 5-year overall survival rate of approximately 70% [36]; thus, it is important to identify new molecular targets for the diagnosis, prognosis, and treatment of NPC.

LncRNAs participate in many biological processes and many studies have implicated that abnormal expression of lncRNAs is closely related to the occurrence and development of malignant tumors [37-40]. Several lncRNAs were shown to be associated with NPC and indicate poor prognosis, including *LINC00312* [23], *H19* [41], *NEAT1* [42], *HNF1A-AS1* [43], *HOTAIR* [44], *AFAP1-AS1* [34] and *lncRNA-LET* [45]. *LINC01420* is a new lncRNA which is firstly reported in this paper.

In this study, we reported a novel functional lncRNA *LINC01420* which was significantly high expressed in NPC samples and correlated with a poor prognosis of NPC patients. *LINC01420* was poly (A)-positive and locates on chr X (p11.21). As we all know, there is a famous lncRNA, *Xist* (X inactive specific transcript), locating in the X inactivation center and its product is transcribed from the inactive X chromosome [46]. It is typically expressed by all female somatic cells but lost in female breast, ovarian, and cervical cancer cell lines [47-49]. In mammals, X chromosome inactivation (XCI) is triggered by *Xist* to equalize gene expression between the sexes [50]. Studies demonstrated that *Xist* directly interacts with SHARP to silence transcription through HDAC3 [51] and the histone deacetylase inhibitor abexinostat induces cancer stem cells differentiation in breast cancer with low *Xist* expression [52]. While another research reported that *Xist* reduction in breast cancer upregulated AKT phosphorylation via HDAC3-mediated repression of *PHLPP1* expression [53]. *Xist* also play important role in cervical cancer [54], non-small cell lung cancer [55] glioblastoma [56] and so on. In our study, we found that *LINC01420* is significantly high expressed in male NPC patients. The role of *LINC01420* as an lncRNA in gender is worth of our further study.

In summary, the findings presented in this study suggested that *LINC01420* expression was commonly high expressed in NPC and significantly correlated with the distant metastasis in NPC patients. Furthermore, high expression of *LINC01420* was an independent poor prognostic for NPC patients. We also found that the *LINC01420* knockdown significantly suppressed the invasive abilities of tumor cells, indicating that further investigation of *LINC01420* might lead to the development of novel tumor therapies.

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Conflicts of interest

The authors declare that there are no conflicts of interest in this work.

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