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Research paper

Long non-coding RNA 319 facilitates nasopharyngeal carcinoma carcinogenesis through regulation of miR-1207-5p/KLF12 axis

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ABSTRACT

Long non-coding RNAs (lncRNAs) have been wildly verified to modulate multiple tumorigeneses, especially nasopharyngeal carcinoma (NPC). In present study, we aim to investigate the role of LINC00319 in the NPC carcinogenesis. It was indicated that LINC00319 was markedly increased in NPC tissues and cells in comparison to their corresponding controls. Moreover, the aberrant overexpression of LINC00319 indicated the poor prognosis of NPC patients. Silence of LINC00319 was able to suppress NPC cell growth in vitro while overexpression of LINC00319 inversed this process. Moreover, in vivo tumor xenografts were established using CNE-1/SUNE-1 cells to investigate the function of LINC00319 in NSCLC tumorigenesis. Rescue assay was performed to further confirm that LINC00319 contributed to NPC progression by regulating miR-1207-5p/KLF12 signal pathway. Taken together, our study discovered the oncogenic role of LINC00319 in clinical specimens and cellular experiments, showing the potential LINC00319/miR-1207-5p/KLF12 pathway. This results and findings provide a novel insight for NPC tumorigenesis.

1. Introduction

Nasopharyngeal carcinoma (NPC), occurred in the epithelial lining of the nasopharynx, is one of the most common types of head and neck tumors that has an extremely uneven endemic distribution within Southern China and Southeast Asia (Hu et al., 2012; Song and Yin, 2016). Although the benefit of adjuvant chemotherapy is still open to debate, adjuvant chemotherapy is commonly prescribed for patients with loco-regionally advanced NPC (Jiang et al., 2016; Song and Yin, 2016). The addition of concurrent chemotherapy to radiotherapy prolongs the survival of patients with NPC and renders it a controllable and treatable chronic disease. Approximately 76–80% of patients survive for at least 5 years (Lee et al., 2014). But up to date, the detail mechanism for NPC tumorigenesis is still remaining largely unclear. Hence, a thorough understanding of the molecular mechanisms involved in the development and progression of NPC could provide more effective diagnostic markers and targets for NPC patient therapy.

The completion of the human genome and GENCODE projects revealed that < 3% of the human genome codes for proteins, while at least 75% is transcribed into non-coding RNAs, including miRNAs and lncRNAs. Many studies have shown that miRNAs can act either as tumor suppressors or oncogenes in various cancers through regulating target gene expression by binding 3′ untranslated regions and repressing transcription (Huang et al., 2015; Sun et al., 2015a, 2015b; Sun et al., 2016a, 2016b, 2016c, 2016d, 2016e, 2016f, 2016g, 2016h, 2016i; Pan et al., 2017a, 2017b; C. Sun et al., 2017; K.Y. Sun et al., 2017). Recently, lncRNAs have drawn attention as potential biological regulators involved in a wide range of cellular activities such as cell proliferation, apoptosis, migration, and invasion [8]. The underlying mechanisms of lncRNAs are varied, and include acting as competitive endogenous RNAs similar to miRNA sponges, chromatin remodelling, and histone protein modification (Fang et al., 2016; Sun et al., 2016a, 2016b, 2016c, 2016d, 2016e, 2016f, 2016g, 2016h, 2016i). Recently, several lncRNAs, including LOC100129148 (C. Sun et al., 2017), CCAT1 (Wang et al., 2017), EWSAT1 (Song and Yin, 2016), and PCAT7 (D. Liu et al., 2017; Y. Liu et al., 2017) are related to NPC carcinogenesis. Nevertheless, the detail molecular mechanism during NPC carcinogenesis is still sustained unclear.

Long intergenic non-protein coding RNA 319, LINC00319 (NR_152722), is located on chromosome 21q22.3 and has a transcript

Abbreviations: lncRNAs, long noncoding RNAs; NPC, nasopharyngeal carcinoma; EMT, epithelial-to-mesenchymal transition; ceRNA, competing endogenous RNAs; miR-1207-5p, hsa-miRNA-1207-5p; ANOVA, One-Way Analysis of Variance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IHC, immunohistochemistry; GBC, gallbladder cancer; RCC, renal cell carcinoma; LINC00319, long intergenic non-protein coding RNA 319; GC, gastric cancer; NSCLC, non-small cell lung cancer; CC, colorectal cancer

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length of 2901 nucleotides. It was first reported to be highly expressed in lung cancer (LC) (Zhou et al., 2017; Zhang et al. 2018). Further work showed that LINC00319 was involved in cell proliferation, apoptosis, migration, and invasion in non-small cell lung cancer (NSCLC), suggesting that it could be viewed as a potential target in both cancers (Zhou et al. 2017; Zhang et al. 2018). However, the expression pattern, functional role, underlying mechanism, and clinical significance of LINC00319 in NPC remain unclear.

In this study, we evaluated the expression level of LINC00319 in NPC tissues and cells by analyzing gene profiling data from the TCGA database and validating these in a cohort of 50 paired tissues. We found that LINC00319 was significantly up-regulated in NPC tissues compared with normal nasopharyngeal epithelial tissues, and that increased LINC00319 levels were associated with poor prognosis and short survival time of NPC patients. LINC00319 was also showed to regulate the miR-1207-5p targeted gene KLF12 by functioning as a competitive endogenous RNA (ceRNA) for miR-1207-5p in NPC, thereby inhibiting the expression of KLF12 and promoting NPC cell proliferation.

2. Materials and methods

2.1. Ethical statement and tissue collection

50 patients diagnosed as NPC were involved in this study. All histologic diagnoses were conducted by the pathology department at the Zhongnan Hospital of Wuhan University. Normal nasopharynx epithelial tissues were obtained from non-tumor adjacent tissues. Informed consent was received from all subjects. All experimental protocols were permitted by the Ethics Committee at the Zhongnan Hospital of Wuhan University.

2.2. Cell lines and plasmids

Six human NPC cell lines (SUNE-1, CNE-1, HNE-1, CNE-2, C666-1 and HONE-1) were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Kibbutz BeitHaemek, Israel). The human immortalized nasopharyngeal epithelial cell line NP69 was cultured in keratinocyte/serum-free medium (Invitrogen) supplemented with bovine pituitary extract. All cells were cultured at 37 °C in a 5% CO_2 atmosphere and maintained in 10% FBS (Kibbutz BeitHaemek, Israel). Plasmid pcDNA3.1-KLF12 and pcDNA3.1-LINC00319 were prepared by ourselves. RNAi sequence: **LINC00319**: sh-1 targets CCAGCAGCACCTGCGGAGAATCCCA; sh-2 targets GAGAA TCCCACCAGCTCCCACGCCT; sh-3 targets GCACCTGCGGAGAATCCCA CCAGCT.

2.3. qRT-PCR

Total RNA was extracted from cultured cells or frozen tissues by the TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. The isolated RNA was reverse transcribed to cDNA using a PrimeScript RT reagent Kit (Takara, Dalian China). Real-time PCR analysis was conducted using SYBR Green (Takara, Dalian China). Results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primers used were as follows: LINC00319 forward, 5′-GGAAGCCGGATAAGCACCTC-3′, and reverse, 5′-GCTACG CTGCAGTCACAAAC-3′; GAPDH forward, 5′-AGCCACATCGCTCAGA CAC-3′, and reverse, 5′-GCCCAATACGACCAAATCC-3′. qRT-PCR were performed on an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). $2^{-\Delta\Delta Ct}$ method was used to quantify LINC00319.

2.4. Cell viability analysis and colony formation assay

Viability of cells transfected with indicated shRNA or plasmid constructs was determined by trypan blue dye exclusion and CCK-8 assay (Sun et al., 2016a, 2016b, 2016c, 2016d, 2016e, 2016f, 2016g, 2016h, 2016i; Xi et al. 2016). Colony formation assay was performed with CNE-1 and SUNE-1 cells transfected with sh-NC and sh-LINC00319 for 14 days followed by crystal violet staining.

2.5. Luciferase reporter assays

Luciferase reporter assays was conducted as described previously (Stiuso et al. 2015; Catapano et al. 2016; C.Y. Li et al., 2016; J. Li et al., 2016; Miao et al. 2016; Sun et al., 2016a, 2016b, 2016c, 2016d, 2016e, 2016f, 2016g, 2016h, 2016i; Wang et al. 2016; Xi et al. 2016).

Biotinylated RNA pull-down assay.

The biotinylated RNA pull-down assay was conducted as described previously (Tsai et al. 2010; Hung et al. 2014). Biotin-labelled RNAs was in vitro transcribed with AmpliScribe T7-Flash Biotin-RNA Transcription Kit (Epicentre), treated with RNase-free DNase I and purified with an RNeasy Mini Kit (Qiagen). The lambda transcript was generated with the control plasmid provided by the Transcription Kit. To form the proper secondary structure, biotinylated RNA supplied with RNA structure buffer (10 mM Tris pH 7, 0.1 M KCl and 10 mM $MgCl_2$) was heated to 90 °C for 2 min, incubated on ice for 2 min and then shifted to room temperature (RT) for 20 min. The RNA was then mixed with cell nuclear extract or purified proteins and incubated at RT for 1 h, followed by incubating with Streptavidin Mag Sepharose (GE Healthcare) at RT for 1 h. After subsequent washes, the pull-down complexes were analyzed by standard western blot technique.

2.6. Tumor formation in nude mice

5 weeks old male BALB/c nude mice were maintained under specific pathogen-free conditions and were manipulated according to protocols approved by the Wuhan Medical Experimental Animal Care Commission. CNE-1/SUNE-1 cells were stably transfected with sh-LINC00319 or empty vector using Lipofectamine 2000 (Invitrogen). After 48 h, the cells were collected and harvested at a concentration of 1×10^8 cells/mL 100 μL of the cell suspension was subcutaneously injected into a single side of the posterior flank of each mouse. Tumor volumes and weights were tested every 4 days in mice from the sh-NC (six mice) or sh-LINC00319 (six mice) groups. The tumor volumes were measured (length × width^2 × 0.5). 36 days after injection, the tumors were isolated from all of the animals and used for further analysis.

2.7. Immunohistochemical (IHC) analysis

Primary tumors were immunostained for Ki-67 and KLF12 as previously described (Sun et al., 2015a, 2015b, 2015c, 2015d; Sun et al., 2016a, 2016b, 2016c, 2016d, 2016e, 2016f, 2016g, 2016h, 2016i).

2.8. Statistical analysis

The results were analyzed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA) and the SPSS 23.0 software (SPSS, Chicago, IL, USA). The significance of differences was assessed using Student's *t*-test or a one-way ANOVA. All data are expressed as the means ± SEM. $P < 0.05$ indicates a significant difference.

3. Results

3.1. LINC00319 is overexpressed in NPC tissues and cells, and correlates with poor prognosis

Using the bioinformatics tool lncRNAtor (http://lncrnator.ewha.ac.kr/index.htm), we analyzed RNA-Seq data (from TCGA: The Cancer Genome Atlas) for lncRNAs from Head and Neck squamous cell carcinoma tissues (n = 341). Results showed that LINC00319 was up-regulated in NPC tissues (Fig. 1A). We also analyzed LINC00319 expression in 519 NPC tissue samples and 44 normal tissue samples using the