Long non-coding RNA nuclear-enriched abundant transcript 1 (LncRNA NEAT1) upregulates Cyclin T2 (CCNT2) in laryngeal papilloma through sponging miR-577/miR-1224-5p and blocking cell apoptosis

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ABSTRACT
Long non-coding RNA nuclear-enriched abundant transcript 1 (Lnc-NEAT1) is a crucial mediator in cancer progression, which is associated with poor prognosis of patients with laryngeal papilloma (LP). Herein, we aimed to determine how Lnc-NEAT1 promotes LP development. q-PCR, MTT, EDU and Western blotting were performed to determine that Lnc-NEAT1 facilitates LP cell proliferation and hinders cell apoptosis. LncBase database, q-PCR, GEPIA online database, Dual luciferase reporter and RIP assays were utilized to confirm that Lnc-NEAT1 sponged miR-577/miR-1224-5p and negatively mediated CCNT2. Western blotting, MTT and EDU were used to confirm that Lnc-NEAT1 promoted LP cell proliferation and inhibited cell apoptosis through CCNT2. Lnc-NEAT1 was highly expressed in LP, and enhanced LP cell proliferation, and it was inhibited by Lnc-NEAT1 depletion. Concerning the underlying mechanism, it was found that Lnc-NEAT1 could functionally sponge microRNA-577 (miR-577) and microRNA-1224-5p (miR-1224-5p) and up-regulate Cyclin T2 (CCNT2) in LP cells. Notably, CCNT2 knockdown blocked Lnc-NEAT1-induced LP cell proliferation, and rescued cell apoptosis, which was specifically indicated by restoration of Bax, Cleaved caspase 3 and Cleaved caspase 9. Lnc-NEAT1 played a carcinogenic role in LP through mediating miR-577 or miR-1224-5p/CCNT2 axis, which may provide promising insights for the treatment of LP.

Introduction
Laryngeal papilloma (LP) is a common benign tumor mainly affecting children under 10 years old [1], and human papilloma virus (HPV) infection is generally considered as the main cause [2]. Clinically, patients with LP may present with symptoms of foreign body sensation, dysphagia, dyspnea, or slurred speech [1]. Currently, surgical treatment is the standard therapy of LP, including surgical resection, CO₂ laser resection and endoscopic resection [3−5]. Although LP is a benign tumor, the tumor grows faster in children, and recurrence is very common. Thus, it is necessary to identify biomarkers for prediction and amelioration of LP.

Long non-coding RNAs (LncRNAs) are RNA molecules consisting of over 200 nucleotides [6,7]. The crucial roles of LncRNAs in oncogenesis and tumor progression have been indicated. For instance, Wang et al. [8] revealed the carcinogenic role of LINC00922 in tumor cell proliferation and tumorigenesis through sponging miR-361-3p in ovarian cancer. Dong et al. [9] suggested that H3K27 acetylation-activated Lnc-TINCR enhanced the resistance of Trastuzumab and accelerated EMT of breast cancer via sponging microRNA-125b. Apart from the carcinogenic role, several LncRNAs also inhibited cancer progression, e.g., LINC01272 inhibited lung cancer [10], FUT8-AS1 inhibited melanoma [11] and HCG11 suppressed non-small cell lung cancer [12]. In addition, Sun et al. [13] reported that ARST acted as an emerging lncRNA and suppressed the development of glioma through restraining ALDOA-modulated actin cytoskeleton integrity. However, the functional roles and related mechanisms mediated by long non-coding RNA nuclear-enriched abundant transcript 1 (Lnc-NEAT1) in LP remain to be investigated.

MicroRNAs can mediate the transcriptional and post-transcriptional expression of targeting proteins via binding to the 3′-UTR of the downstream genes [14]. MicroRNA-577 (miR-577) and
microRNA-1224-5p (miR-1224-5p) are microRNAs, which have been proved to participate in the progression of various cancers. For example, miR-577 suppressed progression of multiple cancers, such as non-small cell lung cancer [15], liver cancer [16] and gastric cancer [17]. In addition, miR-1224-5p has been determined as an inhibitor in malignant development of rectal cancer [18] and esophageal squamous cell carcinoma [19]. Interestingly, previous evidence has indicated that miR-1224-5p-targeted OGFOD1 could facilitate the malignant development of laryngeal papilloma, whereas the role of miR-577 in LP has not been reported, and whether miR-577 and miR-1224-5p mediated by Lnc-NEAT1 affect the biological function of LP remains elusive.

Cyclin T2 (CCNT2) is a protein coding gene, and is highly associated with diverse diseases, such as myocardial ischemia reperfusion injury [20], acute myeloid leukemia [21] and chronic kidney disease [22]. In addition, many studies have elaborated the functional role of CCNT2 in cancers. For instance, microRNA-216b-inhibited cyclin T2 promoted tumor development and facilitated cell cycle progression of gastric cancer [23], and Wang et al. [24] suggested that MiRNA-188-5p hindered tumor growth and metastasis of osteosarcoma through binding to the 3′-UTR of CCNT2. However, whether CCNT2 plays a role in LP remains unknown.

The aim of the present study was to explore the expression levels of Lnc-NEAT1, miR-577, miR-1224-5p and CCNT2 in LP, and to further investigate the potential underlying molecular mechanism by which they regulate the progress of laryngeal papilloma development.

**Methods and materials**

**Tissue collection**

Laryngeal papillomatosis (n = 15) and normal tissues (n = 10) were harvested from Fourth Affiliated Hospital of Harbin Medical University, and Declaration of Helsinki was followed. The protocol was approved by the Ethics Committee of Fourth Affiliated Hospital of Harbin Medical University.

**Cell culture and transfection**

Laryngeal papillomatosis cells were supplied by ATCC (American Type Culture Collection, Manassas, VA, USA) and were cultured on indicated medium based on ATCC’s instruction. The pcDNA-NEAT1, si-NEAT1-1, si-NEAT1-2, miR-1224-5p mimics, miR-577 mimics, miR-1224-5p inhibitors, miR-577 inhibitors, si-CCNT2-1, and si-CCNT2-2 were designed by Gene Chem (Shanghai, China) (Table 1), and were transfected with indicated plasmids based on lipofectamine’s (11668–019, Invitrogen, Carlsbad, CA, USA) references.

**Cell proliferation assays**

To assess cell viability, MTT and Ethylenediurea (EDU) assays were performed. MTT assay was conducted using MTT Cell Proliferation and Cytotoxicity Assay Kit supplied by Beyotime (Shanghai, China). Briefly, LP cells were cultured for 4 h, and then maintained in Formazan for another 4 h after mixed with MTT reagent. A multi-plate reader (model 680 Bio-Rad, Hercules, CA, USA) was used to measure the absorbance at 570 nm. For EdU assay, a Cell-Light EdU Imaging detecting kit (Ruibo Biotech, Guangzhou, China) was used according to the manual instructions. The EdU-positive cells were examined using a fluorescence microscope.

**RNA Immunoprecipitation Assay (RIP)**

To determine the relationship of Lnc-NEAT1 with miR-1224-5p and miR-577, the RIP assay was performed based on EZ-Magna RIP kit (Millipore, MA, USA). RIP lysis buffer was used to disrupt LP cells, and then the lysates in the RIP buffer were cultured with magnetic beads pre-coated with Ago2 (ab186733, 1/30, Abcam, Cambridge, MA, USA) or IgG (ab205719, Abcam,

**Table 1. Sequences of siRNA against specific targets.**

| siRNA     | Sequence (miRNA) | Sequence (miRNA) |
|-----------|------------------|------------------|
| si-NEAT1-1| 5′-GAGGAUGAGGGUGAAGAA-3′ | 5′-GGAGAGUCAGGAGGAAUA-3′ |
| si-NEAT1-2| 5′-GAGGAUGAGGGUGAAGAA-3′ | 5′-UACUAACUGGGUACAUUCAC-3′ |
| si-CCNT2-1| 5′-GAGGAUGAGGGUGAAGAA-3′ | 5′-UACUAACUGGGUACAUUCAC-3′ |
| si-CCNT2-2| 5′-GAGGAUGAGGGUGAAGAA-3′ | 5′-UACUAACUGGGUACAUUCAC-3′ |
Cambridge, MA, USA) antibody. RT-qPCR was performed to estimate the NEAT1 RNA enrichment.

**Luciferase reporter assay**

Luciferase reporter assay was performed to investigate the relationship of Lnc-NEAT1 and CCNT2 with miR-1224-5p and miR-577. In brief, miR-1224-5p and miR-577 mimics along with corresponding NC-mimics were co-transfected with Lnc-NEAT1-MUT or Lnc-NEAT1-WT/CCNT2-MUT or CCNT2-WT into cells. Finally, using Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA), the luciferase activities of Lnc-NEAT1 and CCNT2 were analyzed in accordance with indicated references.

**Western blotting**

To examine the apoptosis-related proteins levels, LP cells were lysed using RIPA buffer (Beyotime, Shanghai, China), and the collected lysates were isolated using SDS-PAGE. They were then transferred onto PVDF membranes (Roche, Basel, Switzerland). Then these membranes were blocked for 2 h using 5% skim milk, and then cultured with apoptosis-related primary antibodies, including Bcl-2 (ab32124, 1:1,000, Abcam, Cambridge, MA, USA), Bax (ab32503, 1:2000, Abcam, Cambridge, MA, USA), Cleaved caspase 3 (ab32042, 1:500, Abcam, Cambridge, MA, USA), total caspase 3 (ab32351, 1:5,000, Abcam, Cambridge, MA, USA), cleaved caspase 9 (ab2324, 1 μg/ml, Abcam, Cambridge, MA, USA), and total caspase 9 (ab32539, 1:1,000, Abcam, Cambridge, MA, USA). They were subsequently cultured with secondary antibody. The internal control was determined as glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The electrochemiluminescence (ECL) (Bio-Rad, Hercules, CA, USA) was used to observe protein bands.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

To perform the q-PCR assay, laryngeal papillomatosis tissues and cells were collected and the total RNA was extracted. The PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan) was used to synthesize the complementary deoxyribose nucleic acid (cDNA) based on the instructions. SYBR Green RealTime PCR Kit (TaKaRa, Tokyo, Japan) was used to detect the relative expression levels of NEAT1, miR-577, miR-1224-5p and CCNT2. GAPDH and U6 were respectively considered as internal control for corresponding RNA expression. The primers sequences are listed in Table 2.

**Statistical analysis**

In this work, all data were presented as mean ± standard deviation (SD), and GraphPad Prism (vision 5.01, La Jolla, CA, USA) was adopted for the data calculation and analysis. To compare differences among groups, analysis of variance (ANOVA) or the Student’s t-test was used. p-value < 0.05 indicated significant difference.

**Results**

**Lnc-NEAT1 drives LP cell proliferation and blocks apoptosis**

To determine the role of Lnc-NEAT1 in LP, Lnc-NEAT1 expression levels in LP tissues and normal tissues were compared, and Lnc-NEAT1 in LP cells and normal cells were also compared. q-PCR displayed that Lnc-NEAT1 was highly expressed in LP tissues (LP, n = 15, 2.500 ± 0.8478; NL, n = 10, 1.016 ± 0.1825; Figure 1(a)) and LP cells (LP cells, 3.028 ± 0.8150; NL cells, 1.057 ± 0.3445; Figure 1(b)). Subsequently, to disclose the biological function of Lnc-NEAT1 in LP cells, Lnc-NEAT1-overexpressed and Lnc-NEAT1-depleted LP cells were constructed respectively (Vector, 1.001 ± 0.000; NEAT1, 3.534 ± 0.000; si-NC, 0.9851 ± 0.000; si-NEAT1-1, 0.2790 ± 0.000; si-NEAT1-2, 0.2081 ± 0.000; Figure 1(c)). MTT assay

**Table 2. Sequences of PCR primers used in this study.**

| Gene   | Primer sequences                  |
|--------|----------------------------------|
| NEAT1  | Forward (5′-3′) GAGAAGGGTTGAGTAAAGAT  |
|        | Reverse (5′-3′) CCTTCAACTCTGATTTCTTA  |
| GAPDH  | Forward (5′-3′) TGCCATGTAGCCCTTGAAG  |
|        | Reverse (5′-3′) AATGATCATGACAAAGCTGCGG  |
| miR-577| Forward (5′-3′) GGACUUUUCUUACUACACCG  |
|        | Reverse (5′-3′) GACCCUCAGGGUUAAGGGCA  |
| miR-1224-5p| Forward (5′-3′) GATGTAAAGTCCGCGGTATATAC  |
|        | Reverse (5′-3′) TGCAAGGCTGGCCGAGGAG  |
| CCNT2  | Forward (5′-3′) GGAGTGCCGAGGATTAAGAG  |
|        | Reverse (5′-3′) AGAGACATTGAGCGCTGTCC  |
| U6     | Forward (5′-3′) CTGGCCCTCGCCAACACA  |
|        | Reverse (5′-3′) AAGCGTCTCACGAATTTCGCT  |
showed that Lnc-NEAT1 overexpression enhanced LP cell viability, which was inhibited by Lnc-NEAT1 depletion (Figure 1(d)). In addition, EDU assay showed significantly increased EDU-positive cells after the upregulation of Lnc-NEAT1, whereas Lnc-NEAT1 depletion caused a striking drop (Figure 1(e)). Moreover, it was found that Lnc-NEAT1 affected the expression levels of apoptosis-related proteins. Specifically, Lnc-NEAT1 overexpression induced the upregulation of Bcl-2 and the downregulation of Bax, Cleaved caspase 3 and Cleaved caspase 9, while Lnc-NEAT1 depletion resulted in a contrary effect (Figure 1(f)), suggesting that Lnc-NEAT1 hindered LP cell apoptosis. Taken together, our findings indicated upregulated Lnc-NEAT1 in LP, and its carcinogenic role in LP progression was demonstrated.

**Lnc-NEAT1 sponges miR-577 and miR-1224-5p in LP**

To investigate the fundamental mechanism, LncBase database was analyzed, and it was found that Lnc-NEAT1 contained the complementary sites for miR-577 and miR-1224-5p. q-PCR assay clarified that miR-577 (NL, n = 10, 1.040 ± 0.2997; LP, n = 15, 0.4825 ± 0.1708) and miR-1224-5p (NL, n = 10, 1.011 ± 0.1642; LP, n = 15, 0.4247 ± 0.1333) were downregulated in LP tissues (Figure 2(a)). Additionally, q-PCR assay showed that miR-577 and miR-1224-5p were downregulated in LP cells (NL cells, 1.025 ± 0.2415; LP cells, 0.3730 ± 0.0882; Figure 2(b)). Noticeably, there was a negative correlation between Lnc-NEAT1 and miR-577/miR-1224-5p (Figure 2(c)), and the expression levels of miR-577 (Vector, 1.011 ± 0.000; NEAT1, 0.5292 ± 0.000; si-NC, 1.130 ± 0.000; si-NEAT1-2, 4.023 ± 0.000) and miR-1224-5p (Vector, 1.011 ± 0.000; NEAT1, 0.3048 ± 0.000; si-NC, 0.9484 ± 0.000; si-NEAT1-2, 3.094 ± 0.000) were significantly inhibited by Lnc-NEAT1 overexpression and enhanced by Lnc-NEAT1 depletion (Figure 2(d)). Furthermore, the luciferase activity of Lnc-NEAT1-WT was suppressed by transfecting with miR-577-mimics (Figure 2(e)) or miR-1224-5p-mimics (Figure 2(g)) in LP cells, and there were no changes in Lnc-
For further validation, RIP results demonstrated that Lnc-NEAT1 was enriched inAGO2 complexes through upregulating miR-577 or miR-1224-5p, in comparison with that in IgG group (Figure 2(f,h)). Collectively, these data confirmed that Lnc-NEAT1 could negatively mediate miR-577 or miR-1224-5p through sponging with them.

**miR-577 and miR-1224-5p negatively mediate CCNT2 in LP**

Next, to determine the downstream genes of miR-577 and miR-1224-5p, an intersected network was constructed based on bioinformatics StarBase database, and IGF2BP1, CCNT2 and RSBN1 were identified as the co-targeting genes of miR-577 and miR-1224-5p (Figure 3(a)). Among them, CCNT2 was selected as the targeted protein for further investigation due to its role in cell proliferation and cell cycle [23]. q-PCR results exhibited that CCNT2 was highly expressed in LP tissues (LP, \( n = 15; \) NL, \( n = 10; \) Figure 3(b)) and LP cells (NL cells, 1.021 ± 0.2329; LP cells, 2.653 ± 0.7575; Figure 3(c)). In addition, it was uncovered that the expression of CCNT2 was negatively correlated with miR-577 and miR-1224-5p, and positively associated
miR-577 and miR-1224-5p negatively mediate CCNT2 in LP. A. StarBase database was used to identify the co-targeting genes of miR-577 and miR-1224-5p. q-PCR was performed to determine CCNT2 expression in LP tissue samples (LP, n = 15; NL, n = 10) (b) and cells (c). (d) GEPIA database was used to verify the correlation between CCNT2 and miR-577, miR-1224-5p and Lnc-NEAT1. q-PCR was performed to examine CCNT2 expression in miR-577 overexpressed or miR-577 silenced (e), miR-1224-5p overexpressed or miR-577 silenced (f) and Lnc-NEAT1 overexpressed or Lnc-NEAT1 silenced (g) LP cells. Dual luciferase reporter (h and j) and RIP (i, k) assays were performed for further confirmation.
with Lnc-NEAT1 (Figure 3(d)). q-PCR further verified the online analytical results that CCNT2 was inhibited by the upregulation of miR-577 or miR-1224-5p, but was enhanced by the inhibition of miR-577 (NC mimics, 1.014 ± 0.000; miR-577 mimics, 0.4680 ± 0.000; NC inhibitors, 1.142 ± 0.000; miR-577 inhibitors, 3.755 ± 0.000; Figure 3(e)) or miR-1224-5p (NC mimics, 1.012 ± 0.000; miR-1224-5p mimics, 0.2377 ± 0.000; NC inhibitors, 0.9624 ± 0.000, miR-1224-5p inhibitors, 2.570 ± 0.000; Figure 3(f)). On the contrary, Lnc-NEAT1 overexpression upregulated CCNT2 while Lnc-NEAT1 depletion downregulated CCNT2 (Vector, 1.009 ± 0.000; NEAT1, 3.653 ± 0.000; si-NC, 1.127 ± 0.000; si-NEAT1-2, 0.3192 ± 0.000; Figure 3(g)). Furthermore, dual luciferase reporter assay displayed that miR-577 or miR-1224-5p mimics weakened the luciferase activity of wide-type CCNT2, rather than CCNT2-MUT (Figure 3(h,j)). RIP assay further confirmed that miR-577 or miR-1224-5p mimics could remarkably enrich CCNT2 in anti-AGO2 complexes, but not in anti-IgG group (Figure 3(i,k)). These findings deduced that CCNT2 was negatively regulated by miR-577 or miR-1224-5p, and activated by Lnc-NEAT1.

**Lnc-NEAT1 drives LP cell proliferation and restrains cell apoptosis via CCNT2**

Finally, to validate whether Lnc-NEAT1 promoted the malignancy in a CCNT2-dependent manner, Lnc-NEAT1 overexpressed and CCNT2 siRNA plasmids were co-transfected into LP cells (Figure 4(a)). MTT (Figure 4(b)) and EDU (Figure 4(c)) assays testified that Lnc-NEAT1 overexpression promoted LP cell viability and increased the number of EdU incorporated cells, which could be hindered by CCNT2 silencing. Additionally, CCNT2 knockdown also played a critical role in rescuing the apoptosis impaired by Lnc-NEAT1, which was specifically confirmed by the recovery of Bax, Cleaved caspase 3 and Cleaved caspase 9, and the inhibited Bcl-2 (Figure 4(d)). These data demonstrated that Lnc-NEAT1 facilitated malignancy of LP cells through upregulating CCNT2.

**Discussion**

HPV-induced LP is a common benign tumor, which mainly affects children. In the current

**Figure 4.** Lnc-NEAT1 drives LP cell proliferation and inhibits cell apoptosis via CCNT2. LP cells were co-transfected with Lnc-NEAT1 overexpressed and CCNT2 siRNA plasmids, and Western blotting was performed to verify the transfection efficacy (a). MTT (b) and EDU (c) assays were performed to detect cell proliferation, and the expression of apoptosis-related proteins was determined by Western blotting (d).
research, Lnc-NEAT1 was identified as an oncogene of LP, and it was found that Lnc-NEAT1 facilitated LP cell proliferation and blocked cell apoptosis through activating CCNT2 by sponging miR-577 or miR-1224-5p.

Up to date, there are still few reports about the role of LncRNAs in LP. Recently, Liu et al. [25] unraveled that LINC00174 promoted BZW2 expression via sponging miR-4500, hence accelerating LP cell proliferation and inhibiting apoptosis. In our work, it was indicated that Lnc-NEAT1 was highly expressed in LP tissue samples and cells. According to functional experiments, it was found that Lnc-NEAT1 overexpression enhanced LP cell viability and hindered cell apoptosis, while Lnc-NEAT1 depletion significantly prevented cell proliferation. These results confirmed the oncogenic role of Lnc-NEAT1 in LP, indicating that Lnc-NEAT1 may be implicated in the regulation of LP progression. Many studies have revealed critical roles of Lnc-NEAT1 in a variety of cancers. For instance, Fan et al. [26] demonstrated that cancer-related fibroblasts-secreted exosomal Lnc-NEAT1 promoted endometrial cancer development through upregulating miR-26a/b-5p-targeted STAT3 signaling pathway, and Shen et al. [27] testified that LncRNA NEAT1 stimulated malignant behaviors of colorectal cancer through mediating KDM5A/Cul4A and Wnt pathways. Noticeably, consistent with these reports, our findings further validated the oncogenic role of Lnc-NEAT1 in LP.

LncRNAs play a biological role through sponging microRNAs. It has been reported that Lnc-408 upregulates LIMK1 signaling pathway via sponging miR-654-5p, thereby accelerating breast cancer metastasis and invasion [28], and Lnc-MT1J1P has been proved to enhance the resistance of hepatocellular carcinoma cells to Lenvatinib via sponging miR-24-3p [29]. In addition, Duan YR and colleagues highlighted that Lnc-ISG20 facilitated NFAT5 through inhibiting miR-486-5p and induced renal fibrosis in diabetic nephropathy [30]. Consistently, our results verified that Lnc-NEAT1 contained the complementary sites of miR-577 and miR-1224-5p, and these two miRNAs were lowly expressed in LP. In addition, given the results of bioinformatics analysis and verified experiments in LP cells, it was concluded that Lnc-NEAT1 could functionally sponge miR-577 and miR-1224-5p and inhibit their expression in LP. Furthermore, our study found that CCNT2 was a co-targeting gene of miR-577 and miR-1224-5p, and also could be directly upregulated by Lnc-NEAT1. Based on the rescue experiments, CCNT2 depletion played an indispensable role in inhibiting LP cell proliferation and rescuing the lost cell apoptosis induced by Lnc-NEAT1 overexpression, highlighting that Lnc-NEAT1 promoted malignant behaviors of LP via CCNT2 signaling pathway. Although the functional role of Lnc-NEAT1/miR-577 or miR-1224-5p/CCNT2 axis in LP has been identified, the regulatory role in tumor growth in vivo remains to be validated in further studies.

Conclusions

In summary, it was found that Lnc-NEAT1 played an oncogenic role in LP, facilitated cell proliferation and inhibited cell apoptosis via miR-577 or miR-1224-5p/CCNT2 signaling pathway, which is a potential candidate for the diagnosis and treatment of LP.

Highlights

- Lnc-NEAT1 drives proliferation and blocks apoptosis in LP cells.
- Lnc-NEAT1 sponges miR-577 and miR-1224-5p in LP.
- CCNT2 was negatively regulates by miR-577 or miR-1224-5p.
- Lnc-NEAT1 facilitates malignancy of LP cells via upregulating CCNT2.

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