Upregulation of IncRNA LINC00473 promotes radioresistance of HNSCC cells through activating Wnt/β-catenin signaling pathway

P.-B. HAN¹, X.-J. JI², M. ZHANG¹, L.-Y. GAO¹

¹Department of Radiotherapy, Gansu Provincial Cancer Hospital, Qilihe District, Lanzhou City, Gansu Province, China
²Department of Radiotherapy, Xigu District of Lanzhou City People’s Hospital, Xigu District, Lanzhou City, Gansu Province, China

Abstract. – OBJECTIVE: LncRNA LINC00473 was reported to be upregulated in human cancers. Whereas, the role of LINC00473 in head and neck squamous cell carcinoma (HNSCC) and radiotherapy remains elusive.

PATIENTS AND METHODS: Gene array analysis was performed to detect lncRNA LINC00473. Then, the expression of LINC00473 in HNSCC 78 pairs of tissues and cell lines was measured by qRT-PCR assay. To explore the detailed functions of LINC00473 on cell proliferation, MTT and colony formation assays were conducted. We also investigated the influence of LINC00473 expression on radioresistance of HNSCC cells. Western blot assay was used to confirm the relationship between LINC00473 and Wnt/β-catenin signaling pathway. Furthermore, the effects of x-ray treatment on LINC00473 expression and Wnt/β-catenin signaling pathway were detected by Western blot assay.

RESULTS: LncRNA LINC00473 was upregulated in HNSCC tissues and cell lines. Functional assays showed that LINC00473 acted as oncogene to promote cell proliferation and inhibit apoptosis. In addition, downregulation of LINC00473 enhanced the sensitivity of radiotherapy for HNSCC cells. Furthermore, Western blot assays demonstrated that Wnt/β-catenin signaling pathway was inhibited by LINC00473 knockdown. Notably, Western blot assay revealed that x-ray treatment suppressed the activity of Wnt/β-catenin signaling pathway after LINC00473 knockdown.

CONCLUSIONS: These data suggested that the upregulation of IncRNA LINC00473 promotes the radioresistance of HNSCC cells through activating Wnt/β-catenin signaling pathway.

Key Words: LINC00473, Wnt/β-catenin, Radioresistance, HNSCC.

Introduction

As the sixth most common malignant cancer, head and neck squamous cell carcinoma (HNSCC) remains major threat of healthy. Head and neck squamous cell carcinoma (HNSCC) arises more from mucosal linings of the oral cavity, oropharynx, and larynx. The incidence of HNSCC is elevating all over the world. About 550 000 new cases were diagnosed as HNSCC, leading to more than 350 000 deaths annually. Smoking and alcohol abuse has been regarded as a key risker of HNSCC. In the USA, the incidence of HNSCC has increased more than 25%, which driven more by HPV during the past decade. Surgery and radiation therapy are the common treatment of HNSCC. However, the impact of radiotherapy on HNSCC is still limited because of its radioresistance. It is crucial for us to understand the etiology and molecular heterogeneity of HNSCC. As a class of transcripts, long non-coding RNAs (lncRNAs) contains more than 200 nucleotides and highly reserved without a protein-coding capacity. Evidence elucidated that the expression of lncRNAs was closely interacted with tumor process in various cancer. A large number of lncRNAs have been reported to play key roles in tumor growth and metastasis. Thus, it is critical for us to investigate the role of lncRNAs in cancers. For instance, HULC, a well-known lncRNA, promotes hepatocellular carcinoma growth and metastasis by targeting miR-200a-3p/ZEB1 signaling pathway. While HOTAIR accelerates cervical cancer cells invasion through regulating the Notch pathway. Reports showed that Wnt/β-catenin signaling pathway contributes to tumorigenesis...
and chronically activated in numerous human cancers. Previously, studies suggested that activation of Wnt/β-catenin signaling pathway inhibited cell apoptosis. Evidence also showed that Wnt/β-catenin signaling pathway enhance radiation resistance in mouse mammary gland progenitor cells. Hence, we extended our view to detect whether the Wnt/β-catenin signaling pathway involving in radioresistance in HNSCC cells. In this study, microarray analysis was performed to identify lncRNA LINC00473 by using three paired of HNSCC tumor tissues and adjacent tumor tissues. We then measured the biological role and clinical significance of lncRNA LINC00473 in HNSCC. Here, the impact of LINC00473 knockdown on radioresistance was measured by Western blot assay. In the process, Wnt/β-catenin signaling pathway was activated by LINC00473. Furthermore, the effect of x-ray treatment on the activity of Wnt/β-catenin signaling pathway was measured. Therefore, the specific role of LINC00473 in HNSCC needs to be further elucidated.

Materials and Methods

Human Tissues and Cells Culture

78 paired of clinical HNSCC and matched adjacent normal tissue were obtained from patients with HNSCC at Gansu Provincial Cancer Hospital. No patients have received irradiation or chemotherapy treatment. This study was approved by the Ethics Committee of Gansu Provincial Cancer Hospital. All patients signed the informed consent. Human HNSCC cell lines (SCC9, SCC15, SCC25, and CAL27) and the oral keratinocyte cell line (OKC) were obtained from ATCC (Rockville, MD, USA). SCC9, SCC15, and SCC25 were incubated in Dulbecco’s Modified Eagle Medium/F12 (DMEM/F12; Thermo Fisher Scientific, Waltham, MA, USA) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA) and 400 ng/ml hydrocortisone (Thermo Fisher Scientific, Waltham, MA, USA). CAL27 were cultured in DMEM/high glucose (Thermo Fisher Scientific) with 10% fetal bovine serum. These HNSCC cell lines were maintained at 5% CO2 and 37°C in a humidified incubator. OKC was maintained in a defined keratinocyte serum-free medium.

Microarray Analysis

The lncRNA expression of HNSCC was detected by Arraystar Human lncRNA Microarray V2.0. The microarray and data collection were assessed by KangChen Bio-tech (Shanghai, China). Gene spring software (version 13.1; Agilent Technologies) was employed to conduct the basic analysis with the raw data. The data were normalized with the quantile algorithm. The two-side t-test was conducted to assess the differentially expressed lncRNAs between three paired of tumor tissues and adjacent normal tissues (fold change > 2.0 and p-value < 0.05).

Quantitative Real-Time PCR

Total RNAs were extracted from HNSCC tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). NanoDrop 2000 (Quawell, San Jose, CA, USA) was used to detect the RNA concentration and quality. After that, RNA was reversely transcribed into High Capacity cDNA using PrimeScript™ RT Master Mix (Perfect Real-time) (TaKaRa Biotechnology, Dalian, China). Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) was adopted for quantitative Real-Time PCR. Furthermore, results were normalized to the expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH). The primers used for LINC00473 were: 5’-GGCAGCCTCAGGTTACAAAT-3’ (forward) and 5’-AGGAGCAGGTAGGGAA-TGA-3’ (reverse); for GAPDH, 5’-CCCACCTC-CTCCACCTTTGAC-3’ (forward) and 5’-ATAC-CAGGAAATGAGCTTGACAA-3’ (reverse). LINC00473 relative expression was observed and normalized with the 2−ΔΔCt method by qRT-PCR. Each treatment group was carried in triplicate.

Cell Transfection

The suppression of LINC00473 expression was achieved by transfection of short-hairpin RNA plasmid in different sequences (sh-LINC00473#1, sh-LINC00473#2) (GenePharma, Shanghai, China) and control shRNA (sh-NC) (GenePharma, Shanghai, China) in SCC25 and CAL27 cells with Lipofectamine® 2000 agent (Invitrogen, Carlsbad, CA, USA). A pcDNA3.1/LINC00473 (LINC00473) (GeneCopoecia, Guangzhou, China) was used to overexpress LINC00473 expression in SCC9 cell line in accordance with the manufacturer’s protocol. After 48 h transfection, cells were used for further analyses.

Radiotherapy Treatment of Cells

The 3×10⁵ cells were cultured in 25 cm² tissue culture dishes. After 48 h transfection, cells were irradiated with different single radiation doses (0, 2, 4, 6 Gy) x-ray treatment. Then, cells were ob-
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served after irradiation for further experiments. Experiments were implemented in triplicate.

**MTT Assays**

Cell proliferation was explored using Cell Proliferation Reagent Kit I (MTT; Roche Applied Science, Basel, Switzerland). sh-LINC00473 was transfected into SCC25 and CAL27 cells. LINC00473 transfected into SCC9. Each well was added to Ten microliters of MTT solution (5 mg/ml, Sigma-Aldrich, St. Louis, MO, USA). Then, the medium containing MTT was removed and replaced with 100 μl DMSO to solubilize the formazan. Optical absorbance was detected at 570 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). Cell proliferation was observed every 24h following the manufacturer’s protocol. Experiments were implemented in triplicate.

**Colony Formation Assay**

At 48 hours’ post-transfection, HNSCC cells at a density of 500 cells per well were placed in six-well plate and cultured in media containing 10% FBS, replacing the medium every 3 days. After 2 weeks, the cells were fixed with methanol and stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA). The number of colonies was calculated with microscope (Olympus Corporation, Tokyo, Japan). Each treatment group were carried in triplicate.

**Analysis of Apoptosis**

Cells were stained with Annexin V-FITC and propidium iodide (PI; BD Biosciences, San Jose, CA, USA) at 37°C in dark. Then, the cell-apoptosis rate was investigated by using flow cytometry (BD Biosciences, San Jose, CA, USA). All the samples were performed in triplicate.

**Western Blot Assay**

Total proteins were isolated from HNSCC tissues and cells by using RIPA lysis buffer. Then, extracted proteins for each sample were resolved by 10% SDS-PAGE and transferred to PVDF membranes (Sigma-Aldrich, St. Louis, MO, USA). The PVDF membranes were incubated in 5% non-fat milk dissolved in 0.1% TBS for 2 h. Primary antibody was incubated including rabbit anti-β-catenin (1:500; Abcam, Hong Kong), rabbit anti-c-myc (1:750; Abcam, Cambridge, MA, USA), mouse anti-β-actin (1:500; Santa Cruz, CA, USA). Protein bands were visualized by the ECL detecting system (Applygen, Beijing, China). β-actin was used as an internal control. All the samples were performed in triplicate.

**Statistical Analysis**

All assays were carried out in triplicate and data were shown as the mean ± standard deviation (SD). Statistical analysis was performed by SPSS 20.0 software. Furthermore, survival curve was generated and analyzed by using the Kaplan-Meier method and log-rank test. Differences between groups were measured by using the Student’s t-tests and one-way ANOVA. Furthermore, Bonferroni corrected Mann-Whitney U tests were utilized for post-hoc comparisons. p-value less than 0.05 were regarded as statistically significant.

**Results**

**LncRNA LINC00473 is Upregulated in HNSCC Tumor Tissues and Cell Lines**

To identify the lncRNA profiles of HNSCC tissues, microarray analysis was performed using three clinical HNSCC tissues and adjacent tissues. LINC00473 exhibited the biggest fold change among these lncRNAs (Figure 1A). Then, we evaluated the expression of LINC00473 in 78 pairs of HNSCC tissues and adjacent tumor tissues by qRT-PCR assay. Results demonstrated that LINC00473 was significantly upregulated in HNSCC tissues compared to adjacent tumor tissues (Figure 1B). We then examined the expression of LINC00473 in four human HNSCC cell lines (SCC9, SCC15, SCC25, CAL27) and oral keratinocyte cell line (OKC). Experiments revealed that LINC00473 was significantly increased in HNSCC cell lines, especially in SCC25 and CAL27 cell lines (Figure 1C). While LINC00473 exhibited the smallest fold change in SCC9 cell line. Furthermore, survival analysis demonstrated that high expression of LINC00473 was positively associated with the poor overall survival of HNSCC patients (Figure 1D). These data revealed that LINC00473 was upregulated in HNSCC, which may affect the development of HNSCC.

**Knockdown of LINC00473 Inhibits HNSCC Cells Proliferation and Accelerates Apoptosis**

To investigate the biological function of LINC00473 in HNSCC cells, we designed qRT-PCR assays in three different HNSCC cells. LINC00473-specific shRNAs (sh-LINC00473#1, sh-LINC00473#2) and sh-NC were transfected into SCC25 and CAL27 cells. The results showed that LINC00473 was remarkably decreased in SCC25 and CAL27 cells (Figure 2a). While sh-LINC00473#2 transfected SCC25 and CAL27 cells showed the biggest
fold change. Simultaneously, the expression of LINC00473 was significantly increased in SCC9 cell after transfecting pcDNA-LINC00473 (Figure 2A). MTT assays were performed to measure the effect of LINC00473 on HNSCC cell proliferation. We found that knockdown of LINC00473 significantly suppressed the proliferation of SCC25 and CAL27 cells. Meanwhile, the proliferation ability of LINC00473-overexpressed SCC9 cell was stronger than that of control group (Figure 2B). Similarly, colony formation assays demonstrated that colony cells were remarkably decreased in SCC25 and CAL27 cells after LINC00473 was silenced. On the contrary, LINC00473 overexpression induced more colony in SCC9 cells than control group (Figure 2C). In addition, flow cytometry analysis was performed to evaluate the effects of knockdown or overexpression of LINC00473 on cell apoptosis. Our results revealed that knockdown of LINC00473 led to a significantly higher apoptosis rate of SCC25 and CAL27 cells (Figure 2D). However, LINC00473 overexpression decreased the percentage of apoptosis in SCC9 cell (Figure 2D). More importantly, Western blot analysis manifested that Bax protein was significantly increased after sh-LINC00473 transfected into SCC25 and CAL27 cells, whereas the Bcl-2 protein level was decreased (Figure 2E). Moreover, the protein level of Bax was significantly decreased after LINC00473 overexpression transfected into SCC9 cell, whereas the Bcl-2 protein level was increased (Figure 2E). Taken together, these findings suggested that LINC00473 promoted HNSCC cell proliferation and inhibited apoptosis.

**LINC00473 Knockdown Reverses Radioresistance of HNSCC Cells**

Radiotherapy was considered as an efficient way for the treatment of cancer. To investigate the potential role of LINC00473 in HNSCC radiotherapy, we firstly measured the expression of LINC00473 with different dose of x-ray treatment (0, 2, 4, 6 Gy). Results revealed that x-ray treatment significantly decreased the expression of LINC00473 in SCC25 and CAL27 cell lines after LINC00473 knockdown (Figure 3A). In addition, the expression of LINC00473 showed significant fold change in 2 and 4 Gy when silenced LINC00473. Then, we further detect the effect of x-ray treatment on cell proliferation and apoptosis after knockdown of LINC00473 in SCC25 and CAL27 cells in colony formation assay (Figure 3C). The apoptosis of cells was measured with flow cytometry analysis. Results suggested x-ray treatment significantly promoted cell apoptosis when LINC00473 down-regulation (Figure 3D). Additionally, Western blot analysis demonstrated that x-ray treatment significantly increased the protein level of Bax.
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LINC00473 promotes radioresistance after sh-LINC00473 transfected into SCC25 and CAL27 cells, whereas the Bcl-2 protein level was decreased (Figure 3E). Therefore, our findings suggested knockdown of LINC00473 enhanced the radiosensitivity of HNSCC cells.

**LncRNA LINC00473 Exerts Functions in HNSCC Through Activating Wnt/β-catenin Signaling Pathway**

We have studied that the Wnt/β-catenin signaling pathway plays vital role in cells proliferation and metastasis in HNSCC. We sought to detect the relationship between LINC00473 and Wnt/β-catenin signaling pathway. The activity of Wnt/β-catenin signaling pathway was investigated by Western blot assay. Results demonstrated that the protein level of β-catenin and c-myc, vital genes of Wnt/β-catenin signaling pathway, was significantly decreased compared with β-Actin when LINC00473 was silenced in SCC25 and CAL27 cell lines (Figure 4A). These results indicated that Wnt/β-catenin signaling pathway was regulated by LINC00473. We further sought to detect whether Wnt/β-catenin signaling pathway involved in the mechanism of LINC00473 knockdown enhancing radiosensitivity in HNSCC. We investigated the activity of β-catenin and c-myc in x-ray treatment when LINC00473 was si-

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**Figure 2.** Knockdown of LINC00473 inhibits HNSCC cells proliferation and accelerates apoptosis. **A,** QRT-PCR assay confirmed the expression of LINC00473 after sh-LINC00473#1 and sh-LINC00473#2 plasmids transfected into SCC25 and CAL27; The expression of LINC00473 was measured by pcDNA/LINC00473 (LINC00473) transfected into SCC9 cell. **B,** MTT assay was performed to determine cell proliferation by transfection sh-LINC00473 into SCC25 and CAL27; SCC9 transfected with LINC00473. **C,** Colony formation assay were performed to detect the proliferation of SCC25 and CAL27 cells after transfected with sh-LINC00473; LINC00473 transfected into SCC9. **D,** Flow cytometry analysis was performed to investigate the apoptosis in SCC25 and CAL27 cells transfected with sh-LINC00473; SCC9 transfected with LINC00473. **E,** The protein level of Bax and Bcl-2 was detected after sh-LINC00473 transfected into SCC25 and CAL27 cells. LINC00473 transfected into SCC9 cell line. **p < 0.01** *p < 0.05.
LINC00473 knockdown reverses radioresistance of HNSCC cells. A, The expression of LINC00473 was measured with x-ray treatment (0, 2, 4, 6 Gy) after sh-LINC00473 transfected SCC25 and CAL27. B, MTT assay was performed to determine the effect of x-ray treatment (2, 4 Gy) on cell proliferation after sh-LINC00473 transfected SCC25 and CAL27 cells. C, The effect of x-ray treatment (2, 4 Gy) on cell colony was investigated after sh-LINC00473 transfected into SCC25 and CAL27 cells. D, sh-LINC00473 cells were transfected with x-ray treatment (2, 4 Gy), the apoptosis rate can be observed by flow cytometry. E, The effect of x-ray treatment (2, 4 Gy) on Bax and Bcl-2 was detected after sh-LINC00473 transfected into SCC25 and CAL27 cells in Western blot assay. **p < 0.01 *p < 0.05.

Figure 3.

Discussion

Currently, chemotherapy and radiotherapy are two main therapeutic approaches for the treatment of human cancer. However, chemotherapy and radiotherapy have limited effectiveness, which lead to recurrence and metastasis in HNSCC patients. Understanding the potential molecular mechanisms of radioresistance is crucial for us to promote effect of therapeutic. Therefore, it is vital to increase the sensitivity of radiotherapy while reduce the resistance of radio treatment. Long non-coding RNAs (lncRNAs) have received increased attention in cancers. Large number of studies showed that lncRNAs play significant role in the occurrence and proliferation of many types of cancer. Reports demonstrated that LINC00473 was upregulated in wilms cancer. Shi et al suggested that LINC00473 upregula-
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gulation was closely connected with bigger tumor size and poorer prognosis of cervical cancer patients. In this study, microarray analysis was performed to identify the lncRNA expression profiles between 78 paired human HNSCC and normal adjacent tissues. The results showed that lncRNA LINC00473 exhibited more than 2-fold expression changes in HNSCC tissues compared to the profiles in adjacent tissues. After that, we discovered that LINC00473 was strikingly upregulated in HNSCC tissues and HNSCC cell lines. For further experiment, survival curve elucidated that high expression of LINC00473 was closely connected with poor overall survival. Therefore, we assumed that lncRNA LINC00473 might be a potential diagnostic marker. Whereas, the biological function and potential mechanism of LINC00473 in HNSCC remained undefined. Interference efficiency assays showed that LINC00473 downregulation significantly reduced the expression of LINC00473 in SCC25 and CAL27 cells. Assays also showed that LINC00473 overexpression elevated the expression of LINC00473 in SCC9 cell. Functional experiments revealed that LINC00473 knockdown strikingly suppressed cells proliferation and accelerated apoptosis. Whereas, overexpression of LINC00473 accelerated cell growth and inhibited apoptosis.

Generally, radiotherapy was regarded as an effective way for cancers. Reports also demonstrated that lncRNA GACAT3 can influence the radiosensitivity of NSCLC cells. However, the effect of x-ray treatment on LINC00473 remains unclearly. We detected the expression of LINC00473 after x-ray treatment (0, 2, 4, 6 Gy).

Results showed that x-ray treatment significantly decreased the expression of LINC00473 in SCC25 and CAL27 cells when LINC00473 knockdown. Furthermore, the effect of x-ray on proliferation and apoptosis were measured in SCC25 and CAL27 cells. Experiments revealed that x-ray treatment significantly decreased cell proliferation when silenced LINC00473. Assays also confirmed that x-ray treatment promoted cell apoptosis after LINC00473 knockdown. These data suggested that LINC00473 knockdown enhanced the sensitivity of radiotherapy.

Wnt/β-catenin signaling pathway has been reported to be closely connected with HNSCC accordance with pathological studies. Reports also demonstrated that Wnt/β-catenin signaling pathway was activated and played significant role in the growth of HNSCC cells. Furthermore, studies suggested that SNHG1 upregulation contributed to progression of non-small cell lung cancer through inhibiting of miR-101-3p and activating of Wnt/β-catenin signaling pathway. TC-1 upregulation promoted cell proliferation in NSCLC via the Wnt/β-catenin signaling pathway to increase susceptibility of NSCLC to radiotherapy. To detect the role of LINC00473 in Wnt/β-catenin signaling pathway. Assays showed that LINC00473 knockdown decreased the protein level of β-catenin and c-myc, indicating that LINC00473 regulated the activity of Wnt/β-catenin signaling pathway. Moreover, research supported that the potential mechanism of radiotherapy may be through inhibiting Wnt/β-catenin signaling pathway when silenced HOTAIR. We detected whether Wnt/β-catenin signaling pathway involving in LINC00473 enhancing radioresi-

![Figure 4](image-url)

**Figure 4.** LncRNA LINC00473 exerts functions in HNSCC through activating Wnt/β-catenin signaling pathway. **A**, The protein level of β-catenin and c-myc can be observed after sh-LINC00473 transfected SCC25 and CAL27 cells by Western blot assay. β-Actin used as an internal loading. **B**, The protein level of β-catenin and c-myc were measured with x-ray treatment (2, 4 Gy) after sh-LINC00473 transfected SCC25 and CAL27 cells in Western blot assay. "*p<0.05", "**p<0.01".
stance. Results suggested x-ray treatment decreased the protein level of β-catenin and c-myc after LINC00473 knockdown. From these experiments we can see, LINC00473 attenuated the effect of radiosensitivity via regulating Wnt/β-catenin signaling pathway. And LINC00473 may be a potential biomarker for HNSCC diagnosis.

Conclusions

We showed that LINC00473 was highly expressed in HNSCC tissues and cell lines, and closely associated with poor overall survival. Furthermore, LINC00473 enhanced radiosensitivity of HNSCC cells through activating Wnt/β-catenin signaling pathway. And LINC00473 may be a potential biomarker for HNSCC diagnosis.


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