LINC01224/ZNF91 Promote Stem Cell-Like Properties and Drive Radioresistance in Non-Small Cell Lung Cancer

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Background: Radioresistance is the main reason for the failure of radiotherapy in non-small cell lung cancer (NSCLC); however, the molecular mechanism of radioresistance is still unclear.

Methods: An RNA-Seq assay was used to screen differentially expressed long non-coding RNAs (lncRNAs) and genes in irradiation-resistant NSCLC cells. RT-PCR and Western blotting assays were performed to analyze the expressions of lncRNAs and genes. The chromosome conformation capture (3C) assay was performed to measure chromatin interactions. Cell cytotoxicity, cell apoptosis, sphere formation and Transwell assays were performed to assess cellular function.

Results: In this study, it was found that LINC01224 increased during the induction of radioresistance in NSCLC cells. LINC01224 was located within the enhancer of ZNF91, and LINC01224 could affect the transcription of ZNF91 by regulating the long-range interactions between the ZNF91 enhancer and promoter. Moreover, upregulation of LINC01224 and ZNF91 could promote irradiation resistance by regulating the stem cell-like properties of NSCLC cells. In addition, high expression levels of LINC01224 and ZNF91 in tissue samples were associated with radioresistance in NSCLC patients.

Conclusion: Our findings demonstrated that LINC01224/ZNF91 drove radioresistance regulation by promoting the stem cell-like properties in NSCLC.

Keywords: non-small cell lung cancer, radioresistance, LINC01224, ZNF91, stem cell-like properties

Introduction

Lung cancer is the leading cause of cancer-related death worldwide, and non-small-cell lung cancer (NSCLC) is the main type of lung cancer.1–3 At present, surgery is the most effective treatment method for NSCLC, but more than 70% of NSCLC patients are diagnosed at an advanced stage, and are thus unsuitable for surgery.3 Radiotherapy is one of the main treatment methods for NSCLC; however, only about 10% of patients receiving radiotherapy can achieve complete remission, as irradiation resistance can result in low rates of cure and treatment failure.4 Therefore, determination of the molecular mechanism of radioresistance and identification of treatment methods to overcome radioresistance is urgently needed.5

Long non-coding RNA (lncRNA) is a functional RNA molecule with a transcript length exceeding 200 nt.6 It has been found that lncRNAs lack protein encoding ability and regulate cell function by regulating target genes.7 Normally,
lncRNAs can regulate target genes at various levels, such as epigenetics, transcription and posttranscription, and the molecular mechanisms related to the regulation of target genes are also different, including interactions with proteins, DNA or miRNAs. Recently, it has been shown that lncRNAs are closely associated with the occurrence and development of cancer including NSCLC. Moreover, it has been found that the expressions of lncRNAs are abnormal during radiotherapy, suggesting that lncRNAs play important roles in the regulation of radioresistance.

In the present study, we found that the expression of LINC01224 increased in irradiation-resistant H1975/IR cells compared with irradiation-sensitive H1975 cells. LINC01224 was transcribed from the enhancer region of ZNF91 and regulated the transcription of ZNF91 by mediating chromatin looping between the ZNF91 enhancer and promoter. Moreover, we also found that LINC01224/ZNF91 was involved in the regulation of irradiation resistance by promoting the stem cell-like properties of NSCLC cells. In addition, the expression levels of LINC01224 and ZNF91 in tissue samples were associated with radiotherapy resistance in NSCLC patients. These findings suggested that LINC01224/ZNF91 was involved in the regulation of radioresistance and was a potential new biomarker of radiotherapy resistance in NSCLC.

Materials and Methods

Cell Culture and Transfection

Human non-small cell lung cancer cells H1299 and H1975 (parental, irradiation-sensitive) were purchased commercially (ATCC cell lines) and H1975/IR (irradiation-resistant) were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO₂. Using a linear accelerator (Primart 6 MV; Siemens), H1975/IR cells were induced from H1975 cells by treating with irradiation (2 Gy) every 3 days until the total dose reached 30 Gy. H1975/IR cells were treated with 2 Gy of irradiation once a week. Irradiation cytotoxicity was determined using CCK8 and represented as ED₅₀ (median effective dose). The ED₅₀ of irradiation cytotoxicity in H1975 cells was 2.83 ± 0.17 Gy, and the ED₅₀ of irradiation cytotoxicity in H1975/IR cells was 10.67 ± 0.36 Gy.

In order to regulate expression levels of target genes, the pCDNA3.1 plasmids transfected by Lipofectamine Transfection Reagent (Invitrogen) were used to upregulate the expression levels, and the shRNAs lentiviral vectors transfected by lentivirus were used to downregulate the expression levels. Cells were harvested 48 or 72 h after transfection for analysis. The sequences of the clone PCR primers and shRNAs were as follows:

- ZNF91 clone PCR primers, forward 5′-ATGGTACATGCCAGGAACCCCTGGAAGCCT-3′ and reverse 5′-CTGAATTTCTTAGAGAAGGGGTTTACTG-3′;
- LINC01224 shRNA, 5′-CCGGGCCTTTCTGAACCAAATCTCGAGATTTGGTTTGGTGTTCAAGAAAAGGCTTTTTG-3′;
- ZNF91 shRNA, 5′-CCGGGCATTTCTATGGTCCTCAACCCAATCTCGAGATTTGGTTTGGTGTTCAAGAAAAGGCTTTTTG-3′.

Collection of Tissue Samples

Fifty-six NSCLC patients (Table 1) were recruited into this study from January 2019 to June 2020 at the Affiliated Cancer Hospital & Institute of Guangzhou Medical University (Guangzhou, China). Inclusion criteria were patients with primary NSCLC; with a histological diagnosis of NSCLC with at least one measurable lesion; with a TNM clinical stage of IIIB to IV; who had undergone radiotherapy. Fresh NSCLC tissues were obtained by aspiration biopsy and immediately snap-frozen in liquid nitrogen and stored at -80°C until use. Tissue samples were divided into two groups according to the Response...
Evaluation Criteria in Solid Tumors (RECIST). That is, patients with a response or partial response to treatment were considered to be “treatment-sensitive” (R, responder), and patients with stable or progressive disease were considered to be “treatment-resistant” (NR, non-responder).

RNA-Seq Assay
Total RNA from H1975/IR cells and H1975 cells was isolated using a Total RNA Purification kit (Qiagen). The RNA concentration and quality were determined by the Qubit 2.0 Fluorometer (Life Technologies) and the Nanodrop One spectrophotometer. Integrity of total RNA was assessed using the Agilent 2100 Bioanalyzer, and samples with RNA integrity number (RIN) values above 7.0 were used for sequencing. One μg RNA was used as input material for the RNA sample preparations. RNA-seq strand-specific libraries were constructed using the VAHTS Total RNA-seq (H/M/R) Library Prep Kit (Vazyme) according to the manufacturer’s instructions. Briefly, RNA was purified by magnetic beads after removal of rRNA. The RNA was then fragmented into small pieces using divalent cations for 8 min at 94°C. The cleaved RNA fragments were copied into first strand cDNA using reverse transcriptase and random primers. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. These cDNA fragments then went through the end repair process, addition of a single “A” base, and ligation of the adapters. The products were purified and enriched by PCR to create the final cDNA library. Purified libraries were quantified and validated by the Qubit 2.0 Fluorometer and Agilent 2100 bioanalyzer to confirm the insert size and calculate the mole concentration. A cluster was generated by cBot after the libraries were diluted to 10 pmol and then sequenced on the Illumina NovaSeq 6000 platform. Reads of the paired H1975/IR cells and H1975 cells RNA samples were obtained and were uniquely mapped to the human GRCh38 genome. Gene abundance was expressed as fragments per kilobase of exon per million reads mapped (FPKM). Stringtie software was used to count the fragment within each gene, and the TMM algorithm was used for normalization. Differential expression analysis of mRNA/lncRNA was performed using R package EdgeR. The original sequencing results have been deposited in Sequence Read Archive (SRA) PRJNA732559.

The Chromosome Conformation Capture (3C) Assay
The 3C assay was performed to analyze the chromatin interactions. Briefly, after cross-linking with 1% formaldehyde, cells were lysed and suspended in EcoRI restriction enzyme solution (NEB), and then, T4 DNA ligase (NEB) was added to perform DNA ligation. After reverse cross-linking with proteinase K, samples were purified using phenol-chloroform extraction and amplified by qRT-PCR analysis. The specific primer sequences were as follows:
Enhancer anchor: 5′-TTTAGGAGAGACAGGGGTTT C-3′;
P1: 5′-ATTAGATGAGTGTGGCAGC-3′;
P2: 5′-TGAATCTTGGTGTCAGAGAC-3′;
P3: 5′-TTTGGGAGACTGAGGTGG-3′;
P4: 5′-GTCTGTAATCCCAGCTACTG-3′.

Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)
The qRT-PCR assay was performed to analyze the mRNA and lncRNA levels of target genes. Briefly, total RNA in cells and tissue samples was extracted by TRIZoL™ (Invitrogen), and 1 μg RNA was used for cDNA reverse transcription by the Reverse Transcription Kit (Takara). The qRT-PCR was performed with the SYBR Green Realtime PCR Master Mix Kit (Toyobo) using the ABI ViiATM7Dx Real-Time PCR System (Life Technologies). The qRT-PCR primers were as follows:
LINC01224, forward 5′-ACGTGCACAGACAGCTAGA-3′ and reverse 5′-ATCATCCACGGGAGTGACGA-3′;
ZNF91, forward 5′-GCAAGGAAAAGAGCCCTGGAG-3′ and reverse 5′-ACTTTGCTCTGGGCAGTTGT-3′;
GAPDH (internal reference), forward 5′-TGACTCCACCGGGATGACG-3′;
A-3′;
ZNF91, forward 5′-GCAAGGAAAAGAGCCCTGGAG-3′ and reverse 5′-ACTTTGCTCTGGGCAGTTGT-3′;
GAPDH (internal reference), forward 5′-TGACTT CAACAGCGACACCCA-3′ and reverse 5′-CACCTGT TGCTGTAATCCCAGCTACTG-3′.

Western Blotting
The Western blotting assay was performed to analyze the protein levels of target genes. First, total proteins in cells were extracted using RIPA buffer for 0.5 h at 4°C, and 50 mg of proteins were loaded onto 15% SDS-PAGE for analysis. Then, rabbit polyclonal primary antibodies (Invitrogen, 1:1000 dilutions) of ZNF91, CD44, ALDH1, cleaved and uncleaved caspase 3 and GAPDH (internal reference) were added and incubated overnight at 4°C. Finally, HRP
(horseradish peroxidase) conjugate goat-anti-rabbit secondary antibody (Invitrogen, 1:1000 dilution) was used and incubated for 4 h. The bound antibodies were detected using the ECL Plus Western Blotting Detection system (GE Healthcare).

Flow Cytometry Assay
The flow cytometry assay was performed to analyze the positive rate of protein expression of the cell surface molecule, CD44. The cells were collected and prepared into a cell suspension, and the final density of the cell suspension was 1×10^5–5×10^5 cells/mL. The fluorescent antibody was added for staining and kept away from light for 10–15 min at room temperature. The cells were washed 2–3 times and analyzed using flow cytometry.

Cell Cytotoxicity Assay
The CCK8 Kit (Beyotime) was used to analyze cell cytotoxicity induced by irradiation. Briefly, 1×10^6 cells were plated in the well of a 6-well plate, after different doses (0, 2, 4, 6, 8, 10 Gy) of irradiation treatment by a linear accelerator (Primart 6 MV; Siemens), the cells were collected for cell survival analysis using the CCK8 Kit. Irradiation-induced cytotoxicity was determined and represented as ED_{50} (Gy). The median effective dose (ED_{50}) was calculated as follows: The exponential form of the equation was used and a curve fitted to the data points [fraction affected (Fa) and irradiation dose]: Fa = 1/[1 + (ED_{50}/irradiation dose)^m], m = slope of the curve, Fa = % reduction from untreated control x 0.01.

Cell Apoptosis Assay
The Annexin V-FITC Apoptosis Detection Kit (Beyotime) was used to analyze cell apoptosis induced by irradiation. Briefly, 1×10^6 cells were plated in the well of a 6-well plate, after 2 Gy irradiation treatment by a linear accelerator (Primart 6 MV; Siemens), and the cells were collected for cell apoptosis analysis. Cell apoptosis was determined by flow cytometry.

Sphere Formation Assay
The sphere formation assay was performed to analyze the sphere formation capacity. Briefly, 5000 cells were plated in the well of a 6-well plate using DMEM F12 serum-free medium reconstituted with 20 ng/mL of EGF, 20 ng/mL of bFGF, 2% B27, and 1% methylcellulose. After 4–7 days, microsphere-like structures were visible, and images of the microspheres were captured using a microscope (Leica).

Transwell Assay
The migration and invasion capability of cells was detected using the Transwell-chamber culture system (Becton Dickinson). After 48 h incubation at 37°C, the cells were transferred into the upper Transwell chamber with Matrigel (1×10^5 cells per well in an 8 μm 24-well Transwell). Following 24 h incubation at 37°C, cells on the upper surface of the upper chamber (non-invasive cells) were removed using cotton swabs, and cells on the lower surface of the filters were fixed and stained with Giemsa stain. The number of invaded cells was counted under a light microscope (Leica).

Statistical Analyses
Values shown are the mean ± standard deviation (SD) of at least three separate experiments. The ED_{50} values were assessed by linear regression analysis. The one-way ANOVA, log-rank statistic, Spearman’s rank correlation tests and receiver operating characteristic (ROC) curves were analyzed by SPSS v.21.0 (IBM, USA). P < 0.05 (two-tailed) was considered significant.

Results
LINC01224 is Upregulated by Irradiation Treatment and Regulates ZNF91 Expression
We compared RNA expression in irradiation-resistant H1975/IR cells with that of parent irradiation-sensitive H1975 cells using RNA-Seq analyses. A total of 831 differentially expressed lncRNAs (log_{2}FC > 2 and q < 0.05) included 423 upregulated lncRNAs, 408 downregulated lncRNAs, and 715 differentially expressed mRNAs (log_{2}FC > 2 and q < 0.05) included 420 upregulated mRNAs and 295 downregulated mRNAs (Figure 1A and B). Of note, the expression of LINC01224 was upregulated (log_{2}FC = 4.9) in H1975/IR cells compared with H1975 cells (Table S1). Consistent with the results of RNA-Seq analyses, qRT-PCR results confirmed increased expression of LINC01224 in H1975/IR cells compared with H1975 and H1299 cells (Figure 1C). These findings suggested that LINC01224 was closely associated with radioresistance in NSCLC.

In order to elucidate the molecular mechanism of LINC01224, we found that LINC01224 was predominantly located in the nuclear region (Figure 1D). The nuclear accumulation of LINC01224 suggested a possible role in local gene expression. LINC01224 was transcribed from an enhancer region of ZNF91, and the expression of ZNF91
was higher in H1975/IR cells compared with H1975 cells as revealed by RNA-Seq assays (log$_2$FC = 1.5) (Table S2). Moreover, qRT-PCR and Western blotting results also confirmed that the expression of ZNF91 was increased in H1975/IR cells compared with H1975 and H1299 cells (Figure 1E and F). In addition, knockdown of LINC01224 led to decreased expression of ZNF91 in H1975/IR cells (Figure 1E and F). Therefore, these findings suggested that LINC01224 regulated the expression of ZNF91.

**LINC01224 Enhances ZNF91 Expression by Mediating Long-Range Chromatin Interactions Between the ZNF91 Enhancer and Promoter**

LINC01224 is transcribed from the enhancer region of ZNF91, and this suggests that LINC01224 is an enhancer-derived RNA (eRNA), which is involved in the regulation of chromatin looping between the enhancer and promoter of target genes. Therefore, we reasoned that LINC01224 may enhance the transcription of ZNF91 by forming a chromatin loop and performed Chromosome Conformation Capture (3C) to measure the chromatin interactions between the ZNF91 enhancer and promoter (Figure 2A). It was observed that the interaction frequencies between the ZNF91 enhancer and promoter locus were higher in H1975/IR cells compared with H1975 and H1299 cells (Figure 2B). Moreover, knockdown of LINC01224 resulted in a reduction of the interaction frequencies in H1975/IR cells (Figure 2C). Collectively, these results suggested that LINC01224 acted as an eRNA to enhance ZNF91 transcription by forming DNA loops between the ZNF91 enhancer and promoter.

**LINC01224/ZNF91 Confers Radioresistance by Promoting Stem Cell-Like Properties**

Given the increased expression of LINC01224/ZNF91 identified in H1975/IR cells, we wondered how LINC01224/ZNF91 influenced the radioresistance of NSCLC cells. It was found that knockdown of LINC01224 or ZNF91 increased irradiation sensitivity (the ED$_{50}$ decreased from 10.67 ± 0.36 to 5.56 ± 0.23 Gy (knockdown of LINC01224), and from 10.67 ± 0.36 to 4.12 ± 0.15 Gy (knockdown of ZNF91)) and irradiation-induced cell apoptosis in H1975/IR.
cells (Figure 3A and B). Moreover, overexpression of ZNF91 reversed the promoting effects of LINC01224 knockdown on irradiation sensitivity (the ED50 increased from 5.56 ± 0.23 to 12.32 ± 0.53 Gy) and irradiation-induced cell apoptosis in H1975/IR cells (Figure 3A–C). In addition, overexpression of ZNF91 decreased irradiation sensitivity (the ED50 increased from 2.83 ± 0.17 to 7.15 ± 0.49 Gy (H1975 cells), and from 2.95 ± 0.28 to 6.52 ± 0.38 Gy (H1299 cells)) and irradiation-induced cell apoptosis in H1975 and H1299 cells (Figure 3D–F). Therefore, these findings indicated that LINC01224/ZNF91 were involved in the regulation of radioresistance in NSCLC.

The results showed that H1975/IR cells increased the sphere formation capacity compared with H1975 cells (Figure 4A and B); thus, we investigated the effect of LINC01224/ZNF91 on sphere formation capacity. In H1975/IR cells, knockdown of LINC01224 or ZNF91 decreased sphere formation capacity, and overexpression of ZNF91 restored the inhibiting effects of LINC01224 knockdown (Figure 4A). In H1975 and H1299 cells, overexpression of ZNF91 increased sphere formation capacity (Figure 4B). Moreover, in H1975/IR cells, knockdown of LINC01224 and ZNF91 decreased the stem cell marker CD44 (intracellular and cell face expression) and ALDH1, and overexpression of ZNF91 restored the inhibiting effects of LINC01224 knockdown (Figure 4C). In H1975 and H1299 cells, overexpression of ZNF91 increased stem cell marker CD44 (intracellular and cell face expression) and ALDH1 (Figure 4D). In addition, in H1975/IR cells, knockdown of LINC01224 and ZNF91 decreased cell invasion and migration, and overexpression of ZNF91 restored the inhibiting effects of LINC01224 knockdown (Figure 4E). In H1975 and H1299 cells, overexpression of ZNF91 increased cell invasion and migration (Figure 4F). Therefore, these data suggested that LINC01224/ZNF91 influenced radioresistance by regulating the stem cell-like properties of NSCLC cells.

**LINC01224/ZNF91 as Potential Prognostic Biomarkers of Treatment Outcomes After Radiotherapy in NSCLC**

In order to analyze the correlation between the expressions of LINC01224/ZNF91 and the outcome of radiotherapy in NSCLC patients, we collected tissue samples from 56 NSCLC patients who received radiotherapy and detected the expressions of LINC01224 and ZNF91 by qRT-PCR. It was found that the expressions of LINC01224 and ZNF91 were significantly higher in radioresistant NSCLC tissues (NR tissues) compared with radiosensitive NSCLC tissues (R tissues), suggesting that the poor therapeutic effect of radiotherapy was related to the high expressions of LINC01224 and ZNF91 in NSCLC patients (Figure 5A). Moreover, there was
a positive correlation between the expression of ZNF91 and LINC01224 \((r = 0.54, P < 0.01)\), suggesting that LINC01224 and ZNF91 were also positively regulated in vivo (Figure 5B). Finally, we performed receiver operating characteristic (ROC) analyses to evaluate the potential of LINC01224 and ZNF91 as biomarkers. The area under the curve (AUC) score of the combined analyses of LINC01224 and ZNF91 was 0.79 (95% CI, 0.66–0.91), which was higher than that when LINC01224 and ZNF91 were analyzed separately (Figure 5C).
Radiotherapy is one of the main treatments for NSCLC, and radioresistance is the main cause of radiotherapy failure. However, the molecular mechanism of radioresistance is still unclear. Recently, lncRNAs have been found to be involved in the regulation of radioresistance by affecting radioresistance-associated cell functions, such as cell proliferation, cell apoptosis, and DNA repair. In this study, abnormal high expression of LINC01224 was found in irradiation-resistant H1975/IR cells, and knockdown of LINC01224 enhanced irradiation-induced cell apoptosis. Based on recent studies, LINC01224 has been shown to have an important role in ovarian cancer, breast cancer and hepatocellular cancer. In this study, abnormal high expression of LINC01224 was found in irradiation-resistant H1975/IR cells, and knockdown of LINC01224 enhanced irradiation-induced cell apoptosis. Based on recent studies, LINC01224 has been shown to have an important role in ovarian cancer, breast cancer and hepatocellular cancer. 

Moreover, expression levels of LINC01224 were associated with radiotherapy response in NSCLC patients, and LINC01224 has potential as a biomarker for the therapeutic response to radiotherapy in NSCLC. Therefore, these results suggested that LINC01224 was involved in the regulation of radioresistance in NSCLC.

LINC01224 is transcribed from a locus 21 kb upstream of ZNF91, an enhancer region of ZNF91. Enhancers are a class of DNA regulatory sequences that can activate target gene expression by forming long-range chromatin loops with the promoters of target genes. Recently, it was found that a large portion of enhancers can be transcribed into RNAs, as eRNAs. Moreover, eRNAs have been proposed to contribute to gene activation, presumably by the establishment or maintenance of enhancer-promoter looping. According to our results, knockdown of LINC01224 inhibited the interaction frequencies between ZNF91 enhancer and promoter. ZNF91 is a member of the zinc finger protein family, which can act as a transcription factor to regulate the transcription of target genes, and the abnormal expression of ZNF91 is related to the
occurrence and development of ovarian cancer and bladder cancer.\textsuperscript{34–36} Here, we found that the expression of ZNF91 was increased in irradiation-resistant H1975/IR cells compared with irradiation-sensitive H1975 cells and knockdown of LINC01224 inhibited the expression of ZNF91 in H1975/IR cells. Therefore, LINC01224, which was transcribed from the enhancer region of ZNF91, regulated the transcriptional activation of ZNF91 by promoting long-range interactions between the ZNF91 promoter and enhancer.

In recent years, the “cancer stem cell” theory proposes that a few tumor cell subpopulations have stem cell-like self-renewal ability and strong DNA damage repair, which can resist irradiation-induced cell apoptosis, and cancer stem cells can lead to the failure of radiotherapy to completely remove the tumor and finally produce radioresistance.\textsuperscript{37–39} Here, we demonstrated that LINC01224 and ZNF91 promoted radioresistance by regulating the stem cell-like properties of NSCLC cells. Therefore, LINC01224/ZNF91 were involved in the regulation of radioresistance by promoting stem cell-like properties.

**Conclusion**

Taken together, the present results revealed a novel connection between the chromatin organization regulated by LINC01224 and ZNF91 expression in NSCLC, and LINC01224/ZNF91 were involved in the regulation of radioresistance by promoting the stem cell-like properties of NSCLC cells. Thus, these findings provided a novel insight into the molecular mechanisms of radioresistance, and provided a promising strategy for predicting and reversing radioresistance in the future.

**Ethics Approval**

All patients provided written informed consent, and the collection of NSCLC tissues for research purposes was approved by relevant human research ethics committees of the Affiliated Cancer Hospital & Institute of Guangzhou Medical University (Approval no. (2014) 100). This was conducted in accordance with the Declaration of Helsinki.

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Disclosure

The authors declare that they have no competing interests.

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