Radiotherapy induced Lewis lung cancer cell apoptosis via inactivating β-catenin mediated by upregulated HOTAIR

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Abstract: Objective: HOTAIR, a long intervening non-coding Hox transcript antisense intergenic RNA, negatively regulates transcription on another chromosome and is reported to reprogram chromatin organization and promote tumor progression. Nevertheless, little is known about its roles in the development of radiation therapy of lung cancer. In this study, we established a xenografed model of Lewis lung carcinoma in C57BL/6 mice and investigated the possible involvement of HOTAIR in this radiotherapy. Methods: C57BL/6 mice were subcutaneously transplanted with Lewis lung carcinoma cells and locally irradiated followed by measurement in tumor volume. Levels of HOTAIR and WIF-1 mRNA expression were determined by using Quantitative Real-Time PCR. Levels of WIF-1 and β-catenin were determined by using western blot assay. Cell viability was evaluated by MTT assay. Cell apoptosis was examined by using TUNEL assay. Results: In mice bearing Lewis lung carcinoma tumor, local radiotherapy suppressed tumor growth and it also reduced level of HOTAIR but increased WIF-1 expression. When HOTAIR was overexpressed, radiosensitivity was reduced. In vitro experiments, irradiation inhibited HOTAIR transportation to the nucleus. However, it was reversed by over-expressed HOTAIR. Cells transfected with pcDNA-HOTAIR or siRNA-HOTAIR resulted in decline or increase in radiosensitivity, which was abrogated by co-tansfected with siRNA-β-catenin. Conclusion: Radiotherapy induced Lewis lung cancer cell apoptosis via inactivating β-catenin mediated by upregulated HOTAIR.

Keywords: β-catenin, lewis lung cancer, radiotherapy, xenograft model, WIF-1

Introduction

At present, lung cancer is still one of the most prevalent malignancies worldwide and is the leading cause of death in both men and women [1]. Despite advances made in therapy, the median overall survival is only 15 months, indicating an urgently need for new strategies to improve curative effect. Traditionally, for conveniently be located, approximately 70% of patients with lung cancer receive external beam radiation treatment as one component of their treatment [2]. However, individuals respond to radiotherapy differently and the efficacy of irradiation treatment is often impairs by the emergence of radioresistance. Thus, a better understanding the molecular mechanisms underlying the development of radiotherapy would promote our understanding of lung cancer development and treatment failure.

Recently, evidences have been accumulating to suggest a significant relationship between radiotherapy and epigenetic alterations exists [3-5]. Both microRNA (miRNAs) and long non-coding RNAs (lncRNAs) are the major regulatory noncoding RNAs that regulate gene expression at epigenetic, transcriptional, and post-transcriptional processing levels [6, 7]. An increasing number of contemporary studies have shown that altered microRNA expression may play an important role in the radiotherapy of cancer cells by impairing cellular responses that affect cell cycle arrest, apoptosis, and DNA damage repair [8]. The increasing prevalence of miRNA mediated radiotherapy data has led to an increase in studies focused on targeting miRNAs as strategies for therapeutic intervention. Unfortunately, the correlations between IncRNAs and tumor chemoresistance are rarely reported, and need to be more clearly elucidat-
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ed before these therapeutic strategies can be fully developed and undergo clinical assessment. HOTAIR is one of the few biologically well-documented lncRNAs, with a length of 2158 bp and a functional role in the mediation of trans-silencing [9]. HOTAIR has been found to be overexpressed in a variety of human cancers and determined to be a negative prognostic indicator in breast, colon, liver, and pancreatic cancer patient survival, evidencing a close association with increase in cancer cell metastasis [9-12]. Zhuang and his colleagues showed that tumor-promoting Col-1 up-regulates the expression of HOTAIR in NSCLC cells, suggesting that HOTAIR might play critical roles in lung tumorigenesis [13]. Although numerous studies have demonstrated the critical functions of HOTAIR in tumor development and metastasis, whether HOTAIR plays an important role during development of radiotherapy in lung cancer is still unclear.

In the present study, we established mice bearing tumor model. qRT-PCR assay was performed to detect the expression of HOTAIR in radio-cured tumor and irradiated Lewis lung cancer cells. Then, functional relationship analyses between HOTAIR and β-catenin nuclear translocation was conducted to evaluate the molecular signaling on the radiotherapy of lung cancer both in vivo and in vitro. This study explores the validity of HOTAIR as a valid therapeutic target for the reversal of radiotherapy in lung cancer.

Material and method

Mice, cells and reagents

C57BL/6 male mice (aged 6 weeks; n=20) were obtained from Institute of Laboratory Animal Science (Chinese academy sciences, Shanghai) and housed in polycarbonate cages containing woodchip bedding in a room with controlled temperature (20 to 24°C) and relative humidity (50% to 65%). Mice were fed rodent maintenance diet and provided with water ad libitum. The protocol was approved by the Guide for the Care and Use of Laboratory Animals.

The Lewis cancer cell line was cultured in RPMI 1640 (Gibco, Invitrogen Company, USA) supplemented with 10% FCS and human insulin (10 μg/ml). The cell cultures were maintained in a water humidified 37°C incubator with 5% CO₂.

Quantification of IncRNAs and real-time PCR reactions

Total RNA isolated from plasma samples were used for cDNA synthesis using the First Strand cDNA Synthesis kit (Thermo Scientific, USA) according to the manufacturers' instructions. The real-time amplification of IncRNA molecules was performed using the Light Cycler 480 (Roche, Germany). SYBR Green (Roche) was used as the fluorescent molecule. The endogenous reference gene for real-time PCR reactions was selected by comparing the basal expression levels of the GAPDH (glyceraldehyde-3-phosphate). The GAPDH gene was chosen in view of its higher stability and consistency in the control plasma samples.

The PCR program included an initial “hot start” for 10 min, followed by 45 cycles of amplification. Each cycle consisted of a denaturation step at 95°C for 10 s, annealing starting at 60°C for 20 s and decreasing 2°C every 2 cycles until 55°C, and amplification at 72°C for 30 s. For quantification of IncRNAs, the ΔΔCt method was used by coamplifying GAPDH for each sample and by comparing the Ct values. All experiments were performed triplicate and the mean values were calculated. Primers sequences are as followed: HOTAIR: sense, 5'-CAGTGGGGAACTCTGACTCG-3'; and reverse, 5'-GTGCCTGGTGCTCT-CTTACC-3'. WIF-1: sense, 5'-AGTGTCCTGATGGGTTCCAC-3'; and reverse, 5'-TGGTTG-AGCAGTTTGCTTTG-3'.

Tumor volume assessment

Lewis lung cancer cells were used in a xenograft model in female C57BL/6 mice. A suspension of 1×10⁶ cells in 100 μL volume was injected subcutaneously into the right flank of mice using a 1-cc syringe with 27½-gauge needle. Tumors were grown for 7 days. Tumors on the flanks of the mice were irradiated using an X-ray irradiator (Therapax, Agfa NDT, Inc., Lewis Town, PA). The non-tumor parts of the mice were shielded by lead blocks. Tumors were measured 2 or 3 times weekly in 3 perpendicular dimensions using a Vernier caliper. Tumor volumes were calculated using the modified ellipse volume formula (Volume = (Height × Width × Depth)/2). Growth delay was calculated as the number of days required to reach a tumor volume of 1.75 cm³ for treatment groups relative to the control.
Protein extraction and Western blot

Proteins in Lewis lung cancer cells and tumors were extracted by inM-PER mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA). The nuclear protein fraction was extracted using the NE-PER Nuclear and cytoplasmic protein was extracted using Cytoplasmic Extraction Kit (Pierce Biotechnology, Rockford, IL, USA) from Lewis lung cancer cells and Lewis lung cancer -HOTAIR cells, according to the manufacturer's instructions. Protein concentration of cell lysates was measured by using DC protein assay kit (Bio-Rad). Proteins (10-20 mg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane (Milipore, Bedford, MA, USA). Two hours after blocking in 10% non-fat milk, the protein blots were then incubated with primary antibodies in 5% bovine serum albumin at 4°C overnight, followed by incubation with secondary antibodies at room temperature for 2 h. The protein signals were detected by ECL method. For Western blot, antibodies against WIF-1 (1:500; Santa Cruz, CA, USA), β-catenin (1:1000; Millipore, Bedford, MA, USA) and GAPDH (1:5000; D16H11, CST) were used.

TUNEL assay

The TUNEL reaction, based on labeling of DNA strand breaks, was conducted to detect cells apoptosis and performed with an in situ cell detection kit (Roche Applied Science, Indianapolis, IN, USA), according to the manufacturer’s instruction. In brief, sections in 2 changes of xylene for 5 minutes each, and hydrate with 2 changes of 100% ethanol for 3 minutes each, and 95% ethanol for 1 minute followed by rinsing in distilled water. After pretreated with proteinase K, cells were cultured in TdT Reaction Buffer for 10 minutes and then conducted TdT Reaction in TdT Reaction Mixture for 1-2 hours at 37-40°C in humidified chamber. The reaction was stopped with washing buffer and the sample was resuspended in PBS containing FITC-Avidin D for 30 minutes at room temperature. The reaction mixture was counterstained with PI or DAPI for 20 minutes. TUNEL-positive nuclei (stained brown) were counted by Motic Images Advanced 3.2 in 10 random fields (×200), and then averaged.

Transfection of siRNAs and plasmid vectors

The cells were seeded into 6-well plates and transfected with 50 nM siRNAs targeting HO-TAIR (siRNA/HOTAIR1: 5'-UUUUCUACCAGGUCG- GUAC-3'; siRNA/HOTAIR2: 5'-AAUUCUAAAUUG- GGCUUG-3') (GenePharma, Shanghai, China) or 50 nM siRNAs targeting siRNA/β-catenin (siRNA/β-catenin, 5'-AGCUGAUAUGUGACA- G-3'; 5'-CATGUUGUGUAAAGCUCUAdC-3') or nM siRNAs targeting β-catenin siRNA/control (5’-CUACAACACCCAGACGUCdTdT-3') (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturers’ instructions.

Overexpression HOTAIR in Lewis lung cancer cells was performed by retrovirus mediated gene transfer. Briefly, the HOTAIR gene was subcloned into pcDNA3.1 (+) (Invitrogen, USA) by PCR method using the following primers: HOTAIR, sense, 5'-CATGGATCCACATTCTGCCCTGATTTCCGGAACC-3'; reverse, 5'-ACTCTCGAGCACACACACACAACCTACAC-3'. The PCR products were separated by electrophoresis on a 1% agarose gel and the gel was stained with ethidium bromide and imaged. The identity of the PCR product was confirmed by direct sequence analysis. The recombinant vector was confirmed by the digestion analysis of restriction endonuclease and DNA sequencing. The stable HOTAIR-expressing cells were generated by transfection with either pcDNA/HOTAIR or pcDNA/control vectors using Lipofectamine 2000, followed by selection with G418.

MTT assay

Lewis lung cancer cells were seeded at a density of 1400 cells/well. On the next day, cells were irradiated with dose 5 Gy for 24 h. After irradiation, 50 mL of 5 mg/mL 3-(4,5-Dimeth- ylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) was added to wells for 4 h, and then the reaction was stopped by media replaced with DMSO. Optical density analysis was performed by using an mQuant Microplate Spectrophotometer (BioTek, UK) at a wavelength of 540 nm.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (Chicago, IL). One-way Analysis of Variance (ANOVA) was used to analyze the significance differences between control groups and irradiation treatment groups as well as different time points. Student’s test was used to test the significance of the mRNA and protein expression. Error bars represent SD.
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**Results**

**HOTAIR expression in lung tumor radiotherapy**

We subcutaneously transplanted C57BL/6 mice with Lewis lung carcinoma cells and locally irradiated tumor with a single dose of 12 Gy/1f/1d for 14 days. After radiation treatment, HOTAIR expression levels were assessed in xenografted tumor by qRT-PCR normalized to the GAPDH. Compared with no treatment, irradiation substantially attenuated HOTAIR expression (Figure 1A). Inversely, WIF-1 expression in levels of both mRNA and protein was enhanced (Figure 1B). During radiotherapy, tumor size was regularly measured and the result showed a time-dependent inhibition on tumor growth by irradiation (Figure 1C).

**HOTAIR is essential for lung cancer radiotherapy**

To identify the role of HOTAIR in radiotherapy, the Lewis lung cancer cells were transfected with pcDNA-HOTAIR before tumor xenograft. After Normal breeding for 7 days, tumor-bearing mice were locally irradiated with 12 Gy/1f/1d for 14 days and tumors were measured regularly per 2 days. As showed in Figure...
Characterization of HOTAIR and β-catenin expression after irradiation in Lewis lung cancer cells

The above results suggested the essential role of HOTAIR for lung cancer radiotherapy. To ascertain the underlying mechanism, Lewis lung cancer cells were irradiated with 5 Gy. Level of HOTAIR expression was assessed by using western blot assay. In accordance with in vivo, the result showed a much lower level of HOTAIR in cells exposed to irradiation (Figure 3A). Consistently, levels of WIF-1 mRNA and protein were promoted by x ray irradiation (Figure 3B). WIF-1 is an inhibitor for Wnt/β-catenin signaling pathway and its combination to Wnt proteins can leads to β-catenin degradation. Thus, we respectively evaluated β-catenin expression level in whole-cell, cytoplasm and nuclear, respectively, by using western blot. As represented in Figure 3C, irradiation did not alter total β-catenin expression, but prevented protein transportation to the nucleus, which indicating inactivation action of β-catenin by irradiation.

HOTAIR promoted β-catenin transportation to the nucleus

To verify the regulator role of HOTAIR on β-catenin activity, the cells were transfected with si-HOTAIR or pcDNA-HOTAIR and expression of WIF-1 and β-catenin was represented by using western blot assay. Consequently, silenced HOTAIR up-regulated WIF-1 expression but inhibited nuclear translocation of β-catenin protein (Figure 4A). While HOTAIR over-expression by pcDNA-HOTAIR attenuated WIF-1 expression but increased nuclear β-catenin concentration (Figure 4B).

β-catenin interference reversed HOTAIR-reduced radiosensitivity

Irradiation treatment decreased cell viability. While pcDNA-HOTAIR transfection reduced cancer cells radiosensitivity and increased cell viability, which was reversed by cells co-transfected with pcDNA-HOTAIR and si-β-catenin (Figure 5A). Additionally, apoptosis of cancer cells were evaluated by TUNEL assay. The result showed that irradiation promoted lung cancer cells apoptosis but the radiation effect was suppressed by HOTAIR over-expression. While cell apoptosis responding to radiotherapy was recovered when cells was combining treated with pcDNA-HOTAIR and si-β-catenin (Figure 5B).

Discussion

Radiotherapy, based on its activation effect on DNA-damaged cellular signaling molecular, is proved to be the major therapeutic option for cancer treatment. However, many tumors have shown radioresistance which greatly limits the effect of radiotherapy. Cancer cells tend to be resistant to radiotherapy due to defects in apoptosis, overexpression of genes related to cell survival [14]. Therefore, fully exploration of the therapeutic targets and mechanisms underlying radiotherapy might provide better curative effect. In our current studies, we demonstrated that long non-coding RNAs-HOTAIR are the negative regulator for lung cancer radiosensitivity inhibits and functioned as an inhibitor for nuclear localization of β-catenin molecule signaling.

As an important regulator for chromosome remodeling, transcription and post-transcription-
al processing, long noncoding RNAs (lncRNAs) have received increased attention, especially in cancers [15]. HOTAIR has been found to be overexpressed in a variety of human cancers and determined to be a negative prognostic indicator in breast, colon, liver, and pancreatic cancer patient survival, supporting a close association with increase in cancer cell metastasis [9-12]. In lymph node metastasis, HOTAIR is overexpressed and showed as a promoter for lung cancer cell motility and invasion [16]. HOTAIR is also relevant to cellular prolifer-
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HOTAIR is one of long noncoding RNAs and plays an important role in carcinogenesis via being associated with storage or modification of the pre-mRNA of gene function regulation [20]. Our data showed that, along with HOTAIR downregulation, radiotherapy increased both Wnt inhibitory factor 1 (WIF-1) mRNA level and WIF-1 protein expression in irradiation-treated tumor tissue. WIF-1, as a key inhibitor of the Wnt/β-catenin signaling pathway, binds directly to extracellular Wnt ligands, preventing their interaction with the receptors and leading to degradation of cytosolic β-catenin by the APC/ Axin1 destruction complex [21]. Thus, we evaluated nuclear β-catenin in irradiated-Lewis lung cancer cell and the data showed an inhibition for β-catenin transportation to the nucleus from cytoplasm. Being the key transcriptional activation factor of Wnt/β-catenin signaling pathway, upon upstream activation, β-catenin often translocates to the nucleus from cytoplasm to activate its target genes, which in turn play pivotal role in tumor initiation and development [22-25]. These data indicated that the underlying mechanism of radiotherapy may be through a repression of β-catenin signaling, via down-regulation of HOTAIR expression. This was further demonstrated in vitro by Lewis lung cancer cells transfected with pcDNA-HOTAIR that resulted in accumulation of β-catenin in nuclear and in vivo by co-transfection of siRNA-β-catenin and pcDNA-HOTAIR reduced cancer cell apoptosis.

In conclusion, our results uncovered the mechanisms by which radiotherapy down-regulates HOTAIR, in turn alters the nuclear localization of β-catenin, resulting in lowered β-catenin signaling and eventual inhibition of proliferation. This discovery will contribute to a better understanding of not only the molecular mechanisms of radiotherapy in lung cells, but also the devel-

![Graph](image)

**Figure 5.** Effect of β-catenin on radiosensitivity of Lewis lung cancer cells. Lewis lung cancer cells were treated with pcDNA-HOTAIR, β-catenin or their control lentivirus, followed by irradiation with 5 Gy. Twenty-four hours after radiation, (A) MTT assay was conducted to assess cell viability and (B) TUNEL was performed to evaluate cell apoptosis. Columns, mean of three individual experiments in each clone; bars, SD; *P<0.05 as compared with the corresponding control group.
opment of combination between new gene therapies and radiotherapies for types of malignancies.

Disclosure of conflict of interest

None.

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