

Knockdown of long non-coding RNA HOTAIR inhibits proliferation and invasiveness and improves radiosensitivity in colorectal cancer

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Abstract. Colorectal cancer (CRC) is still one of the most important neoplasias causing human death. Multidisciplinary therapy has won consensus in the management of CRC, of which, radiotherapy occupies an important position. However, radioresistance is still a major obstacle in local control of CRC. Overexpression of long non-coding RNA HOTAIR has been found to correlate with tumorigenesis and poor prognosis in several types of cancer. In the present study, we analyzed HOTAIR expression levels of 53 CRC patients in tumor and adjacent normal tissue by real-time quantitative PCR. Knockdown of HOTAIR by RNA interference was performed to explore its roles in cell proliferation, migration, invasion, apoptosis and radiosensitivity. Results showed that CRC patients had higher HOTAIR expression in tumor tissues compared with adjacent normal tissues. *In vitro*, down-regulation of HOTAIR reduced proliferation, migration and invasiveness while enhanced apoptosis and radiosensitivity of CRC cells. Taken together, our findings suggest that long non-coding RNA HOTAIR expression is closely associated

with tumor invasion and radiosensitivity, indicating the potential role in diagnostics and therapeutics of CRC.

Introduction

Colorectal cancer (CRC) has high morbidity and mortality as one of the most common malignant tumors of human (1,2). It is the second ranking newly diagnosed cancer and the second leading cause of overall cancer deaths (3). Radiotherapy occupies an important position in the treatment of CRC. Radiotherapy decreases the rate of local relapse and improves survival for stage II and III CRC (4). However, radioresistance is still an obstacle of radiotherapy in CRC. Therefore, it is necessary to improve our understanding of the occurrence and development of the disease and accurately predict tumor radioresistance.

Molecular techniques reveal that several genes are differently activated in CRC. Most attention has focused on the biological characteristics of protein-coding genes while ignoring non-coding RNA (ncRNA), the majority of human genome (5,6). ncRNAs are divided into long ncRNAs (lncRNAs) and small ncRNAs based on size (7). lncRNAs are frequently >200 nt in length (8). Increasing research suggests that aberrant lncRNA expression may be a major contributor to tumorigenesis in numerous cancer types (9-12).

Recent finding suggested that HOTAIR functioned as a molecular scaffold to target PRC2 and LSD1 and ultimately caused epigenetic gene silencing promoting cancer metastasis (13). Aberrant HOTAIR expression significantly affects survival and prognosis of various types of cancers such as hepatocellular, breast, gastric, and pancreatic cancer (14). Upregulation of HOTAIR promotes metastasis and poor prognosis of esophageal squamous cell carcinoma (15). A recent study showed that suppressed expression of HOTAIR inhibited proliferation and tumorigenicity in renal carcinoma

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cells (16). However, the potential involvement of HOTAIR in radiosensitivity of CRC is so far unknown. In the present study, therefore, we explored the influence of HOTAIR knockdown on cell proliferation, migration, invasion, apoptosis and radiosensitivity.

Materials and methods

Clinical specimens. Fifty-three cases of fresh colorectal cancer (CRC) tissues and matched adjacent normal colorectal tissues were collected immediately after surgical resection and stored at -80°C for further analysis. This study was conducted in accordance with the Declaration of Helsinki and with approval from the Ethics Committee of Soochow University. Table I shows the clinical characteristics of these patients.

Cell cultures and irradiation. All the cell lines (FHC, CCL244, HCT116, SW480 and LOVO) were purchased from Shanghai Institute of Cell Biology (Shanghai, China) and maintained in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin G, 100 U/ml streptomycin sulfates; Gibco, Grand Island, NY, USA) at 37°C in a humidified atmosphere containing 5% CO₂. Cells were exposed to a single dose of X-ray irradiation from the linear accelerator (RadSource, Suwanee, GA, USA) at a dose rate of 1.15 Gy/min.

siRNA construction and transfection. The siRNA sequences targeting human HOTAIR (si-HOTAIR) or negative control (NC) sequence were designed and synthesized by Genepharma (Shanghai, China) as shown in Table II. Cells were transfected with siRNA by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

RNA extraction, reverse transcription and real-time RT-PCR. Total RNA from tissues and cells was extracted using TRIzol reagent (Invitrogen). The reverse transcription of mRNAs was performed using a reverse transcription kit (Thermo-Fisher, Waltham, MA, USA). Amplification of cDNA template was performed by real-time RT-PCR using the SYBR Green kit (Tiangen, Beijing, China). Real-time RT-PCR was performed by the ABI7500 system (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an endogenous standard, and HOTAIR values were normalized to GAPDH. The relative expression of HOTAIR was calculated by the 2^{-ΔΔCt} method. The primer were designed and synthesized by Genepharma and the primer sequences are listed in Table III.

Cell proliferation assays. The effect of HOTAIR knockdown on cell viability was monitored by the MTT assay. Cells were seeded in 96-well plates and cultured for 24, 48 or 72 h. Four hours before termination of culture, 20 μl of 0.5 mg/ml MTT solution was added into each well, then the MTT solution was removed and 200 μl DMSO was added. The supernatant solution was vibrated for 15 min and then placed in a microplate reader (Bio-Rad, Hercules, CA, USA) to measure the optical density (OD) value at the wavelength of 490 nm. The viability index was calculated as the experimental OD value/the control OD value.

Colony-forming assay. Cell colony formation ability of CRC cells treated with siRNA transfection was measured by plate

Table I. Clinical characteristic of the CRC patients.

Clinical parameters	Numbers
Cases	53
Age	
<65	30
≥65	23
Tumor size	
Small size (<5 cm)	25
Large size (≥5 cm)	28
Gender	
Male	35
Female	18
Invasion levels	
Mucosa	4
Submucosa	3
Muscle	10
Serosa	36
TNM stage	
Stage 1-2	34
Stage 3-4	19
Lymph node metastasis	
Positive	26
Negative	27
Grade of tumors	
Low grade	13
Intermediate grade	33
High grade	7
Vascular invasion	
Positive	22
Negative	31
Perineural invasion	
Positive	17
Negative	36

colony formation assay. Cells were seeded into 6-well plates at 300-8,000 cells/well depending on the dose of radiation. After irradiated with 0, 2, 4, 6 or 8 Gy X-ray irradiation, cells were incubated at 37°C for 10-14 days to allow for colony formation. Then cells were fixed with 4% paraformaldehyde for 15 min and stained with Giemsa for 20 min. Colony-forming efficiency (CFE) was calculated as follow: CFE = (number of colonies/number of seeded cells) x100%. Only viable colonies containing at least 50 cells were counted.

Cell cycle progression analysis. The detection of CCL244 cell cycle was assessed by flow cytometry 48 h after siRNA transfection. Cells were harvested by trypsin digestion, pelleted by centrifugation at 114 x g for 5 min, washed with ice-cold PBS, then fixed with 75% cold ethanol overnight. The staining solution containing propidium iodide and DNase-free RNase

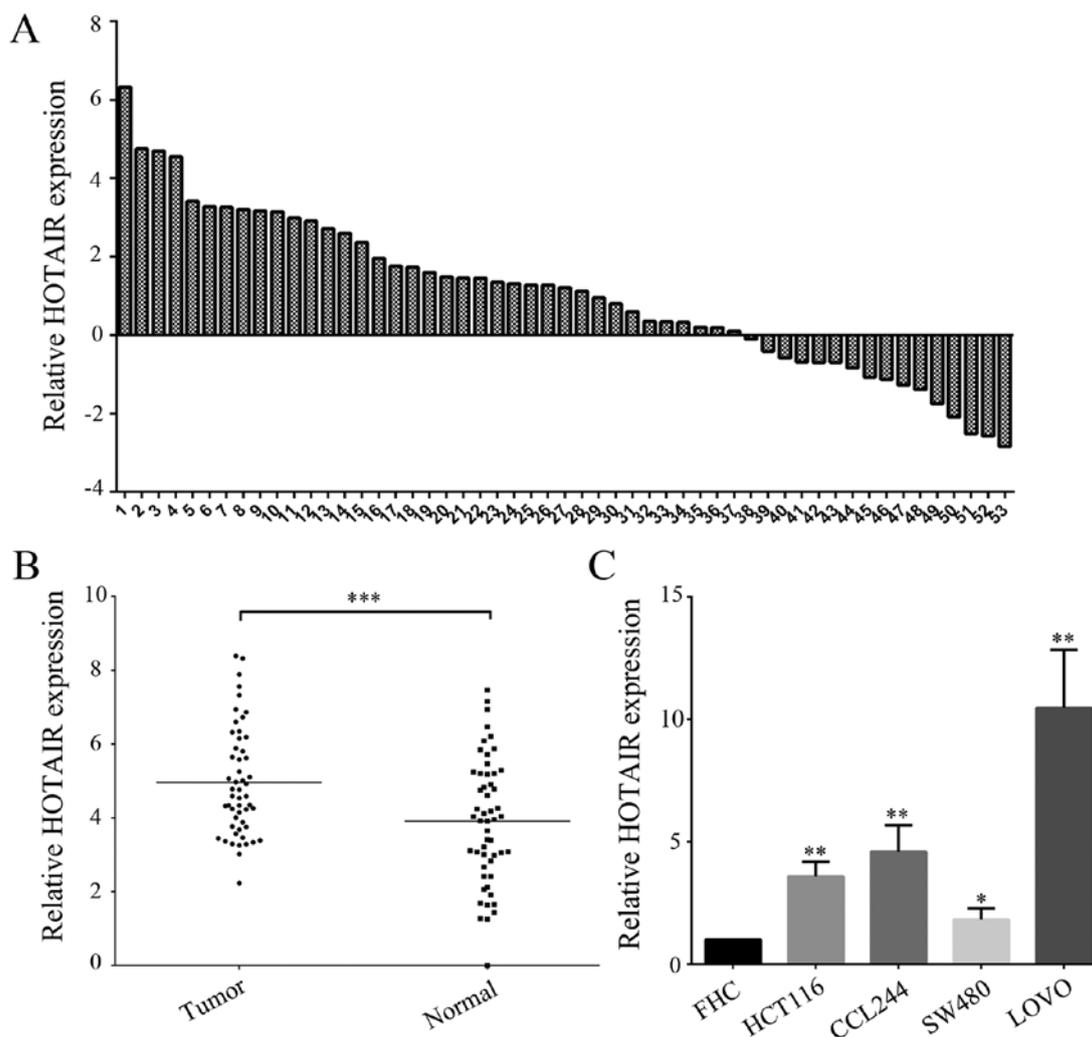


Figure 1. HOTAIR expression in CRC tissues and cells. (A) Real-time RT-PCR was used to detect the relative expression of HOTAIR in 53 pairs of human CRC cancerous and adjacent noncancerous tissues. GAPDH was used as an internal control. The $-\Delta\Delta Ct$ ($-\Delta Ct$ [Tumor (CtHOTAIR-CtGAPDH) - Normal (CtHOTAIR-CtGAPDH)]) of HOTAIR in 53 pairs of human CRC cancerous and matched noncancerous tissues. (B) The mean $-\Delta Ct$ [$-(CtHOTAIR-CtGAPDH)$] of HOTAIR in the 53 CRC tissues was compared with that in the matched noncancerous tissues. (C) Relative expression of HOTAIR ($2^{-\Delta\Delta Ct}$) in 4 CRC cell lines (HCT116, CCL244, SW480, LOVO) and in one normal colonic mucosal cell line (FHC). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with normal tissues or normal colonic mucosal cell line.

was added 30 min before detection. Flow cytometric analysis was performed to determine the fraction of the population in each phase of the cell cycle using a Coulter flow cytometry (Beckman-Coulter, Brea, CA, USA).

Wound healing assay. CRC cells transfected with siRNA were seeded into 6-well plates. Then the confluent cell monolayers were scratched using a 200- μ l pipette tip, followed by removal of the supernatant and addition of fresh culture solution. The wound healing was detected using an inverted microscope (Olympus, Tokyo, Japan). The analysis of the cell migration assay was performed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell invasion assay. Cells (5×10^4) transfected with siRNA were seeded into the upper part of a Transwell® chamber (Corning Incorporated, Corning, NY, USA), which was pre-coated with 50 μ l matrigel for 1 h, and 500 μ l DMEM medium containing 10% FBS was added into the lower part of the chamber. Cells

were cultured at 37°C with 5% CO₂ for 24 h to allow the cells to migrate through the matrigel. The remaining cells on the upper surface were removed with a cotton swab moistened with PBS. Penetrated cells on the lower surface were fixed in ice-cold methanol for 30 min followed by staining with 2% crystal violet in methanol for 15 min and counted under an inverted microscope (Olympus, Tokyo, Japan). The cell number represented migration activity.

Cell apoptosis analysis. Cells were transfected with siRNA 24 h prior to treatment with sham or 6 Gy X-ray irradiation. The apoptosis rate of CRC cells was detected by flow cytometry. Cells (5×10^5) were washed with cold PBS at 179 x g for 5 min and then resuspended in 1X Annexin V binding buffer. The solution (500 μ l) was transferred to a culture tube and Annexin V-FITC and PI, 5 μ l each, was added. The solution was mixed and then incubated for 10 min at room temperature in the dark. Flow cytometric analysis was performed after 30 min of staining.

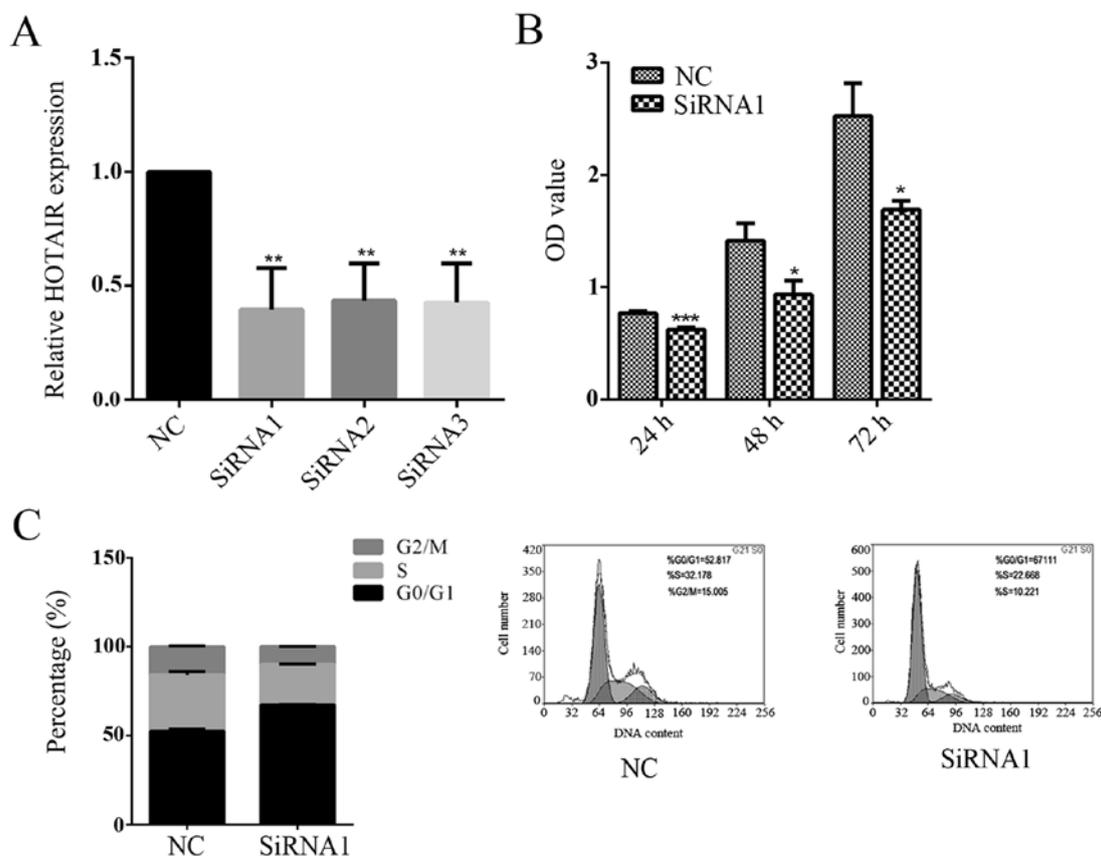


Figure 2. Effect of HOTAIR silencing on cell proliferation. (A) The silencing efficiency of three siRNA sequences targeting HOTAIR gene was confirmed by real-time RT-PCR. (B) The effect of HOTAIR silencing on cell viability of CCL244 cells was detected by MTT assay. (C) Calculated cell cycle distribution and representative charts in HOTAIR knock-down and the control cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with negative control (NC) siRNA group.

Table II. Target sequences of HOTAIR siRNAs or negative control (NC) siRNA.

siRNA	Target sequences
si-HOTAIR1	
Sense	5'-GCGCCUCCUUAUAAGUAUTT-3'
Antisense	5'-AUACUUAUAAGGAAGGCGCTT-3'
si-HOTAIR2	
Sense	5'-GCACAGAGCAACUCUAUAATT-3'
Antisense	5'-UUAUAGAGUUGCUCUGUGCTT-3'
si-HOTAIR3	
Sense	5'-GAGGCGCAAUUAUUGAUTT-3'
Antisense	5'-AUCAAUAAUAGCGCCUCTT-3'
NC	
Sense	5'-UUCUCCGAACGUGUCACGUTT-3'
Antisense	5'-ACGUGACACGUUCGGAGAATT-3'

Western blotting. Cells were transfected with siRNA 24 h prior to treatment with sham or 6 Gy X-ray irradiation. The cells were washed with PBS and then harvested in lysis buffer with 1 mM PMSF on ice. The proteins extracted from supernatants were separated on 10% polyacrylamide gels and subsequently transferred onto PVDF membranes. For

immunoblotting analysis, PVDF membranes were incubated with specific antibodies recognizing target proteins at 4°C overnight. After washing with TBST for three times, the membranes were then incubated with corresponding HRP conjugated secondary antibody (1:1000) for 1 h at room temperature. Finally the membranes were analyzed by ECL detection system and visualized by FluroChem MI imaging system (Alpha Innotech Corp., Santa Clara, CA, USA). The primary antibodies were MMP2, MMP9 (1:1000; Cell Signaling Technology, Boston, MA, USA), Bax and Bcl-2 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Protein GAPDH (1:1000; Sigma-Aldrich, St. Louis, MO, USA) was used as loading controls.

Statistical analysis. Statistical analysis was performed using SPSS 19.0. Data are presented as mean \pm SEM. The Student's t-test was used to measure the difference between two groups. The differences among three or more groups were tested for significance using one-way analysis of variance. $P < 0.05$ was considered to be statistically significant.

Results

Overexpression of HOTAIR in human CRC. The expression level of HOTAIR in 53 pairs of human CRC and matched adjacent normal tissues was detected by real-time RT-PCR. Fig. 1A and B showed the overexpression of HOTAIR in 53 CRC tissues compared to their non-tumorous counterparts.

Table III. Primer sequences for qRT-PCR analysis.

Gene	Forward sequence	Reverse sequence
HOTAIR	5'-AAACAGAGTCCGTTTCAGTGTCA-3'	5'-ATTCTTAAATTGGGCCTGGGTC-3'
GAPDH	5'-CATGAGAAGTATGACAACAGCCT-3'	5'-AGTCCTTCCACGATACCAAAGT-3'

Furthermore, the expression level of HOTAIR in 5 cell lines including one normal colonic mucosal cell line (FHC) and 4 CRC cell lines (HCT116, CCL244, SW480, LOVO) was also determined by real-time RT-PCR. Our results showed that the expression of HOTAIR in HCT116, CCL244, SW480 and LOVO CRC cells was higher than that in FHC cells (Fig. 1C). These results indicated that HOTAIR was highly expressed in CRC tumor tissues and cell lines. Our previous study has demonstrated that the ascending order of these four CRC cell lines in terms of radiosensitivity was CCL244, SW480, LOVO and HCT116 (17). Therefore, CCL244 cell line was selected to perform the following experiments.

HOTAIR silencing inhibits the proliferation of CRC cells. CCL244 cells were transfected with siRNA against HOTAIR to further explore the role of HOTAIR in proliferation. Three different siRNA vectors were designed to knockdown the coding gene of HOTAIR and the silencing efficiency of these siRNA vectors was detected by real-time RT-PCR. The results revealed that the silencing efficiency of siRNA1, siRNA2 and siRNA3 was respectively 39.6, 43.5 and 42.6% (Fig. 2A). Therefore, siRNA1 was selected to perform the following experiments. MTT assay showed that knockdown of HOTAIR caused 18.8% ($P=0.0008$), 33.9% ($P=0.0403$) and 33.1% ($P=0.0242$) reduction in the cell viability at 24, 48 and 72 h after siRNA transfection compared with negative control (NC) siRNA-transfected cells (Fig. 2B). Furthermore, cell cycle progression analysis demonstrated that the population of cells in G0/G1 phase was increased and the percentage of cells in S phase was significantly decreased in HOTAIR knock-down cells (Fig. 2C). Taken together, these results revealed that HOTAIR knock-down obviously inhibited CCL244 cell proliferation through blocking cells in G0/G1 phase.

Knockdown of HOTAIR increases the radiosensitivity of CCL244 cells. Clonogenic survival assay was performed to explore the influence of HOTAIR on radiosensitivity of CCL244 CRC cells. The dose-survival curves of CCL244 cells were obtained according to the multi-target single hit model. Results showed that HOTAIR-silencing caused a significant dose-dependent radiosensitization in CCL244 cells (Fig. 3). The mean lethal dose (D_0) for cells treated with radiation alone was 1.85 Gy, while cells treated with irradiation plus HOTAIR silencing exhibited a D_0 of 1.18 Gy. The quasi-threshold dose (D_q) in radiation alone and radiation plus HOTAIR silencing group was 1.44 Gy and 1.12 Gy, respectively. The sensitizer enhancement ratio (SER) was 1.57 for cells treated with radiation plus HOTAIR silencing, compared to cells treated with radiation alone. Therefore, these results demonstrated that downregulation of HOTAIR significantly increased the radiosensitivity of CCL244 cells.

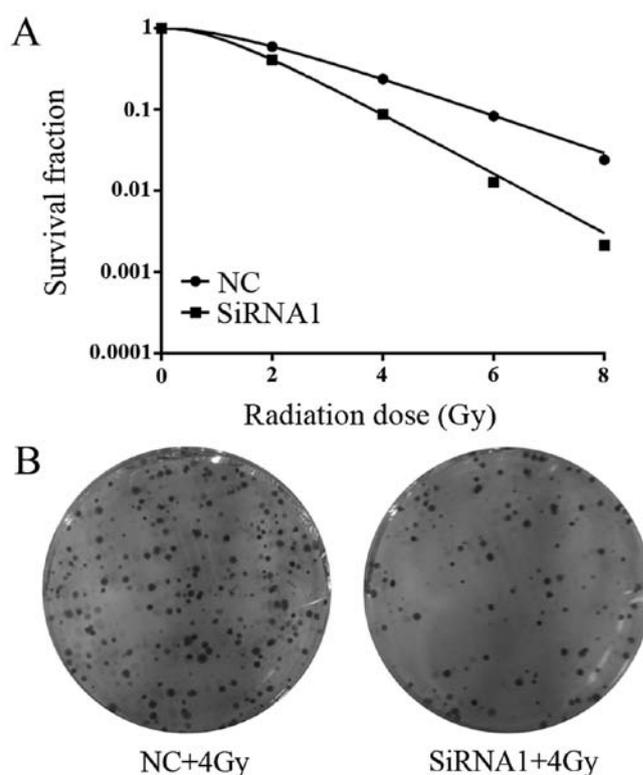


Figure 3. Clonogenic survival rate of CCL244 cells after knock-down of HOTAIR combined with X-ray irradiation. (A) Clonogenic cell survival curve of HOTAIR knockdown and negative control (NC) CCL244 cells exposed to 2, 4, 6 or 8 Gy of X-ray irradiation. (B) Representative colony images of HOTAIR knockdown and NC CCL244 cells exposed to 4 Gy X-ray irradiation.

HOTAIR silencing increases radiation-induced apoptosis in CCL244 cells and regulated the expression of apoptosis-related proteins. To further investigate the role of HOTAIR in radiation-induced apoptosis, flow cytometry was used to detect the percentage of apoptotic cells in HOTAIR silenced CCL244 cells with or without irradiation. As shown in Fig. 4A and 4B, 6 Gy X ray irradiation induced apoptosis in CCL244 cells (NC 6.63% vs. NC+IR 21.99%, $P=0.0033$). HOTAIR silencing further enhanced the apoptosis response to 6 Gy X ray (NC+IR 21.99% vs. siRNA1+IR 44.41%, $P=0.0039$). Furthermore, HOTAIR silencing CCL244 cells combined with irradiation showed downregulation of anti-apoptotic protein Bcl-2 and upregulation of pro-apoptotic protein Bax (Fig. 4C). These results demonstrated that HOTAIR knockdown significantly increased radiation-induced apoptosis in CCL244 cells.

Knockdown of HOTAIR inhibits the invasion and migration of CCL244 cells after irradiation. Metastasis and invasion are the most important prognostic factors in patients with CRC.

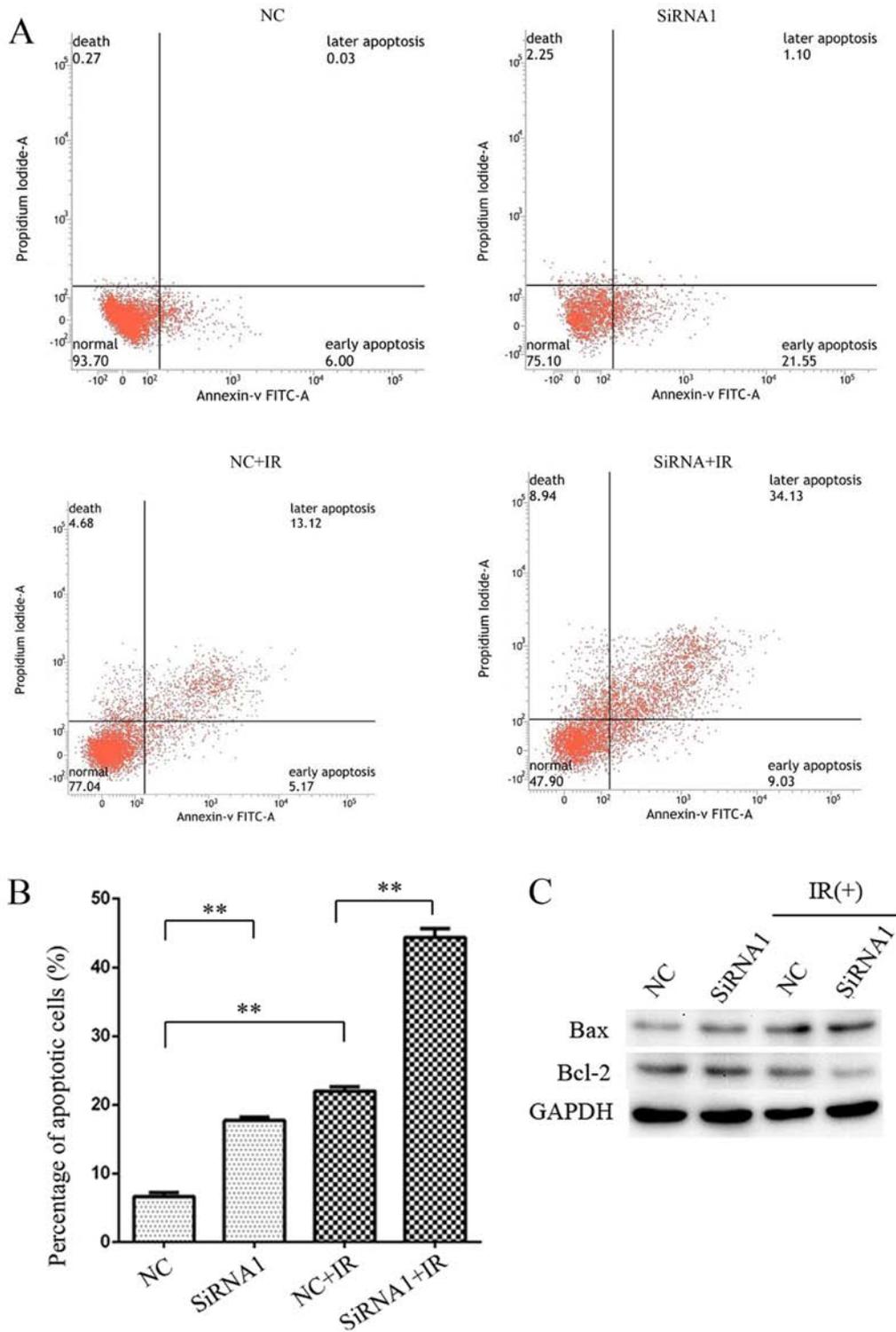


Figure 4. HOTAIR silencing enhanced the radiation-induced apoptosis. (A) Apoptosis were measured using Annexin V/PI staining in HOTAIR silencing CCL244 cells combined with irradiation (IR). (B) Percentage of apoptotic cells in HOTAIR siRNA transfected and negative control (NC) siRNA transfected cells subjected to 0 and 6-Gy X-ray irradiation. (C) Western blot analysis of apoptosis related proteins Bax and Bcl-2 in CCL244 cells. GAPDH was used as an internal control. **P<0.01, compared with negative control (NC) siRNA group or NC siRNA plus irradiation group.

Transwell matrigel invasion and wound healing assays were performed to further explore the participation of HOTAIR in cell metastasis. As shown in Fig. 5A, knockdown of HOTAIR inhibited the invasiveness of CCL244 cells compared with negative control (NC) CCL244 cells (P=0.0005). HOTAIR silencing CCL244 cells combined with 6 Gy X-ray irradiation

showed obvious inhibition in invasiveness compared with cells treated with 6 Gy X-ray radiation alone (P=0.0008). As shown in Fig. 5B, knockdown of HOTAIR inhibited the migration of CCL244 cells compared with (NC) CCL244 cells (P=0.0029). HOTAIR silencing CCL244 cells combined with 6 Gy X-ray irradiation showed obvious inhibition in

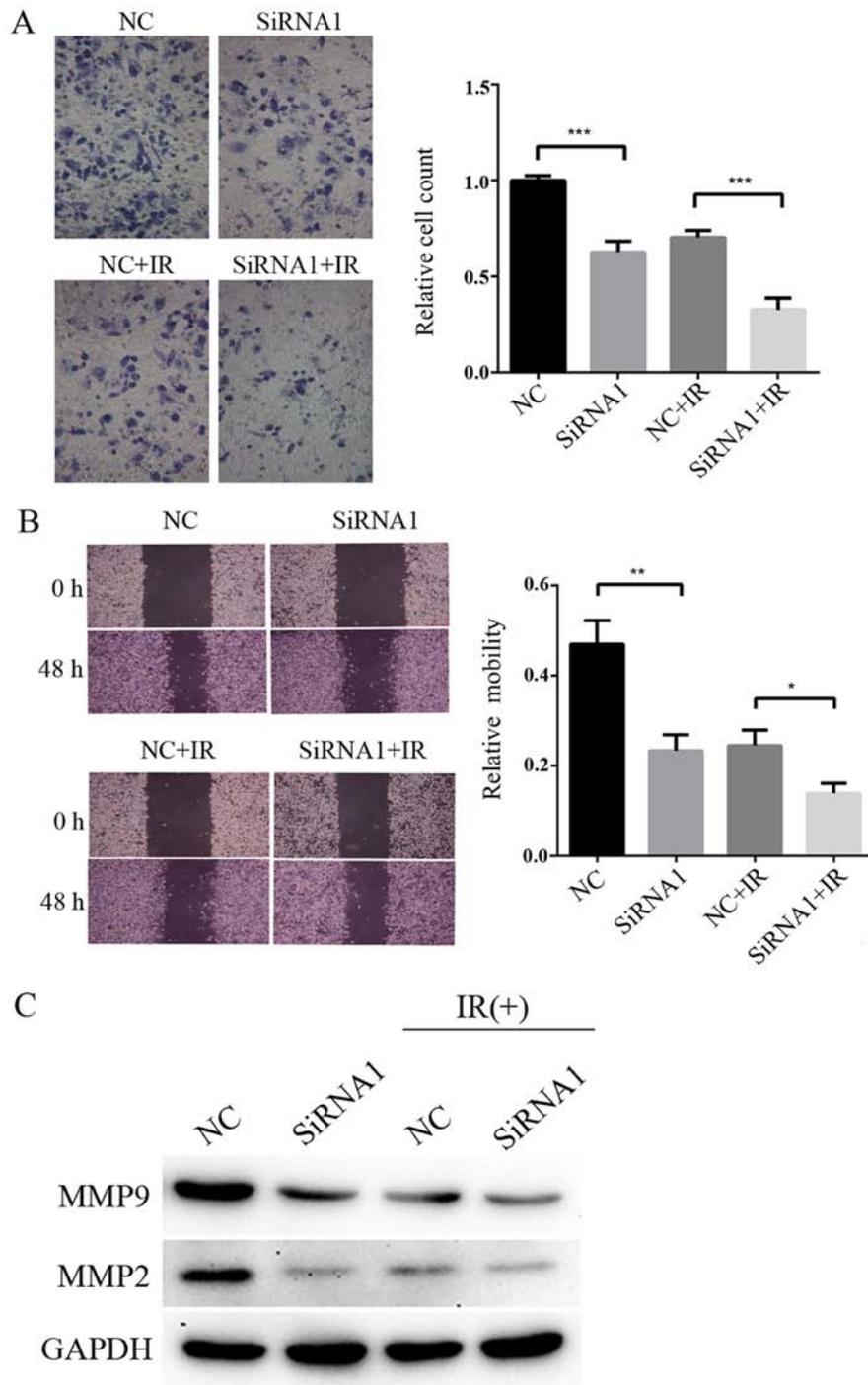


Figure 5. Effect of HOTAIR silencing on cell invasion and migration. (A) Transwell matrigel invasion assays of CCL244 cells after transfected with siRNA1 prior to 6 Gy X-ray irradiation (IR). (B) Wound healing assays of CCL244 cells after HOTAIR silencing combined with 6 Gy X-ray irradiation. (C) The expression level of MMP2 and MMP9 in HOTAIR silencing CCL244 cells combined with 6 Gy X-ray irradiation was detected by western blotting. GAPDH was used as an internal control. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared with negative control (NC) siRNA group or negative control (NC) siRNA plus irradiation group.

migration compared with cells treated with 6 Gy X-ray radiation alone ($P = 0.0108$). As a family of extracellular matrix degrading enzymes, matrix metalloproteinases (MMPs) are expressed in various stages of CRC and participate in tumor invasion and metastasis (18). The expression level of some MMPs was also detected by western blotting. As shown in Fig. 5C, HOTAIR silencing CCL244 cells combined with irradiation showed downregulation of MMP2 and MMP9. Therefore, these results indicated that HOTAIR silencing

significantly inhibited the invasion and migration of CCL244 cells after irradiation.

Discussion

Long non-coding RNAs (lncRNAs) are emerging as key regulators of diverse cellular processes (19,20). As the first found lncRNA, HOTAIR is co-expressed with the HOXC gene cluster to regulate various genes through reverse

transcription (21,22). Numerous evidence has shown that dysregulation of HOTAIR was closely associated with tumor metastasis and poor prognosis (23-26). However, the function role of HOTAIR in CRC proliferation, invasion, radiation-induced apoptosis and radiosensitivity is still being explored.

It is reported that HOTAIR is highly expressed in various types of cancers such as primary breast tumors, hepatocellular carcinoma, and gastrointestinal stromal tumors (27-29). The present study examined the expression level of HOTAIR in CRC tissues and matched normal tissues. Results showed that the HOTAIR level was significantly upregulated in CRC cancer tissues compared with adjacent normal tissues. Furthermore, CRC cell lines showed high expression level of HOTAIR compared with normal colonic mucosal cell line. The above evidence reveals the important role of HOTAIR in CRC tumorigenesis.

To further explore the role of HOTAIR in CRC, we investigated the effects of HOTAIR knockdown on CRC cell proliferation. MTT assay revealed that RNAi-mediated suppression of HOTAIR led to a significant inhibition in CCL244 cell viability. Furthermore, HOTAIR silencing results in a significant inhibition in the proliferation through blocking cells in G0/G1 phase. Therefore, HOTAIR knockdown can inhibit the proliferation in CRC and HOTAIR represents a new promising target and a prognosis marker for CRC therapy.

It was demonstrated that HOTAIR is a negative prognostic factor and HOTAIR silencing results in inhibition of cell invasion in pancreatic tumors (30). Xu *et al* found that HOTAIR promoted epithelial-mesenchymal transition (EMT) through regulating snail expression thus contributing to gastric cancer metastasis (31). Furthermore, HOTAIR may promote tumor aggressiveness through the upregulation of VEGF and MMP-9 and EMT-related genes (32). Additionally, it is reported that HOTAIR promotes tumor cell invasion and metastasis by recruiting EZH2 and repressing E-cadherin in squamous cell carcinoma (33). We performed Transwell invasion and wound healing assays to further explore the influence of HOTAIR knockdown and irradiation treatment on cell migration and invasion. Results showed that irradiation treatment caused an obvious inhibition in cell invasion and HOTAIR silencing further enhanced the inhibition response to irradiation. Furthermore, western blotting was performed to detect the metastasis-related proteins of MMPs to support the role of HOTAIR in CRC metastasis mechanically. Similarly, the present study demonstrated that HOTAIR silencing plus irradiation treatment significantly suppressed the expression of MMP2 and MMP9, indicating the promoting effect of HOTAIR in cancer metastasis. This phenomenon indicates that HOTAIR may function as an oncogene in CRC tumorigenesis.

Apoptotic cell death is involved in almost every mode of cell death. Radiation induced cell death is closely associated with apoptosis. Dysregulation of apoptotic genes such as caspase-3, Bax, and Bcl-2 may play a key role in radiation induced cell death (34). HOTAIR has been found to promote the proliferation of serous ovarian cancer cells through the regulation of cell cycle arrest and apoptosis (35). Recently, it was reported that calyosin and genistein induce apoptosis by inactivation of HOTAIR/p-Akt signaling pathway in human breast cancer MCF-7 cells (36). Consistent with these studies, we also

observed a significant increase in the apoptotic cell population following HOTAIR silencing plus irradiation treatment. Furthermore, HOTAIR silenced CRC cells plus irradiation treatment showed a significant increase in pro-apoptotic protein Bax levels and decrease in anti-apoptotic protein Bcl-2 levels. In conclusion, irradiation treatment induced apoptosis in CRC CCL244 cells and HOTAIR silencing further enhanced the apoptotic response to X ray irradiation.

Few studies have addressed the role of HOTAIR in cancer cell radiosensitivity. Wang *et al* found that Curcumin enhances the radiosensitivity in nasopharyngeal carcinoma cells involving the reversal of differentially expressed long non-coding RNAs (37). Several experiments were performed to further evaluate the role of HOTAIR in CCL244 CRC cell radiosensitivity in our study. Flow cytometry showed that knockdown of HOTAIR significantly increased the radiation-induced apoptosis in CCL244 cells. Furthermore, the SER of HOTAIR knockdown was 1.57 in the clone formation assay. Therefore, our finding indicates that HOTAIR knockdown sensitized CCL244 cells to irradiation. However, the underlying mechanism needs further exploration.

In summary, the present study showed that HOTAIR was overexpressed in CRC and HOTAIR silencing inhibited the proliferation and metastasis and enhanced the radiosensitivity in CCL244 cells. These results indicate that targeting HOTAIR may serve as a potentially novel approach for CRC diagnosis and therapy.

Acknowledgements

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