Original Article
Overexpression of long non-coding RNA HOTAIR enhances breast cancer radioresistance via RhoC-Akt pathway by targeting HOXD10

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Abstract: Background: Radioresistance remains a therapeutic challenge in progress of breast cancer radiotherapy-the main regimen for inoperable and locally advanced breast cancer. Long non-coding RNA HOTAIR (HOX transcript antisense RNA) recently emerged as a carcinogenic promoter and therapeutic targets by inhibiting HOTAIR expression in various cancers including breast cancer. This study is focused to investigate the radioresistance effects of long non-coding RNA HOTAIR on breast cancer and explore the possible underlying mechanisms. Methods: plasmid pLVX-CMV-PGK-puro was transfected into cell line SKBR3 using liposome, thus overexpressing HOTAIR gene in the cells. Furthermore, radiosensivity of each cell lines were assessed following overexpressing HOTAIR gene by cell proliferation/viability tests by means of CCK8, transwell, flow cytometry and colony formation assay in vitro, and tumor formation assay in a nude mouse model treated with a single fraction of 8 Gy irradiation in vivo. Following, HOXD10, p-AKT and p-BAD of the radiated nude mouse were detected by Western blotting. Results: In vivo, mice bearing null vector SKBR3 (Vecotr-SKBR3) tumor and stable over-expressing HOTAIR-transfected SKBR3 (HOTAIR-SKBR3) tumor indicated distinct difference in tumor volume and doubling time in radiation-treated groups (*P*<0.05). Furthermore, the overexpressed HOTAIR inhibited response of SKBR3 xenografts to irradiation with enhancement factor of 0.31 calculated by dividing the normalized tumor growth delay. In vitro, HOTAIR also efficiently enhanced the radioresistance of breast cancer cells, thus increasing cell proliferation via protein HOXD10 and PI3K/Akt pathway. Conclusions: These findings demonstrated that HOTAIR might serve as a valid therapeutic target for the reversal of resistance to radiotherapy in breast cancer.

Keywords: Breast cancer, radioresistance, long non-coding RNA, homemobox transcript antisense RNA, homemobox D10

Introduction

Crucial advances in researching the pathological characterization molecular mechanisms of breast cancer's occurrence and development have allowed breast cancer having more favorable diagnostic and therapeutic prospect, however, over 20% in morbidity and 14% in mortality of breast cancer patients still increasing since 2008 [1]. So far, adjuvant chemotherapy and radiotherapy followed by surgery still remains the top option to prevent and control local recurrence of breast cancer. Promoting the efficacy of recurrence-targeted therapy and improving the overall prognosis in breast cancer patients have evoked oncologists to reveal

further the molecular mechanisms underlying the modulation of local recurrence, although largely unclear at present.

Accumulating evidences showed the potential role of long non-coding RNAs (lncRNAs) as biomarkers and therapeutic targets in solid tumors. The overexpression of homemobox (HOX) transcript antisense RNA (HOTAIR), a novel lncRNAs, in breast adenocarcinoma cells promotes cell proliferation, migration and invasion, with apoptosis significantly inhibited in vitro [2]. Also, two types of ncRNAs, HOTAIR and miRNA-10b (miR-10b), can reportedly suppress the translation of the HOXD10 gene which is a mRNA encoding a transcriptional repressor that

downregulates the expression of cell migration/invasion-associated genes [3], and accelerate cell proliferation via RhoC-Akt pathway by targeting HOXD10 [4].

However, there is no literature available regarding the influence of lncRNA HOTAIR expression and its potential regulatory mechanisms in progress of human breast cancer radiotherapy. Herein, here we focused on the impact of the upregulation of HOTAIR on the radiotherapy sensitivity as well as the underlying mechanisms in human breast cancer cells, and attempt to verify whether HOTAIR can serve as a predictor of radiotherapy resistance in patients with breast cancer.

Materials and methods

Cell lines

Human breast cancer cell lines T47D, MCF-7, SKBR3, BT549 and MDA-MB231 were recruited, with MCF-10A, a cell line of the normal breast epithelium, as normal control. T47D and MCF-7 cells were seeded in RPMI-1640, and the remaining cell lines including normal control MCF-10A were cultured in DMEM, supplemented with 10% fetal bovine serum. All the cells were incubated at 37°C in a humidified environment containing 5% CO_2.

Quantitative real-time reverse transcriptase PCR

Total RNA from fresh tissues was isolated with TRNzol Reagent (TIANGEN Biotech Co., Ltd. Beijing China) according to the manufacturer's instructions with minor modifications. The qualitative and quantitative analyses of RNA were conducted on a NanoDrop® ND-1000 spectrophotometer, followed by reverse transcription of 1 μg total RNA from each sample to synthesize cDNA using the RT reagent Kit (TIANGEN Biotech Co., Ltd. Beijing China) according to the manufacturer's instructions. Real-time PCR was performed to determine the relative expression levels of target genes using the SYBR Green RT-qPCR Kit on the StepOnePlus™ *Real-Time PCR System*. The levels of HOTAIR expression in each sample was normalized to the respective β-actin expression level. The RNA sequences targeting human HOTAIR were customized in Sangon Biotech (Shanghai) Co., Ltd., China, with the primers [5] as follows: HOTAIR sense 5'-ATA GGC AAA TGT CAG AGG

GTT-3' and antisense, 5'-ATT CTT AAA TTG GGC TGG GTC-3'; β-actin sense, 5'-AAA GAC CTG TAC GCC AAC AC-3' and antisense, 5'-GTC ATA CTC CTG CTT GCT GAT-3'. The amplification profile was pre-denatured at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 30 s, respectively. The accuracy of the PCR amplifications was verified by melting curve analyses. The comparative threshold cycle (Ct) method (ΔΔCt) was employed for the quantification of HOTAIR gene expression. The relative expression quantity of HOTAIR to β-actin was calculated using the equation $2^{-\Delta\Delta Ct}$, where ΔCt = Ct (HOTAIR)-Ct (β-actin). The gene expression levels of HOTAIR in tumor tissues were contrasted with normal control. To minimize procedural bias, each sample was analyzed in triplicates, with the mean expression level calculated.

Plasmids and transfection

Full-length human HOTAIR DNA was cloned into a pLVX-CMV-PGK-puro vector (Biowit Technologies Co. Ltd., Shenzhen, China). The DNA and plasmids were transfected as specified above into SKBR3 cells with lipofectamine 2000 (Invitrogen, CA, USA). 48 hours after transfection, cells were subjected to puromycin treatment. At the end of 10-day screening, stable HOTAIR-transfected SKBR3 cell line (HOTAIR-SKBR3) was developed. The tumor cells which did not undergo transfection were designated as the blank control, and those transfected with pLVX-CMV-PGK-puro (null vector, herein Vector-SKBR3) as the negative control.

Clonogenic survival assay

Cells were cultured in 6-well plates for 24 h, followed by X-ray radiation at 2, 4, 6, 8 and 10 Gy (Truebeam, Varian Medical Systems, Inc. USA), cultivation at 37°C for 12 consecutive days, fixation with methanol and staining with Giemsa stain. The counts of colonies containing at least 50 cells were calculated by microscopy.

Cell viability assay

CCK-8 assay (Beyotime, China) was to evaluate the proliferation of breast cancer cells. Then 96-well plates were seeded with cells at a density of 1×10^5 cells/well. After 24 h, cells were treated with radiation therapy, at indicated concentrations for 22 h, 46 h, and 70 h respec-

tively, and then incubated with fresh medium containing 10% CCK8 for another 2 h. Viable cells were detected by measuring absorbance at 450 nm by a microplate reader. Cell viability (%) = (A (Radiotherapy)-A (blank))/(A (Non-radiotherapy)-A (blank)) ×100%. A (Radiotherapy): substance of a well with cells, CCK-8 solution and radiotherapy. A (blank): absorbance of a well with medium and CCK-8 solution, without cells. A (Non-radiotherapy): absorbance of a well with cells and CCK-8 solution, without radiotherapy.

In vitro cell transwell assays

For the migration assays, $2×10^4$ cells in serum-free media were placed into the upper chamber of an insert (8-μm pore size, BD). For the invasion assays, $1×10^5$ cells in serum-free media were placed into the upper chamber of an insert coated with Matrigel (BD, USA). Media containing 10% FBS were added to the lower chamber. After 24 h, cells were treated with radiation therapy, and then 24 hours of incubation, removing the cells remaining on the upper membrane with cotton wool, whereas the cells that had migrated or invaded through the membrane were stained with 0.1% crystal violet in methanol, imaged, and counted using an inverted microscope.

Flow cytometry

Cells were plated into 6-well plates at a specified density ($1×10^5$) and incubated as specified above. At the end of 24-hour incubation, cells underwent X-ray radiation (8 Gy) and subsequent 24-hour culture. With the cells harvested, flow cytometry was conducted using AnnexinV-FITC Apoptosis Detection kit (KeyGEN BioTECH Co. Ltd., Nanjing, China).

In vivo tumorigenesis

All animal experiments were evaluated and approved by the Ethics Committee of Xuzhou Central Hospital. Female BALB/C nude mouse (16-22 g, 4-5 weeks old) were provided by Institute of Zoology Chinese Academy of Sciences (Beijing) and kept under specific pathogen-free (SPF) conditions. After the DNA and plasmid transfected, null vector SKBR3 cell line (SKBR3) cells and stable HOTAIR-transfected SKBR3 cell line (HOTAIR-SKBR3) cells were collected and washed with serum-free DMEM. Two kinds of cells were suspended in PBS and injected into the right axilla of nude mice (1-2×10^6 cells/site) subcutaneously. When tumor volume increased to 100-300 mm³, animals inoculated by above 2 kinds line cells were randomly grouped into two different groups depending on whether receiving irradiation (n = 6): Vector-SKBR3 (Blank control), Vector-SKBR3 plus 8 Gy irradiation (negative control), and HOTAIR-SKBR3, HOTAIR-SKBR3 plus 8 Gy irradiation. Tumors were irradiated at a dose of 8 Gy with 6-MV X-rays. Tumor growth was measured every 2 days and the tumor volume was calculated according to the formula: tumor volume = (length (L)* width (W)²)/2. The tumor doubling time (DT) was calculated as follows: DT = d*lg2/ln(Vd/V0), where d was the length of time between two measurements, Vd was the volume of the tumor treated with X-ray, and V0 was the volume of the tumor before the X-ray. The mice were sacrificed till day 24 and during the period, no mouse died. Mouse was sacrificed by cervical dislocation, and tumors were excised for weighing.

Western blot analysis

Cells were treated with PI3K inhibitor (LY294002) 10 μmol/L for six hours [6, 7]. Cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors (KeyGEN BioTECH). Total protein was quantified by BCA kit (Beyotime Institute of Biotechnology, Shanghai, China). Equal amounts of proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore, USA). The membranes were blocked with 5% skim milk, incubated with primary antibodies against HOXD10 (Abcam, USA), p-AKT and p-BAD (Keygen, China) at 4°C overnight, and incubated with HRP-conjugated secondary antibodies (BioWorld, USA) for 1 h at room temperature. Immunoblotted proteins were visualized by ECL reagents and the signals were detected by ChemiDoc XRS imaging system (Quantity One Quantitation software, BioRad Laboratories, Hercules, CA, USA).

Statistical analysis

Continuous data were analyzed by independent tests or paired T tests between the groups, whereas categorical data were analyzed by the χ^2 test. All values are shown as mean ± SD. Data were analyzed by T test or one-way analysis of variance (ANOVA). All statistical analyses were performed using SPSS for Windows ver.
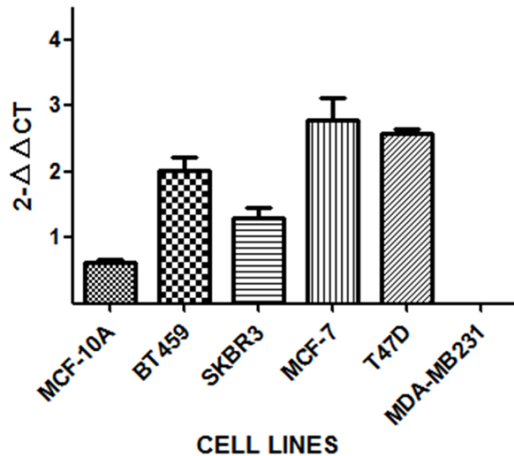
Figure 1. HOTAIR expression in five tumor cell lines (BT549, SKBR3, MCF-7, T47D and MDA-MB231) and one normal breast cell line (MCF-10A), by real-time quantitative PCR (RT-qPCR). We designated the SKBR3 cells, which had moderate HOTAIR expression.
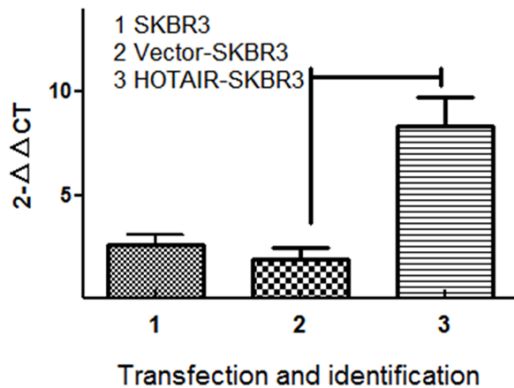

Figure 2. SKBR3 cells transfected by pLVX-CMV-PGK-puro (Vector-SKBR3) and pLVX-CMV-HOTAIR-PGK-puro (HOTAIR-SKBR3). Vector-SKBR3 cells had non-significant variation of expression, while HOTAIR-SKBR3 cells were significantly upregulated, versus blank SKBR3 cells (**$P<0.01$).

16.0 (Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA). $P<0.05$ was considered statistically significant.

Result

In order to test whether HOTAIR also has a expression characters in breast cancer cells, we investigated HOTAIR expression in five tumoral (BT549, SKBR3, MCF-7, T47D, MDA-MB231) and one non-tumoral (MCF-10A) breast cells by using quantitative real-time PCR. We observed that only the HOTAIR expression of
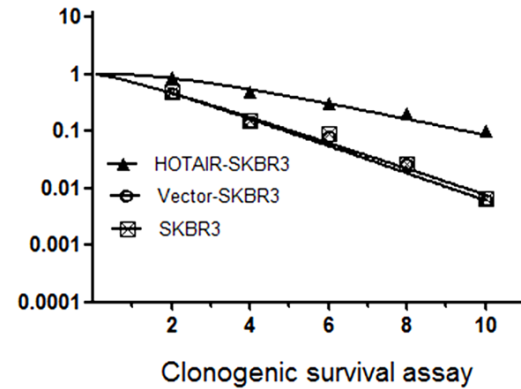
Figure 3. Clonogenic survival assays of SKBR3, Vector-SKBR3 and HOTAIR-SKBR3 cells exposed to radiation at 0-10 Gy. Clonogenic assay showing that cells significantly was a dose-dependent reduction.

Table 1. The Influence of over-expressed HOTAIR on clonogenic survival of SKBR3 cells

Group	D0	Dq	SF2	SERD0
SKBR3	1.90	0.89	0.47	
Vector-SKBR3	1.81	0.98	0.54	
HOTAIR-SKBR3	2.95	4.69	1.59	0.34

HOTAIR: homemobox transcript antisense RNA.

MDA-MB231 was lower than that in MCF-10A cell (**Figure 1**). Finally, the moderate-expressing SKBR3 was selected to continue the experiment in vitro. We labeled control and HOTAIR-overexpressing cells with puromycin resistance. Lipofetmiane 2000 transduction allowed stable over-expression of HOTAIR to several fold over vector-transduced cells. SKBR3 cells transfected by pLVX-CMV-PGK-puro (Vector-SKBR3) and pLVX-CMV-HOTAIR-PGK-puro (HOTAIR-SKBR3). Blank control SKBR3 cells had non-significant variation of expression, while HOTAIR-SKBR3 cells were significantly upregulated, versus negative control Vector-SKBR3 cells (**$P<0.01$) (**Figure 2**). We wondered whether HOTAIR could induce radioresistence of SKBR3. Clonogenic assay showing that cells significantly was a dose-dependent reduction. By the Clonogenic survival assay test, over-expressed HOTAIR accelerated clonogenic survival of SKBR3 cells (SER = 0.34) (**Figure 3**; **Table 1**). The SF data were fitted into the single hit multi target model formula: $SF = 1-(1-e^{-D/D0})^n$. In next experiment, cell viability was determined using a CCK8 assay. We found that HOTAIR enhanced the proliferation of cells and inhibited the damage of radiontherapic

Table 2. Influence of overexpressed HOTAIR on response of SKBR3 xenografts to irradiation

Group	Doubling time (days)	Absolute growth delay (days)	Normalized growth delay (days)	Enhancement factor
Vector-SKBR3	5.28±0.27			
HOTAIR-SKBR3	5.10±0.30	0.18		
Vector-SKBR3+Radiotherapy	7.25±0.89	1.97		
HOTAIR-SKBR3+Radiotherapy	6.08±0.38	0.8	0.62	0.31

HOTAIR: homemobox transcript antisense RNA.
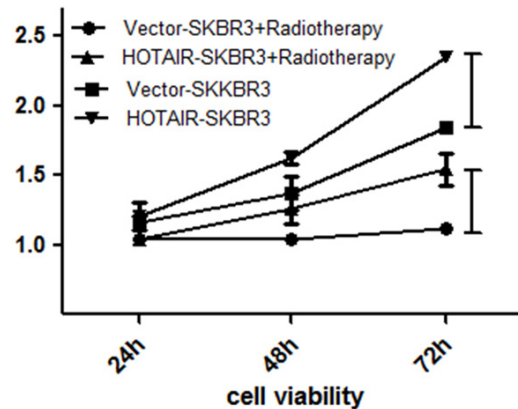
Figure 4. CCK8 assays show vector-SKBR3 and HOTAIR-SKBR3 cells had significantly varying cell viabilities. HOTAIR enhanced the proliferation of cells and inhibited the damage of radiontherapic cells (*P<0.05).

cells (*P<0.05) (**Figure 4**). Furthermore, HOTAIR overexpression enhanced the migratory and invasive abilities of the SKBR3 cells as determined by transwell assay (**Figure 5**). To further ascertain the overexpression of HOTAIR in SKBR3 (HOTAIR-SKBR3), we verified by flow cytometry that HOTAIR-induced radioresistance was contributive to the decreased apoptosis (**Figure 6**).

Finally, to determine the potential radioresistance effect of HOTAIR on breast cancer in vivo, mice bearing null vector SKBR3 (Vecotr-SKBR3) tumor and stable over-expressing HOTAIR-transfected SKBR3 (HOTAIR-SKBR3) tumor were treated with a single fraction of 8 Gy irradiation. Compared to control group, distinct difference in tumor volume was observed in radiation-treated groups (*P<0.05) (**Figure 7**). Furthermore, we analyzed the doubling time required for the tumor to grow twice in size under different HOTAIR expressions. Doubling time for Vecotr-SKBR3 tumor in control group and HOTAIR-SKBR3 alone group was 5.28± 0.27 days and 5.10±0.30 days, respectively.

For irradiation treated groups, the doubling time of Vecotr-SKBR3 extended to 7.25±0.89 days, compared to 6.08±0.38 days in HOTAIR-SKBR3 group. Overexpressed HOTAIR inhibited response of SKBR3 xenografts to irradiation with enhancement factor of 0.31 calculated by dividing the normalized tumor growth delay (**Table 2**). Next Western blot analysis was performed to detect HOXD10 expression, as reflected the effects of HOTAIR of SKBR3 xenograft to irradiation treatment, and showed that HOTAIR leads to the suppression of HOXD10 activation (**Figure 8**).

Discussion

Compared with normal tissues, the expression of lncRNAs in tumors vary remarkably, which might enable these RNAs to be promising biomarkers or more preferably, therapeutic targets [8]. HOTAIR is expressed from the HOXC locus to silence the more distal HOXD locus [9], and involved in the development of primary breast cancer and promotes the invasion and metastasis [10]. The interactions of HOTAIR with the polycomb repressive complex 2 (PRC2) and lysine-specific demethylase 1 (LSD1) decrease the expression of multiple genes, especially metastasis-suppressing genes [9, 10]. HOTAIR is also reportedly overexpressed in a variety of tumors and shown to induce the proliferation and metastasis of the tumor cells [11-16]. Clinically, the overexpression of HOTAIR is a potential predictor of overall survival and progression in several cancers, including colorectal cancers [11], gastrointestinal stromal tumors [12], pancreatic cancer [13], esophageal cancer [14], hepatocellular carcinoma [15] and nasopharyngeal carcinoma [16].

In vitro, we demonstrated that HOTAIR decreased the radiosensitivity of breast cancer cells by colony formation. Next, we detected that HOTAIR-induced radioresistance was related to decreased apoptosis and increased Cell

A

1: Vector-SKBR3
2: Vector-SKBR3+Radiotherapy
3: HOTAIR-SKBR3
4: HOTAIR-SKBR3+Radiotherapy

B
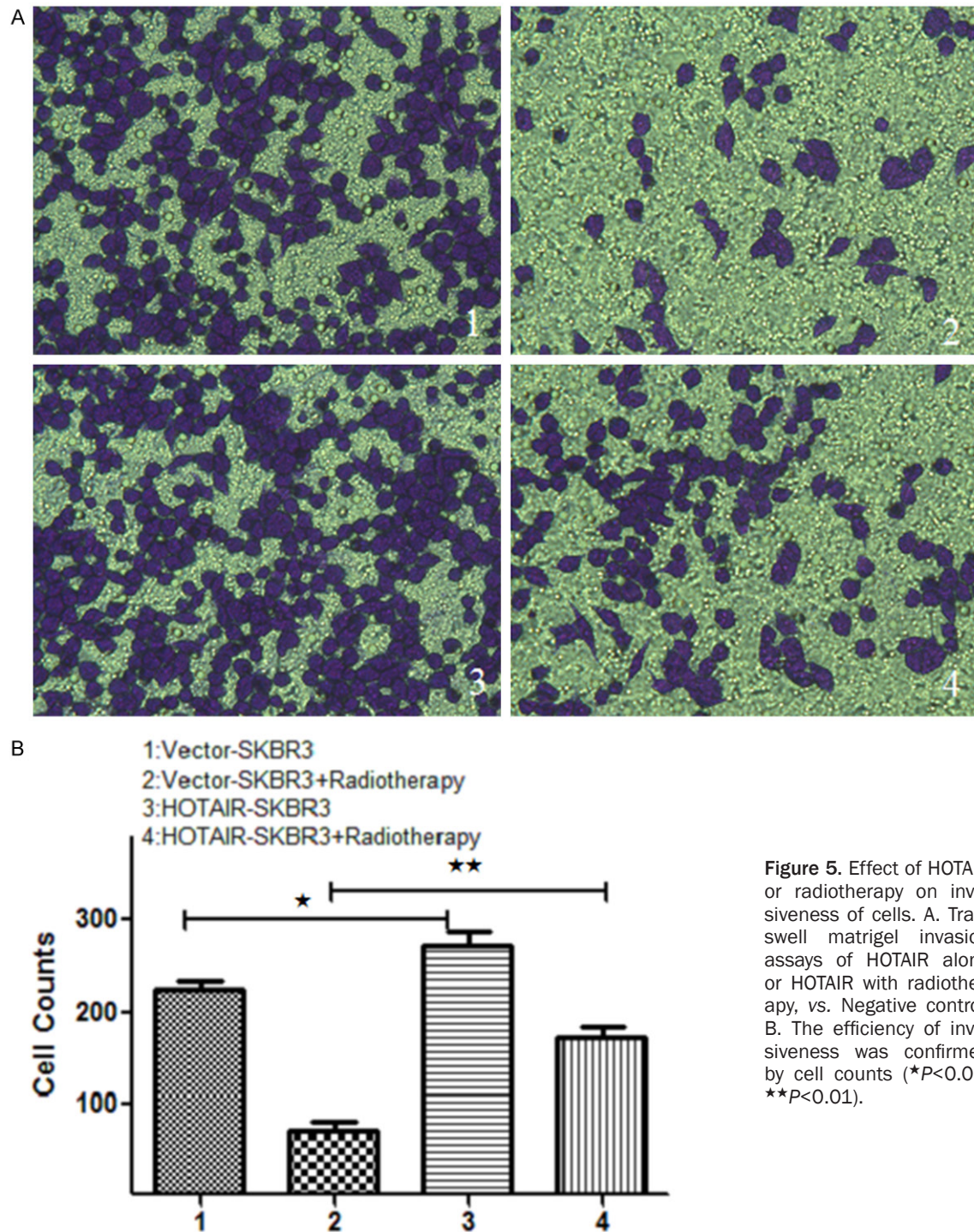
Figure 5. Effect of HOTAIR or radiotherapy on invasiveness of cells. A. Transwell matrigel invasion assays of HOTAIR alone or HOTAIR with radiotherapy, *vs.* Negative control. B. The efficiency of invasiveness was confirmed by cell counts (*$P<0.05$, **$P<0.01$).
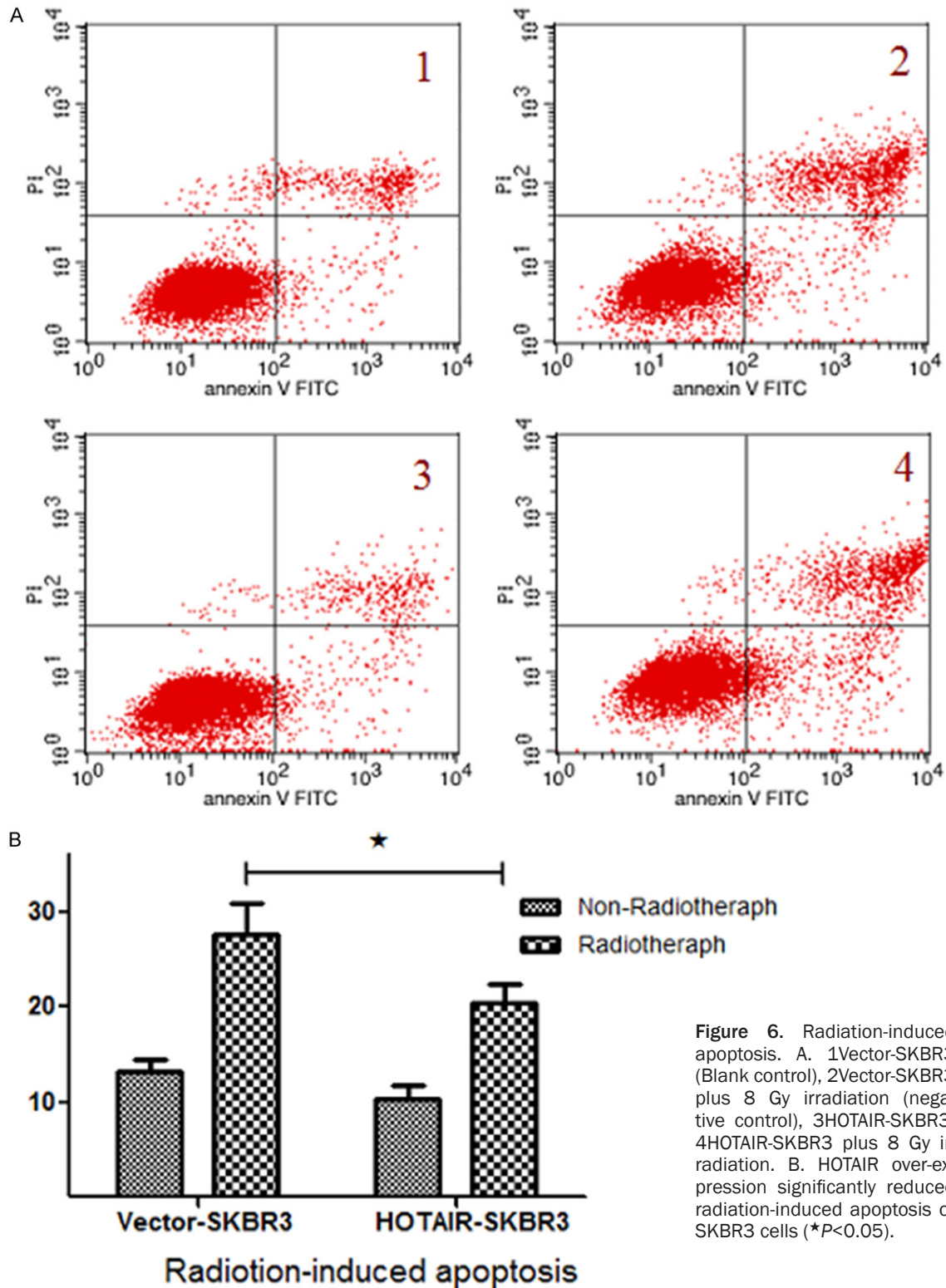
invasion, in which the HOTAIR over-expression in HOTAIR-SKBR3 cells had statistical differences versus the negative control group (Vector-SKBR3).

In our present study, 24 mice were sacrificed, in which the HOTAIR RNA expressions were up-regulated in human breast adenocarcinoma tis-

sues. Our experiment confirmed the association between HOTAIR expression and tumor growth in vivo. Our findings strengthened the clinical feasibility of HOTAIR as a biomarker in the diagnosis and prognosis of breast cancers.

The translation of HOXD10 gene can be reportedly suppressed by two types of ncRNAs,

Figure 6. Radiation-induced apoptosis. A. 1Vector-SKBR3 (Blank control), 2Vector-SKBR3 plus 8 Gy irradiation (negative control), 3HOTAIR-SKBR3, 4HOTAIR-SKBR3 plus 8 Gy irradiation. B. HOTAIR over-expression significantly reduced radiation-induced apoptosis of SKBR3 cells (*$P<0.05$).

homemobox (HOX) transcript antisense RNA (HOTAIR) and miRNA-10b (miR-10b) [3], the latter promoting cell invasion via RhoC-AKT signaling pathway by targeting HOXD10 in gastric cancer [4]. In this study, we investigated the effects of HOTAIR and/or irradiation on the activation of HOXD10 and PI3K/AKT-Bad expression by western blotting. First, we tested the association of HOTAIR overexpression and irradiation-induced HOXD10 expression. Radiothe-
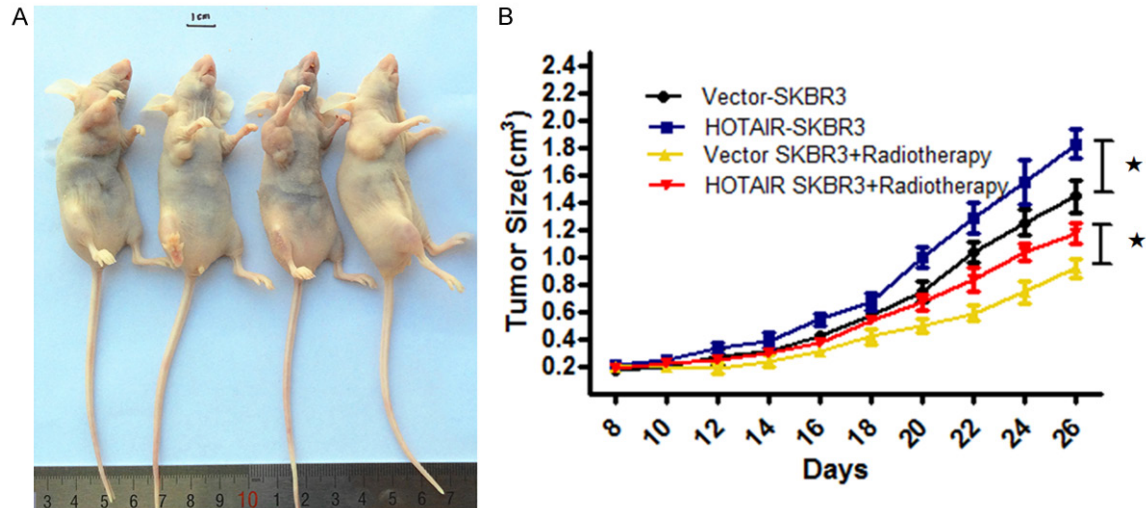
Figure 7. HOTAIR geng resisted SKBR3 to IR in Vivo (A and B). Female BALB/c mice bearing null vector SKBR3 (Vector-skbr3) and over-expressing HOTAIR SKBR3 (HOTAIR-SKBR3) xenograft were divided into four treatment groups (n = 6): (1) Vector-SKBR3, (2) Vector-SKBR3 plus 8 Gy irradiation, (3) HOTAIR-SKBR3, (4) HOTAIR-SKBR3 plus 8 Gy irradiation. 10 days after inoculation of cells (1×10^6 cells/mice), the mice were treated with an 8 Gy-single fraction irradiation. (A) Representative images of mice bearing SKBR3 xenograft. (B) Measurement of tumor size. Data represent average tumor volume; error bars, SD (**Table 2**) (*P<0.05).
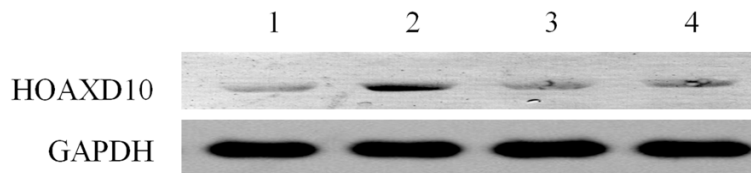
Figure 8. Tumor tissues of every group xenograft treated by Western Blot ECL. Treatment groups (n = 6): (1) Vector-SKBR3, (2) Vector-SKBR3 plus 8 Gy irradiation, (3) HOTAIR-SKBR3, (4) HOTAIR-SKBR3 plus 8 Gy irradiation. Western blot analysis showed that HOTAIR leads to the suppression of HOXD10 activation.

HOTAIR significantly inhibited the irradiation-induced HOXD10 activation, downexpressed p-AKT and upexpressed p-BAD. Finally, over-expressed HOTAIR-SKBR3 cells exhibited diminished apoptosis and augmented proliferation. These findings suggest that HOTAIR increased the radioresistance of breast cancer cells by interfering with HOXD10 and PI3K/AKT-Bad signaling. Nevertheless, puzzles remain as to whether it is via the mediation of HOXD10 and its downstream PI3K/AKT-Bad pathway that HOTAIR promotes breast cancer cell proliferation. Our experiment also suggests that patients with HOTAIR-overexpression tumors might be less responsive to radiotherapy, thus requiring adjuvant or more aggressive regimens in such patients.

In summary, our findings identified patients bearing HOTAIR-overexpression breast tumors have a low sensitivity to radiotherapy by interfering with the activation of HOXD10 and PI3K/AKT-Bad signaling. And, adjuvant or more aggressive treatment should be administered in this group of HOTAIR-overexpression

rapy upexpressed HOXD10 was retarded by the over-expressed HOTAIR. HOXD10 is also known to be inversely correlated with the progression of breast cancers [17]. We might make a bold hypothesis that overexpressed-HOTAIR adversely affected the radiotherapeutic efficacy. Second, we demonstrated that radiotherapy upregulated p-Bad protein expression of PI3K/AKT-Bad pathway, but p-Bad level was lower in the overexpressed-HOTAIR plus radiotherapy group than that in the null-vector plus radiotherapy group, which is supportive of our hypothesis. Next, treatments with Ly294002 10 µmol/L and radiotherapy decreased the levels of p-AKT protein with a corresponding upregulation of p-Bad. We might as well conclude that PI3K/AKT-Bad pathway is involved in HOTAIR-induced radioresistance.

patients, and future targeting of HOTAIR might have added values for therapeutic implications.

Disclosure of conflict of interest

None.

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