Original Article

Knockdown of HOTAIR reduces the malignancy of bladder cancer cells via downregulation of invasions and metastasis-related genes

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Background: Bladder cancer is one of the most common malignant tumors. However, the mechanisms underlying the malignancy of the cancer remain largely unknown. This study was conducted to explore the effect of silencing long non-coding RNA (LncRNA) HOX transcript antisense intergenic RNA (HOTAIR) on the biological features of bladder cancer.

Methods: The *HOTAIR* gene in human bladder cancer cell line 253J was knockdown with siHOTAIR. Cell proliferation and apoptosis were determined using the CCK8 and Hoechst assays, respectively. The expression of Notch1, EpCAM, CXCR2, E-cadherin, vimentin, MMP-2 and α-SMA was measured using qPCR and Western blot analysis.

Results: Compared with the controls, the expression of HOTAIR decreased significantly after the cells were transfected with siHOTAIR, indicating that HOTAIR was down-regulated. After the cells were transfected with siHOTAIR, the OD value was significantly decreased, cell apoptosis was significantly increased, the expression of Notch1, EpCAM, CXCR2, vimentin, MMP-2 and α-SMA was decreased significantly, while the expression of E-cadherin was significantly increased ($P<0.05$). More cells were observed in S stage than in G stage following the siHOTAIR transfection.

Conclusions: siHOTAIR reduces the expression of HOTAIR and inhibits the proliferation of bladder cancer cells. These data further our understanding of the metastasis of bladder cancer and provide new clues for the diagnosis and treatment of bladder cancer.

Keywords: Bladder cancer; HOX transcript antisense intergenic RNA (HOTAIR); malignant biological characteristics

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Introduction

Bladder cancer is one of the most common malignant tumors. The incidence of the cancer ranks fourth in men and tenth in women (1). The most common pathological type of bladder cancer is urothelial carcinoma, which may be non-musculoinvasive and musculoinvasive. After radical resection, about 50% of the musculocutaneous invasive bladder cancer would metastasize with a 5-year survival rate of 50% (2). To understand the mechanisms underlying the metastasis, the molecular aspects of cancer cells have been investigated. Long chain non-coding RNA (LncRNA) generally refers to RNA transcript exceeding 200 nt. Studies have shown that although they do not encode proteins, they regulate the expression of genes in various

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ways (3). LncRNA exerts different biological functions through various molecular mechanisms. For example, HOX transcript antisense intergenic RNA (HOTAIR) plays an important role in the proliferation of breast cancer cells (4). X-inactive specific transcript (Xist) inactivates chromosome X (5) and Nron regulates the nuclear function (6). LncRNA regulates gene expression and stabilizes cell morphology to impact the proliferation of cells. For instance, LncRNA may an oncogenic or tumor suppressor, and may bind to chromatin form complex to silence specific genes (7). HOTAIR is a long noncoding RNA that is transcribed from the antisense strand of the homeobox C gene locus in the chromosome 12. It is overexpressed in various carcinomas including breast cancer and colorectal cancer, suggesting that it is an oncogene (7-9). The regulatory site of the 2,158 nt long antisense LncRNA is not the HOX gene cluster where HOTAIR is encoded (10). In mammals, the HOX family is clustered in 4 different genomic positions (the HOXA, HOXB, HOXC, HOXD gene clusters). HOTAIR is located in the HOXC11 and HOXC12 loci in the human genome, and is transcribed at the HOXC site. HOTAIR can bind to two complexes at the same time, and then recruit them to specific genomic loci to silence the genes in the loci (8,11). Therefore, we studied the effect of silencing HOTAIR on the malignant differentiation, invasion and metastasis of bladder cancer in order to provide new clues for clinical treatment of bladder cancer.

Methods

Cell lines

Human bladder cancer lines 253J and T24 (Cat No. HY-DXT-209, HY-DXT-349) were purchased from the Cell Bank of the Chinese Academy of Science. Cells were maintained in DMEM medium containing 10% fetal bovine serum (FBS) at 37 ℃ in 5% CO_2.

Reagents and instruments

DMEM (Cat No. KGM12400S-500) was purchased from KGI biologicals, Nanjing, China; FBS (Cat No. SKU:04-007-1A) was obtained from Biological Industries, Connecticut, USA; siHOTAIR and negative control were obtained from Shenggong, Shanghai, China; Trizon Reagent (Cat No. CW0580S), ultrapure RNA RNA extraction kit (Cat No. CW0581M) and HiFiScript first strand cDNA synthesis kit (Cat No. CW2569M) were purchased from CWBIO, Beijing, China; rabbit monoclonal antibodies against Notch 1 (Cat No. ab52627, 1:1,500 dilution), EpCAM (Cat No. ab32392, 1:6,000 dilution), E-cadherin (Cat No. ab40772, 1:5,000 dilution), vimentin (Cat No. ab92574, 1:3,500 dilution) were obtained from Abcam, Cambridge, USA; rabbit polyclonal antibodies against CXCR2 (Cat No. bs-12247R, 1:1,500 dilution) and MMP-2 (Cat No. bs-20706R, 1:600 dilution) were obtained from Bioss, Woburn, USA; rabbit monoclonal antibody against α-SMA (Cat No. M0002, 1:800 dilution) was purchased from Boster, Beijing, China. Vertical electrophoresis apparatus (Cat No. 0411312151351), ultrasensitive chemiluminescence imaging system (Chemi DocTM XRS+) and fluorescence PCR instrument (CFX Connect) were purchased from Bio-rad Laboratories, Shanghai, China. Fluorescence microscopy (IX51) was from Olympus, Kyoto, Japan.

Transfection

Cells were grown to 70% confluency, and transfected with lentivirus containing siHOTAIR (HOTAIR KD group), or negative control (Vector group). Non-transfected cells were used as control. For transfection, cells were grown to adhere to the wall, refreshed with new medium, and added with 0.5 μg/mL polybrene and lentivirus (200 μL at a MOI of 5). Twenty-four h after transfection, the culture medium was refreshed. When cells reached a confluency of 70%, they were harvested, washed twice with PBS and digested with 1% trypsin at 37 ℃ for 3 min. The digested cells were suspended in DMEM medium containing 10% FBS and cultured in the wells of 6-well plate as described above. The RNAi sequence and the negative control sequences were as follows:

- siHotair-1F: 5'-GATCCGCCTTTGGAAGCTCTTGAAGGCTCGAGCCTTCAAGAGCTTCCAAAGGC TTTTT G-3';
- siHotair-1R: 5'-AATTCAAAAAGCCTTTGGAAGCTCTTGAAGGCTCGAGCCTTCAAGAGCTTCCAAAGGC G-3';
- ShHOTAIR-2F: 5'-GATCCGAGAAGTGCTGCAACCTAAACCTCGAGGTTTAGGTTGCAGCACTTCTCTTTTTG-3';
- ShHOTAIR-2R: 5'-AATTCAAAAAGAGAAGTGCTGCAACCTAAACCTCGAGGTTTAGGTTGCAGCACTTCTCG-3';
- ShHOTAIR-3F: 5'-GATCCGCAACCTAAACCAGCAATTACCTCGAGGTAATTGCTGGTTTAGGTTGCTTTTTG-3';

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- ShHOTAIR-3R: 5'-AATTCAAAAAGCAACCTAAACCAGCAATTACCTCGAGGTAATTGCTGGTTTAGGTTGCG-3'.

Negative control:

- Top strand 5'-GATCC TTCTCCGAACGTGTCACGTAATTCAAGAGATTACGTGACACGTTCGGAGAATTTTTTg-3';
- Bottom strand 5'-AATTC AAAAAATTCTCCGAACGTGTCACGTAATCTCTTGAATTACGTGACACGTTCGGAGAAg-3'.

CCK8 assay

CCK8 assays were preformed according to the supplier's instruction. Briefly, cells were harvested, inoculated into the wells of 96-well plates, added with 10 μL CCK8 assay solution and cultured for 4 h. OD value was measured at 450 nm using microplate reader. The experiment was repeated 3 times, and the average value of the experimental results was taken as the final experiment result.

Flow cytometry

Cell apoptosis was assayed using Annexin V-PI assay according to the supplier' instructions. Briefly, the cells were seeded in the wells of 6-well plates, grown to a confluency between 50% and 70%, and harvested for apoptosis assay after staining with Annexin V-PI for 30 min in the dark.

For cell cycle analysis, 1×10^6 cells were harvested, washed with PBS and fixed with ice-cold 70% ethanol overnight at 4 ℃. Cells were washed with PBS and incubated with RNase A (100 μg/mL) at 37 ℃ for 30 min. DNA was labeled with PI (50 μg/mL) and the fluorescence was measured with a FACScalibur flow cytometer (Becton Dickinson). Data collection and analysis of the cell cycle distribution were performed using CellQuest and the Modfit software (Becton Dickinson).

Hoechst staining

Cells were fixed and stained with Hoechst 33258 for 5 min at room temperature. The stained cells were added with anti-fluorescence quenching solution and mounted to the slides, and reviewed under fluorescence microscope.

Fluorescent quantitative PCR

RNA was extracted using the TRIzon Reagent and reversely transcribed into cDNA using the HiFiScript first strand cDNA synthesis kit according to the manufacturer's instructions. A total of 2.5 μL of the resultant cDNA was subjected to pre-amplification using the TaqMan Pre-Amp Master Mix (Applied Biosystems) in a total volume of 12 μL. Non-fluorescent probes were used at 1×. Pre-amplification cycling conditions were 10 min at 95 ℃ followed by 14 cycles, each one consisting of 15 s at 95 ℃ and 4 min at 60 ℃. Later on, a 1:5 dilution of the pre-amplified cDNA was performed. RT-qPCR was performed on the 7900HT Fast Real-Time PCR system using TaqMan gene expression assays probes (Applied Biosystems). Human glyceraldehyde-3-phosphate dehydrogenase, *GADPH* (Hs03929097_g1), was used as an internal control. The primers used were as follows:
HOTAIR F: 5'- CTTGCTCTTCTTATCATCTCCATC,
HOTAIR R: 5'- GAACCCTCTGACATTTGCCTA;
Notch1 F: 5'-CTTTGTGCTTCTGTTCTTCGTG,
Notch1 R: 5'-CGCCGCTTCTTCTTGCT;
EpCAM F: 5'-TTCGGGCTTCTGCTTGC,
EpCAM R: 5'-CCCTTCAGGTTTTGCTCTTC;
CXCR2 F: 5'-ATGTCTCAGCATCTGGGGTCT,
CXCR2 R: 5'-GCAGGGTGAATCCGTAGCA;
E-cadherin F: 5'-ATCTGAAAGCGGCTGATACTG,
E-cadherin R: 5'-TGCCCCATTCGTTCAAGTA;
MMP-2 F: 5'-CCGCAGTGACGGAAAGA,
MMP-2 R: 5'-TGGTGTAGGTGTAAATGGGTG;
Vimentin F: 5'-TCGTGATGCTGAGAAGTTTCG,
Vimentin R: 5'-TCTGGATTCACTCCCTCTGGT;
α-SMA F: 5'-GCGTGGCTATTCCTTCGT,
α-SMA R: 5'-TCAGGCAACTCGTAACTCTTCT;
GAPDH F: 5'-CAATGACCCCTTCATTGACC,
GAPDH R: 5'-GAGAAGCTTCCCGTTCTCAG.

The data were managed using the Applied Biosystems software RQ Manager v1.2.1. Relative expression was calculated by using the comparative Ct method and obtaining the fold change value ($2^{-\Delta\Delta Ct}$) according to previously described protocol (12).

Western blot analysis

Cells of 2×10^7 were washed twice with cold PBS and lysed with RIPA buffer that contains protease and phosphatase inhibitors cocktail (Roche, UK). The supernatants were collected after centrifugation at 12,000 rpm for 20 min. The protein was subjected to polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane, and then detected by the proper primary and secondary antibodies before visualization with a chemiluminescence kit. The

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intensity of blot signals was quantitated using Quantity One software (v.4.62, General Electric, UK).

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM) obtained from at least three independent experiments. Statistical comparisons between experimental and control groups were assessed by using the Student's *t*-test. P<0.05 was considered statistically significant.

Results

siHOTAIR inference

Human bladder cancer 253J cells were transfected with three siHOTAIRs. Quantification of mRNA showed that HOTAIR levels were significantly down-regulated compared with control and cells transfected with negative control (P<0.05, *Figure 1*). Among them shHOTAIR1 had the greatest silencing effect, while there was no difference between the control and vector groups, indicating that the gene was silenced by siHOTAIRs specifically. Since siHOTAIR1 was most effective, it was used in subsequent experiments.

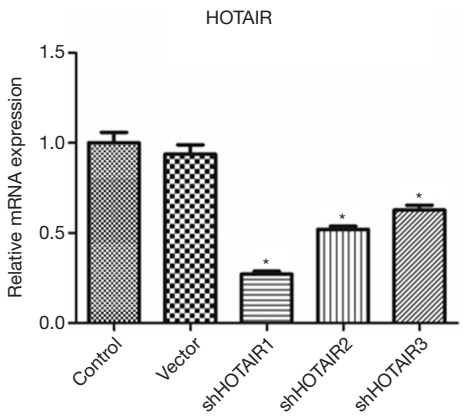

Figure 1 HOTAIR mRNA levels after the cells were transfected with siHOTAIRs. *, P<0.05 *vs.* control or vector.

siHOTAIR reduced cell proliferation

We then measured the proliferation of 253J and T24 cells after siHOTAIR transfection. As shown in *Figure 2*, compared with the control and vector-transfected cells, the OD values of siHOTAIR-transfected cells were significantly decreased (P<0.05), while there was no difference between control and vector-transfected cells, indicating that the proliferation of cells was specifically inhibited by siHOTAIR.

siHOTAIR increased apoptosis

Flow cytometry showed that the percentage of apoptotic cells increased significantly after the cells were transfected with siHOTAIR as compared with the control and vector (P<0.05, *Figure 3*), while these percentages were the same between the control and vector groups. This was further confirmed using Hoechst staining (*Figure 4*).

siHOTAIR arrested cells in S stage

After siHOTAIR transfection, more cells were observed in S stage and less in G1 stage in both 253 and T24 cell lines (*Figure 5*).

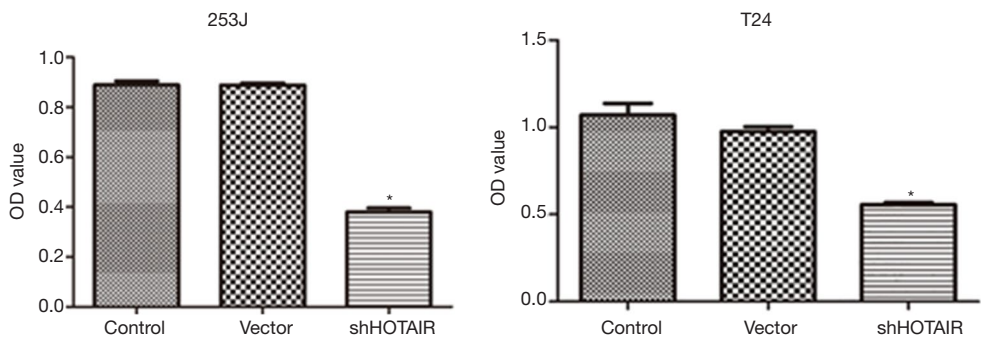

Figure 2 Cell proliferation as measured by OD value after 253J and T24 cells were transfected with siHOTAIR. *, P<0.05 *vs.* control or vector.

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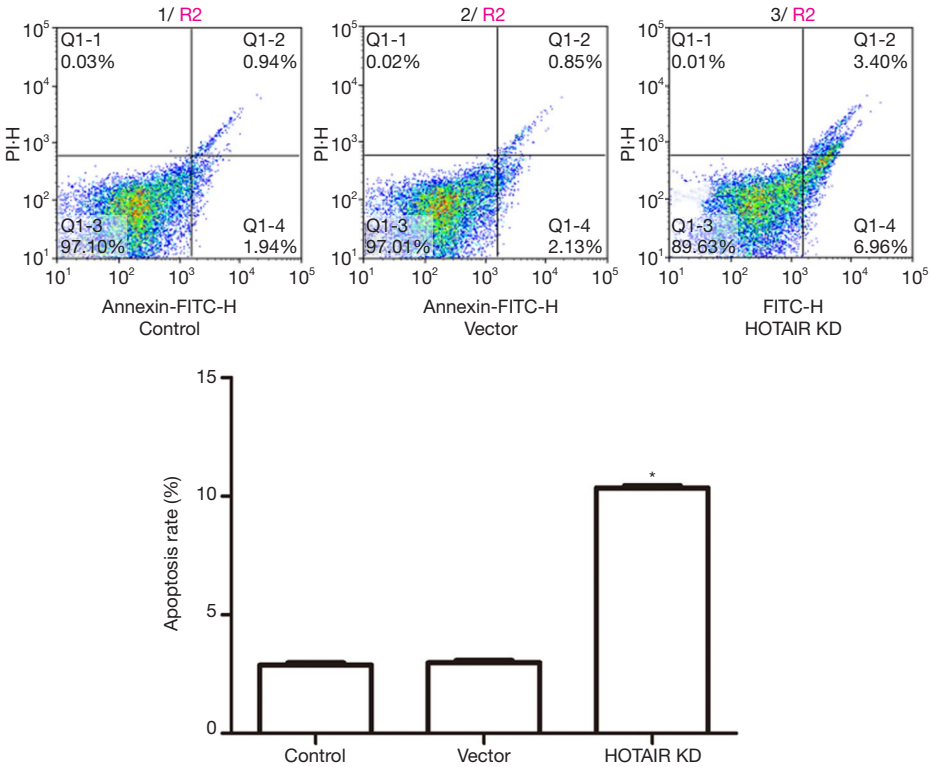

Figure 3 Percentages of apoptotic cells after the cells were transfected with siHOTAIR. Upper pane: flow cytometry; lower pane: percentages of apoptotic cells. *, P<0.05 *vs.* control or vector.

Impact of siHOTAIR on gene expression

We then assayed the expression of genes related to invasions and metastasis of cancer in the cells. The results showed that the mRNA and protein levels of Notch1, EpCAM, CXCR2, E-cadherin, vimentin, MMP-2 and α-SMA were significantly down-regulated, while these of E-cadherin were significantly up-regulated after the cells were transfected with siHOTAIR. The expression of these genes was not affected after transfection with vector at mRNA and protein levels (*Figure 6*).

Discussion

Bladder cancer is one of the most common malignant tumors in the urinary system. It has the biological characteristics of easy recurrence, easy invasion and frequent metastasis. Gupta *et al.* showed that enforced expression of HOTAIR in epithelial cancer cells induced genome-wide re-targeting of Polycomb repressive complex 2 (PRC2) in an occupancy pattern like embryonic fibroblasts. This pattern leads to altered methylation and gene expression of histone H3, and increased cancer invasiveness (which the loss of HOTAIR can inhibit) and metastasis in a manner dependent on PRC2 (8). Chen *et al.* found that HOTAIR is closely related to the metastasis of squamous cell carcinoma of the esophagus (13). In the primary and metastatic tissues of breast cancer, HOTAIR is highly expressed (8) and the high expression of HOTAIR is closely related to the metastasis and recurrence of liver cancer and the drug resistance of hepatoma cells (10). In addition, a significant inverse correlation between HOTAIR and WIF-1 expression was demonstrated in Ta/T1 BC tissues and upregulation of HOTAIR predicts recurrence in stage Ta/T1 bladder cancer (14). After the cells were transfected with siHOTAIR, the viability of cells decreased significantly and the apoptosis was promoted (*Figures 2-4*), demonstrating that the proliferation of bladder cancer cells could be inhibited after the down-regulation of HOTAIR. This is consistent with the previous results (8).

The *NOTCH* gene was first discovered in 1917 in *Drosophila melanogaster*. It is so named because due to its absence, the fly's wings have the notch if the gene

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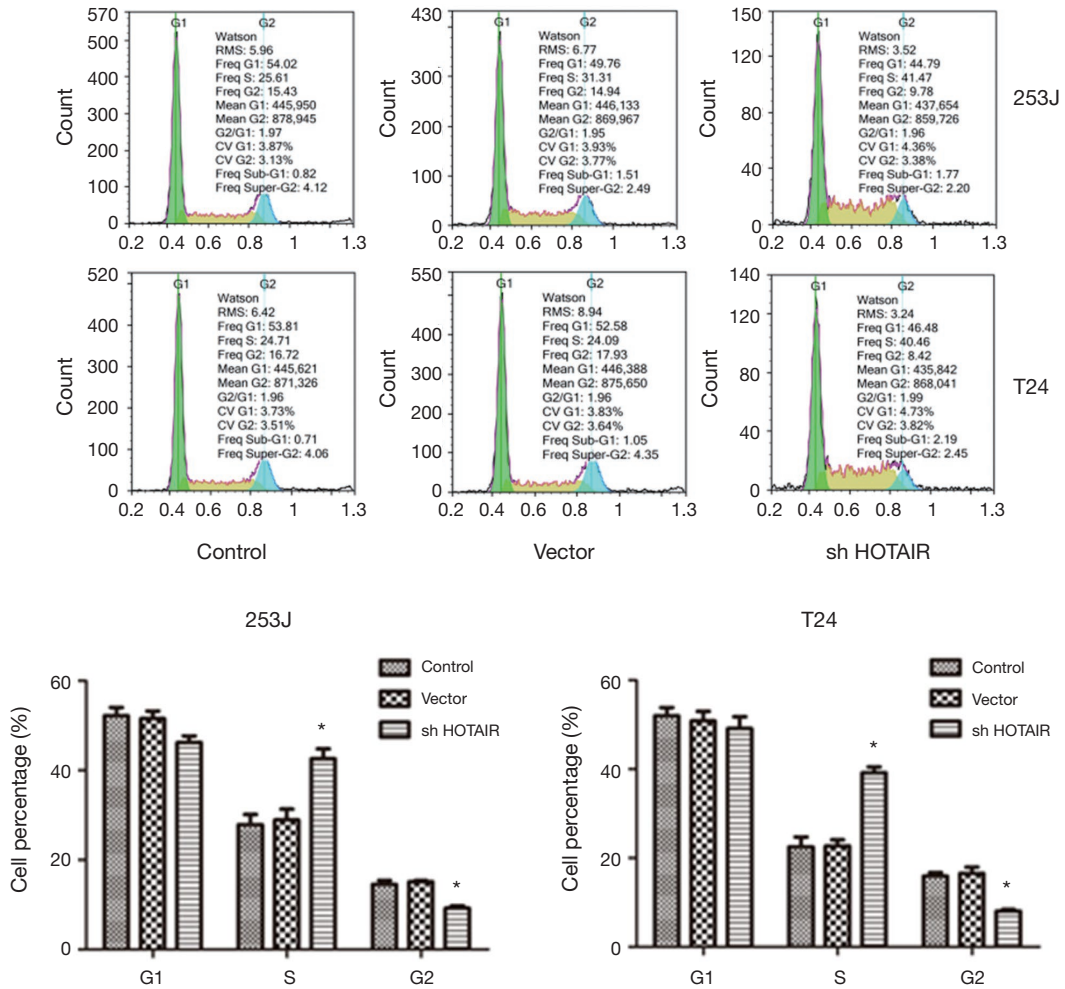

Figure 4 Apoptosis of 253J and T24 cells after siHOTAIR transfection, Upper pane: microphotos of Hoechst staining and lower panels: percentages of apoptotic cells. *, P<0.05 *vs.* control or vector.

is absent. The gene widely exists and is expressed in a variety of organisms (15). The Notch signaling pathway is highly conserved in most multicellular organisms and plays an important role in cell differentiation and embryo development. The signaling pathway also impacts cell apoptosis, proliferation, cell cycle, differentiation, under different physiological and pathological conditions (16). Therefore, we investigated the effect of HOTAIR silence on the expression of genes in the pathways. Notch1 decreased after down-regulating HOTAIR expression, suggesting that HOTAIR may affect the apoptosis through down-regulating the expression of Notch1. We also investigated how the expression of EpCAM, EpCAM stimulates the expression of proto-oncogenes such as cyclin A/E and c-myc to induce tumors such as colon carcinoma (17) and is highly expressed in many epithelial tumors, such as squamous cell carcinoma (18) and breast cancer (19). Therefore, it can be used as a candidate protein for tumor diagnosis and treatment. The expression of EpCAM also decreased after down-regulating HOTAIR in our study. This might suppress the expression of some proto oncogenes and inhibit the growth of tumor cells as we observed.

E-cadherin is a calcium-dependent cell adhesion molecule. It maintains the integrity of cell by binding to identical proteins in adjacent cells. Reduced expression of E-cadherin is an indicator of epithelial-mesenchymal transition (EMT) (20). Vimentin is an intermediate filament protein. Its functions include the maintenance of cell morphology, signal transduction, transplantation immunity

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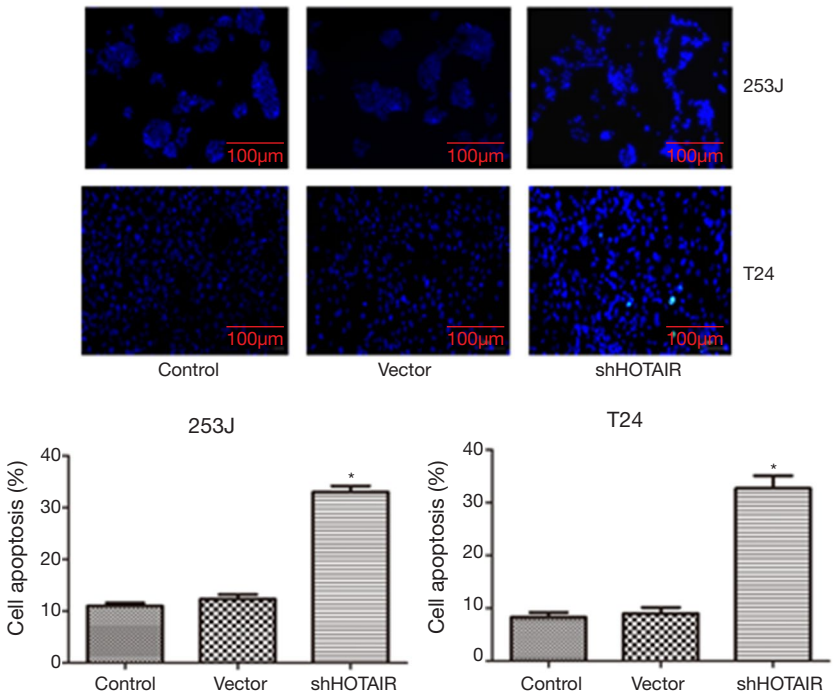

Figure 5 Cell cycle analysis of 253J and T24 cells after siHOTAIR transfection, Upper pane: cytometry results and lower panels: percentages of cells at different cell cycles. *, P<0.05 *vs.* control or vector.

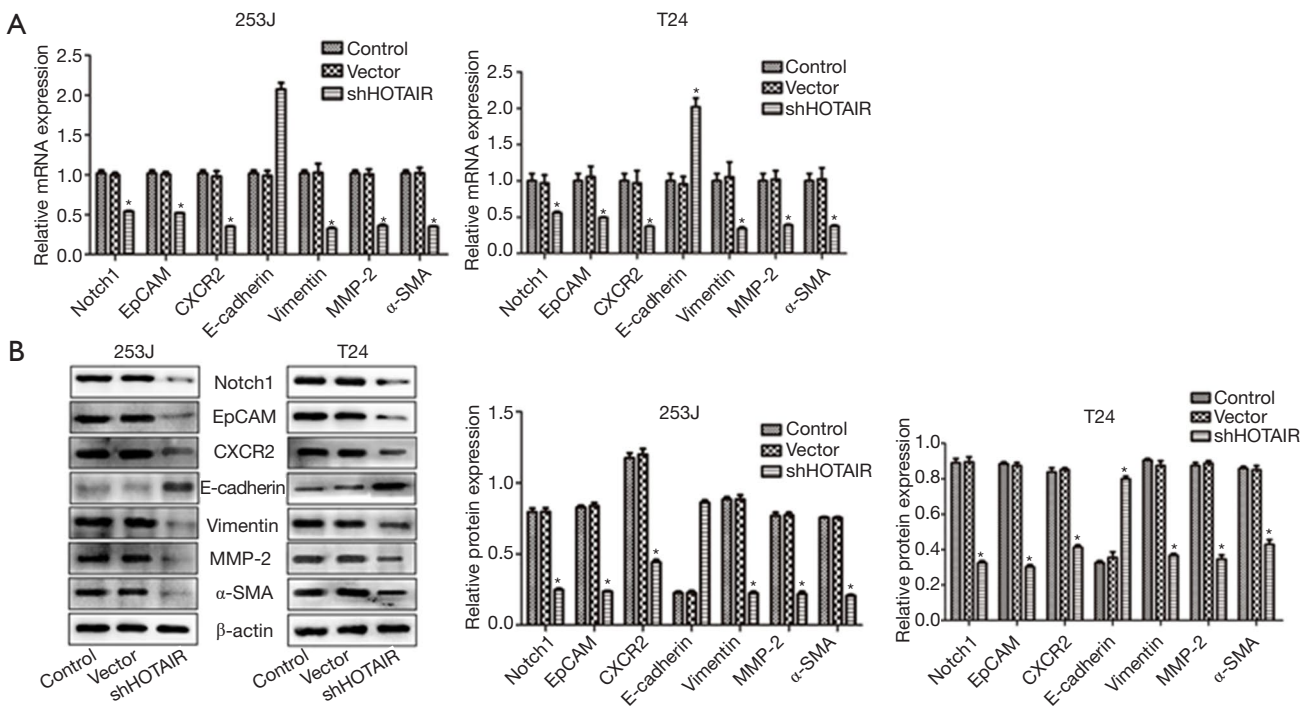

Figure 6 mRNA and protein levels of Notch1, EpCAM, CXCR2, E-cadherin, vimentin, MMP-2 and α-SMA after the cells were transfected with siHOTAIR. (A) mRNA levels; (B) left pane: representative Western blot; right pane: relative protein level. *, P<0.05 *vs.* control or vector.

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and apoptosis (21). α-SMA regulates cell movement. The expression of α-SMA promotes EMT (22), which is mainly involved in growth and development, injury repair, tumor metastasis and the occurrence and development of fibrosis diseases. When HOTAIR was down-regulated, the expression of E-cadherin was up-regulated while the expression of Vimentin and α-SMA was down-regulated. These results suggest that the biological characteristics of bladder cancer may be affected by HOTAIR silence as a result of changed expression of E-cadherin, vimentin and α-SMA.

The chemokine CXC family has 5 receptors, such as CXCR1 and CXCR2. They are overexpressed in breast cancer, colon cancer, bladder cancer and other tumor cells (23). Knockdown of HOTAIR in UBC cell lines reduces *in vitro* migration and invasion. Importantly, loss of HOTAIR expression in UBC cell lines alters expression of EMT genes (24). Our results show that after the downregulation of HOTAIR, the expression of CXCR2 decreased. This might contribute to reduced growth of tumor cells after HOTAIR downregulation. MMPs are zinc-dependent endopeptidases, which degrade the extracellular matrix, effectively leading to tumor invasion and metastasis (25). MMP-2 is one of the most important subtypes of MMPs and is highly expressed in breast cancer, ovarian tumor and nasopharyngeal carcinoma (26). Its expression in the tumor cells was significantly decreased after the cells were transfected with siHOTAIR, suggesting that HOTAIR is related to the biological characteristics of the malignant differentiation in bladder cancer.

Conclusions

siHOTAIR can reduce the expression of HOTAIR, resulting in reduced cell growth and apoptosis. A number of pro oncogenes are up-regulated as a result of HOTAIR down-regulation, providing possible molecular mechanism underlying the inhibition. These results further our understanding of the metastasis of bladder cancer and could help provide new clues for the diagnosis and treatment of bladder cancer.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/tcr.2018.10.03). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). Institutional ethical approval and informed consent were waived.

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