Original Article

The central role of HOTAIR in the malignancy of CD44+ human hypopharyngeal carcinoma cells

Hongxia Cheng¹, Xinhua Cui², Ying Guo², Lintao Gu², Yaning Wang², Qirong Wang², Hui Liang²

¹Department of Pathology, Provincial Hospital Affiliated to Shandong University, 324, Jing 5 Rd, Jinan 250021, Shandong, China; ²Department of Otolaryngology, Shandong Provincial Qianfoshan Hospital, 16766, Jingshi Road, Jinan 250014, Shandong, China

Received June 30, 2016; Accepted October 5, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: Human hypopharyngeal carcinoma is one of the most common malignant tumors. CD44 could serve as a molecular marker to screen for cancer stem cells (CSCs) in hypopharyngeal cancer. The aim of this study was to identify the role of HOX transcript antisense RNA (HOTAIR) on cell proliferation and invasion in CD44+ FADU cells (human hypopharyngeal carcinoma cells). We also explored the underlying mechanism contributing to HOTAIR’s observed effects. CD44+ FADU cells were sorted and purified by flow cytometry and infected with lentivirus stably expressing HOTAIR shRNA. Cell proliferation and invasion analyses were carried out with cell counting kit-8 (CCK-8) and Transwell assays. The expressions of downstream effectors of HOTAIR, including E-cadherin, β-catenin, and vimentin were measured by quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting. Knockdown of HOTAIR markedly inhibited the proliferation and invasion of CD44+ FADU cells in vitro. HOTAIR depletion also increased the expressions of tumor suppressors E-cadherin and β-catenin and decreased the expression of oncogenic vimentin at both mRNA and protein levels. Collectively, our results show that HOTAIR can suppress CD44+ FADU cells proliferation and invasion by regulating the expressions of E-cadherin, β-catenin, and vimentin.

Keywords: Human hypopharyngeal carcinoma, HOTAIR, cancer stem cell

Introduction

Hypopharyngeal squamous cell carcinoma (HSCC) is one of the most common and aggressive head and neck cancers with poor prognosis and high recurrence [1, 2]. Although advances have been made in surgery, radiotherapy and chemotherapy, the 5-year survival rate of patients with HSCC ranges from 40 to 50%, with a morbidity of ~1 case per 100,000 people [3-5]. Recently, the cancer stem cell (CSC) hypothesis proposed that cancers may be originated and amplified by a subset of cells that acquire and maintain stem cell properties, and every cancer may contain a small cell subpopulation with the capacity to differentiate into different cell lineages and self-renew [6, 7]. Importantly, Shen et al revealed the existence of CSCs in HSCC and suggested that CD44 could serve as a molecular marker to screen for CSCs in patients with hypopharyngeal cancer [8]. It is therefore critical to explore the mechanism underlying the malignancy of CD44-positive HSCC.

Long non-coding RNAs (lncRNAs) are non-protein-coding transcripts longer than 200 nucleotides [9]. LncRNAs reportedly play essential roles in many different processes including DNA replication, DNA transcription and post-transcriptional modification [10, 11]. For instance, TINCR was identified as a key lncRNA required for somatic tissue differentiation, which occurs through lncRNA binding to differentiation mRNAs to ensure their expression [12]. Recently, the HOX transcript antisense RNA (HOTAIR) located on chromosome 12 has been indicated to promote tumorigenesis and metastasis in numerous tumors. A growing number of studies have shown that HOTAIR is highly expressed in different cancer types including breast cancer [13, 14], colorectal cancer [15], non-small-cell lung cancer [16], and human colorectal CSCs [17]. Nevertheless, the effects and molecular mechanisms of HOTAIR in CD44-positive HSCC remain unknown.

In the present work, we investigated the role of HOTAIR on cell proliferation and inva-
Role of downregulated HOTAIR in HHC CSC

We investigated the role of downregulated HOTAIR in human hypopharyngeal carcinoma (HHC) cancer stem cells (CSC) in vitro. We also explored the possible mechanisms that might contribute to HOTAIR’s effects.

Materials and methods

Cell culture

The human hypopharyngeal carcinoma cell line FADU was purchased from the Chinese Academy of Sciences Cell Bank. All cells were maintained in minimum essential medium (MEM, Gibco/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco) and routinely passaged at 2-3-day intervals at 37°C under 5% CO₂ in a humidified incubator.

Sorting and identifying CD44-positive cells

For cell sorting, 10⁷ human hypopharyngeal carcinoma cells were dissociated by trypsin and re-suspended in PBS containing 2 mmol/L EDTA and 0.5% bovine serum albumin. Then cells were labeled with CD44 Micro Beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and separated using the magnetic cell separation columns. The sorted cells were stained with CD44-PE (BD Biosciences, Franklin Lakes, NJ, USA) or control IgG isotype (BD Biosciences) monoclonal antibody and then subjected to flow cytometry using a FACS Calibur (BD Biosciences).

RNA extraction and quantitative RT-PCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) following the manufacturer’s instruction. RNA quality and concentration were determined for each sample by the 260/280 nm ratio using a NanoDrop® ND-100 spectrophotometer. The GoScript™ Reverse Transcriptase system was applied for cDNA synthesis. The expression levels of HOTAIR, E-cadherin, β-catenin, and vimentin were tested by quantitative real-time polymerase chain reaction (qRT-PCR) using the All-in-One™ qPCR Mix. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. Fold changes were calculated by the relative quantification (2⁻ΔΔCt) method. The primers are listed in Table 1.

Lentivirus infection and stable cell lines

The shRNA sequence of the human HOTAIR gene was designed with the BLOCK-iT™ RNAi Designer (Invitrogen). The sequence was chemically synthesized and cloned into the pLVX-IRES-ZsGreen1 vector. Lentiviral vectors expressing HOTAIR shRNA or the negative control (NC) together with the packaging vectors were co-transfected into HEK 293T cells using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Virus particles were collected 24 h post-transfection and sustained for 72 h. Cells were then infected with lentivirus expressing HOTAIR shRNA or control shRNA. For stable cell line selection, cells were cultured in 1 μg/mL puromycin (Gibco) for 7 days after infection. Real-time qRT-PCR was performed to assess infection efficiency.

Western blot analysis

Cells were collected and lysed using RIPA lysis buffer (Invitrogen). Protein concentrations were measured with bicinchoninic acid (BCA, Gibco) assays. Equal amounts of total protein mixed with 5× sodium dodecyl sulfate (SDS) loading buffer were loaded into lanes on SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. The membranes were then incubated in 5% milk confling liquid for 2 h at room temperature and incubated overnight with primary antibodies against E-cadherin, β-catenin, vimentin, or GAPDH (1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with an appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Relative pro-

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product length (b.p.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>ACAACTTTGGTATCGTGGAAGG</td>
<td>GCCATCACGCACAGTTC</td>
<td>101</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>CAGAGGTACACGTTGTTG</td>
<td>GGTTGTCGAGGGAAAATAGG</td>
<td>119</td>
</tr>
<tr>
<td>β-catenin</td>
<td>TGTGAATCTCAAGTCCAGTGT</td>
<td>CCGTCAGCACAAGGAGAAACATT</td>
<td>120</td>
</tr>
<tr>
<td>Vimentin</td>
<td>AGTCCACCTAGTCTGCACTGAC</td>
<td>CATTTCAAGCATCTGCGGTC</td>
<td>98</td>
</tr>
<tr>
<td>HOTAIR</td>
<td>CAGTGGGGAACCTGACTCG</td>
<td>GTCCCTGGTCTCTCTTACC</td>
<td>104</td>
</tr>
</tbody>
</table>
Role of downregulated HOTAIR in HHC CSC

protein expression was detected using a Super Signal protein detection kit (Invitrogen).

Cell proliferation assay

Cell proliferation assay was measured using the CCK-8 Cell Counting Kit (Invitrogen) according to the manufacturer’s instructions. Cells were plated at 10,000 cells/well in 96-well plates. After culturing for 24 h, 10 μl CCK8 was added to each well and incubated for 4 h. Absorbance at 450 nm was measured with a microplate reader.

Cell invasion assay

The 24-well Transwell filters (8-mm pore size, Millipore, Billerica, MA, USA) were used to determine cell invasion ability. Briefly, the Transwell filters coated with Matrigel were placed into 24-well plates. Cells were re-suspended in serum-free MEM medium, and then seeded on the top side of membrane and cultured at 37°C for 48 h. Invaded cells on the bottom side of the filter were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The numbers of invaded cells were photographed in six randomly selected regions under an Olympus microscope (Olympus, Tokyo, Japan).

Statistical analysis

Analyses of two groups were performed with Student’s t-tests, and analyses of variance (ANOVAs) followed by Dunnett’s multiple comparison tests were to compare more than two groups. All data were processed with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

Results

HOTAIR is up-regulated in CD44-positive hypopharyngeal carcinoma cells

Previous studies have revealed that CD44 expression is related to poor prognosis of HSCC [8]. Here, we first used a CD44-PE antibody coupled with flow cytometry to examine CD44 expression in FADU cells. As shown in Figure 1A, only 25.9% of FADU cells were CD44 posi-

Figure 1. HOTAIR is up-regulated in CD44-positive hypopharyngeal carcinoma cells. A. To examine CD44 expression level FADU cells by flow cytometry. B. To measure the purity of the CD44-positive cells enriched by flow cytometry with CD44 microbeads. C. To detect differences in HOTAIR expression between CD44-positive FADU and CD44-negative FADU with qPCR.
Role of downregulated HOTAIR in HHC CSC

Next, CD44-positive and -negative cells were sorted with CD44 microbeads. The purity of the CD44-positive cell was examined by flow cytometry. The results indicated that the ratio of CD44-positive cells increased to 94.8% after sorting (Figure 1B) HOTAIR expression levels in CD44-positive and -negative FADU cells were measured by qRT-PCR. Interestingly, HOTAIR expression was significantly higher in CD44-positive FADU cells, indicating that HOTAIR may be a key mediator in CD44-positive FADU cells.

Down-regulated HOTAIR inhibited CD44-positive hypopharyngeal carcinoma cell proliferation and invasion in vitro

To investigate the role of HOTAIR in the tumorigenesis of CD44-positive human hypopharyngeal carcinoma, we infected cells with lentivirus expressing HOTAIR shRNA. The qRT-PCR results indicated that cells infected with HOTAIR shRNA produced significantly lower levels of HOTAIR compared with the blank control and NC groups (Figure 2A). Next, the effect of HOTAIR on cell proliferation was detected with CCK-8 assays. As shown in Figure 2B, HOTAIR knockdown inhibited FADU cell proliferation as soon as 3 days after transfection and persisting for 6 days, suggesting that HOTAIR is a key regulator in hypopharyngeal carcinoma cell proliferation. We also performed Transwell assay to determine whether HOTAIR influenced FADU cell invasive capability. As we expected, compared to control groups, HOTAIR down-regulation markedly restrained FADU cell invasion (Figure 2C).
To further explore how HOTAIR regulates CD44-positive FADU cell malignancy, we performed qRT-PCR and western blot analyses to assay the expression of genes associated with tumor growth and metastasis. As illustrated in Figure 3A and 3B, suppressing HOTAIR increased expression of the tumor suppressors E-cadherin and β-catenin but decreased expression of the oncogenic gene vimentin at both mRNA and protein levels. These data suggest that HOTAIR plays an important role in the proliferation and invasion of CD44-positive hypopharyngeal carcinoma cells in vitro.

**Discussion**

The CSC hypothesis posits that cancer may be originated and amplified by a small number of cells that acquire and maintain properties of stemness, allowing them to differentiate into various lineages and self-renew [6, 7]. These cells might be a primary reason why malignant tumors recur and metastasize. Shen et al revealed that CSCs exist in HSCC and that CD44 could serve as a molecular marker to screen for CSCs [8]. However, how CD44 expression influences HSCC remains unclear. It is critical for us to explore the mechanism underlying the malignancy of CD44-positive HSCC.

A growing body of evidence indicates that lncRNAs are involved in diverse biological processes including cell proliferation, metastasis, apoptosis, cell aging, response to chemotherapy and hypoxia, as well as regulation of...
epithelial-mesenchymal transition (EMT) [18-23]. Numerous clinical studies have linked aberrant lncRNA regulation and mutation to a variety of human cancers, indicating that lncRNAs may contribute to the human tumor development and progression. For example, ANRIL is up-regulated and required to inhibit the tumor suppressors INK4a/p16 and INK4b/p15 in prostate cancer lines [24, 25]. MALAT1 is down-regulated and plays a significant role in the cell growth and metastasis of lung cancer cell lines [26].

As an important member of tumor-associated lncRNAs, HOTAIR plays a crucial role in tumorigenesis and has been related to tumor initiation and poor prognosis [14, 27]. Berrondo et al demonstrated that HOTAIR knockdown reduced the malignancy of UBC cell lines by affecting EMT gene expression [28]. In our experiment, we found that inhibiting HOTAIR expression specifically suppressed the proliferation and invasion of CD44-positive FADU cells in vitro, which is consistent with a previous study.

Metastasis is the leading cause of mortality in cancer patients [29]. The crucial step of cancer metastasis is EMT, a process by which epithelial cells lose their orientation and cell-cell contact and consequently acquire the capabilities of mesenchymal cells to migrate and invade [30]. Therefore, we further determined whether HOTAIR knockdown affected the expression of EMT-related genes in CD44-positive FADU cells. Our results showed that HOTAIR suppression markedly increased protein levels of E-cadherin and β-catenin and decreased expression of the EMT marker vimentin. These findings further confirm that HOTAIR is a tumor suppressor in human cells.

In summary, our results demonstrate that suppression of HOTAIR inhibits the proliferation and invasion of CD44-positive FADU cells. Furthermore, HOTAIR inhibition alters the expression of EMT genes including E-cadherin, β-catenin, and vimentin. These data suggest that HOTAIR may be a useful biomarker of poor prognosis and a possible therapeutic target for hypopharyngeal cancer.

Acknowledgements

This study was supported by a Grant from the Natural Science Funds of Shandong Province (2009ZRB14166).

Disclosure of conflict of interest

None.

Address correspondence to: Hui Liang and Qirong Wang, Department of Otolaryngology, Shandong Provincial Qianfoshan Hospital, 16766, Jingshi Road, Jinan 250014, Shandong, China. Tel: 86-13791123898; E-mail: onlinelh@163.com (HL); Tel: 86-13791120867; E-mail: qirongwagent@hotmail.com (QRW)

References

Role of downregulated HOTAIR in HHC CSC


