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

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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-

Table 1. Primer Sequences for the Tested Genes

Gene name	Forward	Reverse	Product length (b.p.)
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC	101
E-cadherin	CGAGAGCTACACGTTCACGG	GGGTGTCGAGGGAAAAATAGG	119
β-catenin	TGTGAATCCCAAGTACCAGTGT	CCGTCAGACAAAGGAGAAACATT	120
Vimentin	AGTCCACTGAGTACCGGAGAC	CATTTCACGCATCTGGCGTTC	98
HOTAIR	CAGTGGGGAACTCTGACTCG	GTGCCTGGTGCTCTCTTACC	104

sionin human hypopharyngeal carcinoma cells (CD44+ FADU cells) *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% CO_2 in a humidified incubator.

Sorting and identifying CD44-positive cells

For cell sorting, 10^7 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 ($2^{-\Delta\Delta Ct}$) 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 confining 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-
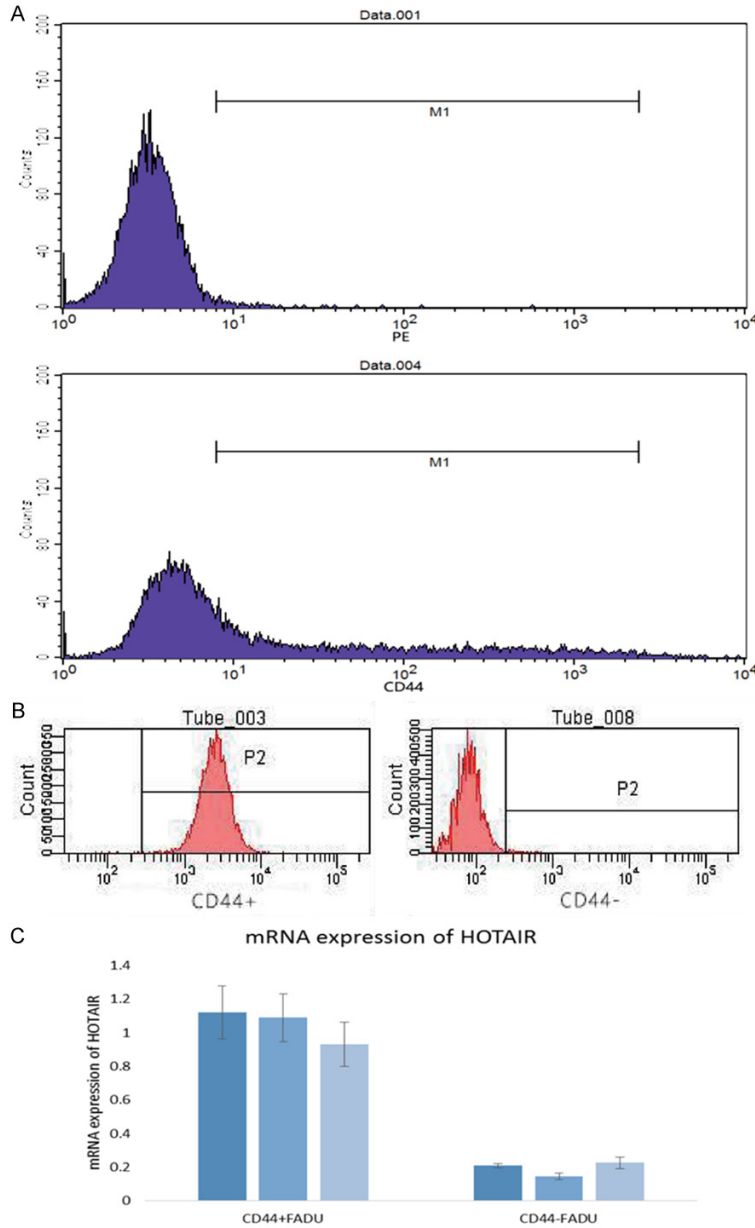
Figure 1. HOTAIR is up-regulated in CD44-positive hypopharyngeal carcinoma cells. A. To examine CD44 expression level FADU cells by flow cytometry. B. To measure the purity of the CD44-positive cells enriched by flow cytometry with CD44 microbeads. C. To detect differences in HOTAIR expression between CD44-positive FADU and CD44-negative FADU with qPCR.

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).
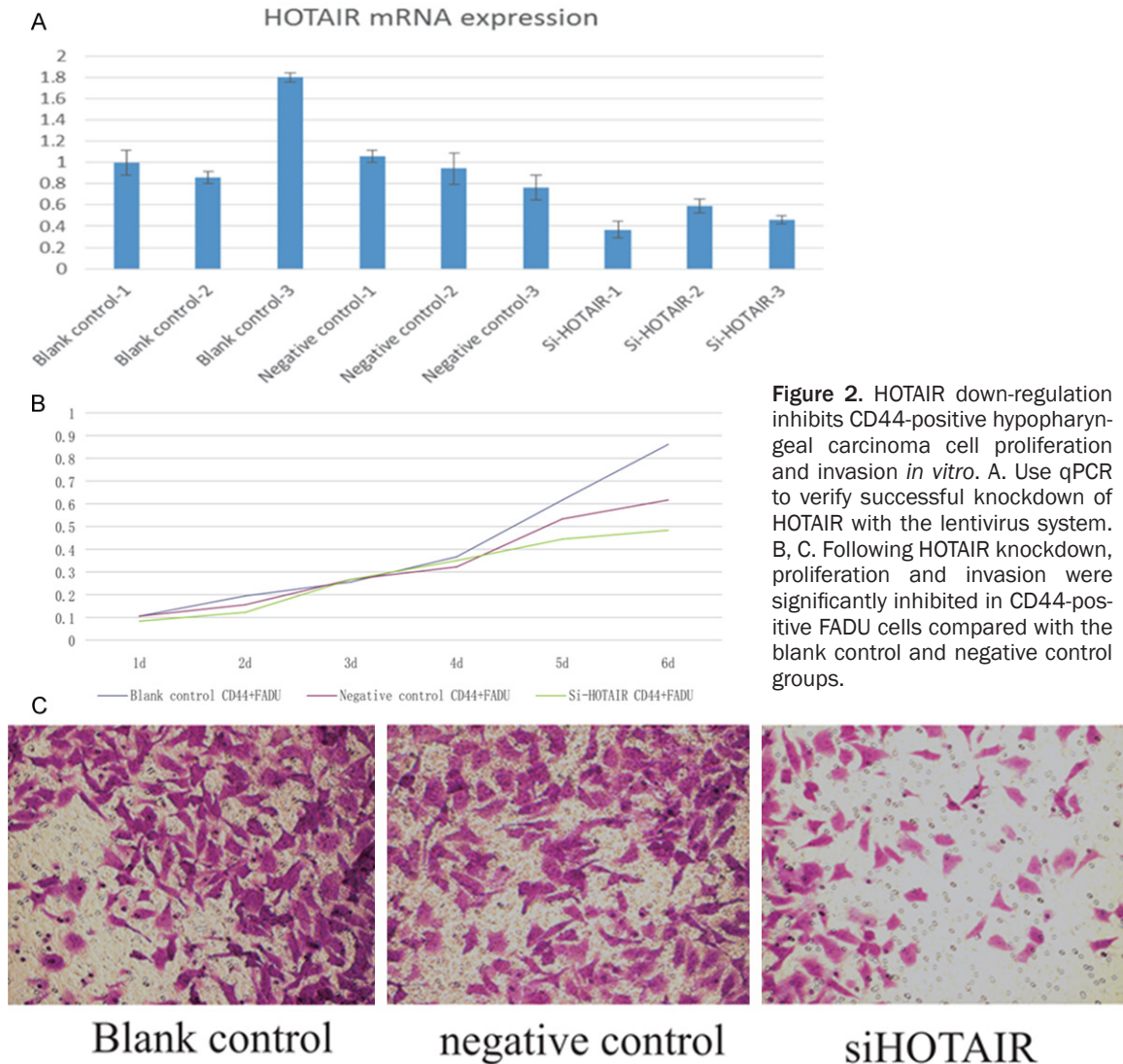
Statistical analysis

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

tein 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

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 2. HOTAIR down-regulation inhibits CD44-positive hypopharyngeal carcinoma cell proliferation and invasion *in vitro*. A. Use qPCR to verify successful knockdown of HOTAIR with the lentivirus system. B, C. Following HOTAIR knockdown, proliferation and invasion were significantly inhibited in CD44-positive FADU cells compared with the blank control and negative control groups.
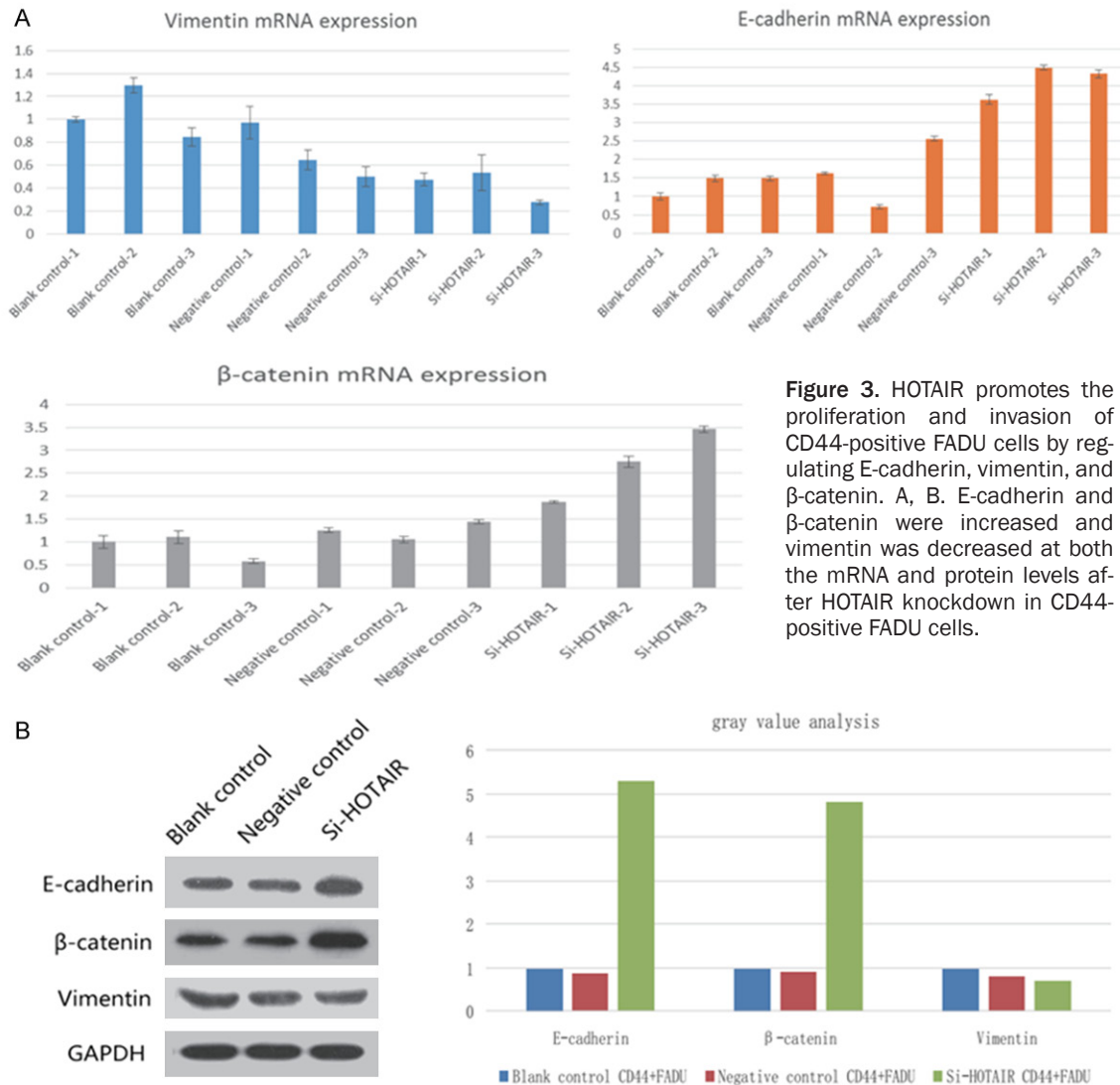
tive. Next, CD44-positive and -negative cells were sorted with CD44 microbeads. The purity of the CD44-positive cell was examined by flow cytometry. The results indicated that the ratio of CD44-positive cells increased to 94.8% after sorting (**Figure 1B**) HOTAIR expression levels in CD44-positive and -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 hypopharyn-

geal 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**).

Figure 3. HOTAIR promotes the proliferation and invasion of CD44-positive FADU cells by regulating E-cadherin, vimentin, and β-catenin. A, B. E-cadherin and β-catenin were increased and vimentin was decreased at both the mRNA and protein levels after HOTAIR knockdown in CD44-positive FADU cells.

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 repressors 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.

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References

[1] Helliwell TR. acp Best Practice No 169. Evidence based pathology: squamous carcinoma of the hypopharynx. J Clin Pathol 2003; 56: 81-85.

[2] Spector JG, Sessions DG, Haughey BH, Chao KS, Simpson J, El MS and Perez CA. Delayed regional metastases, distant metastases, and second primary malignancies in squamous cell carcinomas of the larynx and hypopharynx. Laryngoscope 2001; 111: 1079-1087.

[3] Hoffman HT, Karnell LH, Shah JP, Ariyan S, Brown GS, Fee WE, Glass AG, Goepfert H, Ossoff RH and Fremgen AM. Hypopharyngeal cancer patient care evaluation. Laryngoscope 1997; 107: 1005-1017.

[4] Gupta T, Chopra S, Agarwal JP, Laskar SG, D'cruz AK, Shrivastava SK and Dinshaw KA. Squamous cell carcinoma of the hypopharynx: single-institution outcome analysis of a large cohort of patients treated with primary non-surgical approaches. Acta Oncol 2009; 48: 541-548.

[5] Davies L and Welch HG. Epidemiology of head and neck cancer in the United States. Otolaryngol Head Neck Surg 2006; 135: 451-457.

[6] Jordan CT, Guzman ML and Noble M. Cancer stem cells. N Engl J Med 2006; 355: 1253-1261.

[7] Reya T, Morrison SJ, Clarke MF and Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001; 414: 105-111.

[8] Shen C, Xiang M, Nie C, Hu H, Ma Y and Wu H. CD44 as a molecular marker to screen cancer stem cells in hypopharyngeal cancer. Acta Otolaryngol 2013; 133: 1219-1226.

[9] Perkel JM. Visiting "noncodarnia". Biotechniques 2013; 54: 301, 303-304.

[10] Hung T and Chang HY. Long noncoding RNA in genome regulation: prospects and mechanisms. RNA Biol 2010; 7: 582-585.

[11] Koerner MV, Pauler FM, Huang R and Barlow DP. The function of non-coding RNAs in genomic imprinting. Development 2009; 136: 1771-1783.

[12] Kretz M, Siprashvili Z, Chu C, Webster DE, Zehnder A, Qu K, Lee CS, Flockhart RJ, Groff AF, Chow J, Johnston D, Kim GE, Spitale RC, Flynn RA, Zheng GX, Aiyer S, Raj A, Rinn JL, Chang HY and Khavari PA. Control of somatic tissue differentiation by the long non-coding RNA TINCR. Nature 2013; 493: 231-235.

[13] Tao S, He H and Chen Q. Estradiol induces HOTAIR levels via GPER-mediated miR-148a inhibition in breast cancer. J Transl Med 2015; 13: 131.

[14] Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, Hung T, Argani P, Rinn JL, Wang Y, Brzoska P, Kong B, Li R, West RB, van de Vijver MJ, Sukumar S and Chang HY. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 2010; 464: 1071-1076.

[15] Wu ZH, Wang XL, Tang HM, Jiang T, Chen J, Lu S, Qiu GQ, Peng ZH and Yan DW. Long non-coding RNA HOTAIR is a powerful predictor of metastasis and poor prognosis and is associated with epithelial-mesenchymal transition in colon cancer. Oncol Rep 2014; 32: 395-402.

[16] Liu XH, Liu ZL, Sun M, Liu J, Wang ZX and De W. The long non-coding RNA HOTAIR indicates a poor prognosis and promotes metastasis in non-small cell lung cancer. BMC Cancer 2013; 13: 464.

[17] Dou J, Ni Y, He X, Wu D, Li M, Wu S, Zhang R, Guo M and Zhao F. Decreasing lncRNA HOTAIR expression inhibits human colorectal cancer stem cells. Am J Transl Res 2016; 8: 98-108.

[18] Zhang ZZ, Shen ZY, Shen YY, Zhao EH, Wang M, Wang CJ, Cao H and Xu J. HOTAIR Long Non-coding RNA Promotes Gastric Cancer Metastasis through Suppression of Poly r(C)-Binding Protein (PCBP) 1. Mol Cancer Ther 2015; 14: 1162-1170.

[19] Bolton EM, Tuzova AV, Walsh AL, Lynch T and Perry AS. Noncoding RNAs in prostate cancer: the long and the short of it. Clin Cancer Res 2014; 20: 35-43.

[20] Shi X, Sun M, Liu H, Yao Y and Song Y. Long non-coding RNAs: a new frontier in the study of human diseases. Cancer Lett 2013; 339: 159-166.

[21] Chan KC, Jiang P, Zheng YW, Liao GJ, Sun H, Wong J, Siu SS, Chan WC, Chan SL, Chan AT, Lai PB, Chiu RW and Lo YM. Cancer genome scanning in plasma: detection of tumor-associated copy number aberrations, single-nucleotide variants, and tumoral heterogeneity by massively parallel sequencing. Clin Chem 2013; 59: 211-224.

[22] Hutchinson JN, Ensminger AW, Clemson CM, Lynch CR, Lawrence JB and Chess A. A screen for nuclear transcripts identifies two linked noncoding RNAs associated with SC35 splicing domains. BMC Genomics 2007; 8: 39.

[23] Sun M, Nie FQ, Wang ZX and De W. Involvement of lncRNA dysregulation in gastric cancer. Histol Histopathol 2016; 31: 33-39.

[24] Kotake Y, Nakagawa T, Kitagawa K, Suzuki S, Liu N, Kitagawa M and Xiong Y. Long non-coding RNA ANRIL is required for the PRC2 recruitment to and silencing of p15(INK4B) tumor suppressor gene. Oncogene 2011; 30: 1956-1962.

[25] Yap KL, Li S, Munoz-Cabello AM, Raguz S, Zeng L, Mujtaba S, Gil J, Walsh MJ and Zhou MM. Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. Mol Cell 2010; 38: 662-674.

[26] Gutschner T, Hammerle M, Eissmann M, Hsu J, Kim Y, Hung G, Revenko A, Arun G, Stentrup M, Gross M, Zornig M, MacLeod AR, Spector DL and Diederichs S. The noncoding RNA MALAT1 is a critical regulator of the metastasis phenotype of lung cancer cells. Cancer Res 2013; 73: 1180-1189.

[27] Qi P and Du X. The long non-coding RNAs, a new cancer diagnostic and therapeutic gold mine. Mod Pathol 2013; 26: 155-165.

[28] Berrondo C, Flax J, Kucherov V, Siebert A, Osinski T, Rosenberg A, Fucile C, Richheimer S and Beckham CJ. Expression of the Long Non-Coding RNA HOTAIR Correlates with Disease Progression in Bladder Cancer and Is Contained in Bladder Cancer Patient Urinary Exosomes. PLoS One 2016; 11: e0147236.

[29] Clark AG and Vignjevic DM. Modes of cancer cell invasion and the role of the microenvironment. Curr Opin Cell Biol 2015; 36: 13-22.

[30] Li L and Li W. Epithelial-mesenchymal transition in human cancer: comprehensive reprogramming of metabolism, epigenetics, and differentiation. Pharmacol Ther 2015; 150: 33-46.