BIOMARKERS, GENOMICS, PROTEOMICS, AND GENE REGULATION

Long Intergenic Noncoding RNA HOTAIR Is Overexpressed and Regulates PTEN Methylation in Laryngeal Squamous Cell Carcinoma

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Homeobox (HOX) transcript antisense RNA (HOTAIR) is a long intergenic noncoding RNA (lincRNA) that is significantly overexpressed in breast and hepatocellular cancers. It remains unclear, however, whether HOTAIR plays an oncogenic role in human laryngeal squamous cell cancer (LSCC). We therefore investigated the expression and functional role of HOTAIR in LSCC. HOTAIR levels were significantly higher in LSCC than in corresponding adjacent non-neoplastic tissues, and patients with poor histological grade or advanced clinical stage had higher HOTAIR expression. Log-rank test showed a significant association between high levels of HOTAIR and poor prognosis in LSCC patients. Multivariate Cox analysis suggested that HOTAIR is an independent prognostic factor of LSCC. siRNA-mediated knockdown of HOTAIR led to reduced invasion and increased apoptosis of Hep-2 cells in vitro and significantly reduced growth of LSCC xenograft tumors in mice. Moreover, PTEN methylation was significantly reduced in Hep-2 cells depleted of HOTAIR. Taken together, these results suggest that the oncogenic role of HOTAIR in LSCC is related to promotion of PTEN methylation. HOTAIR could serve as a marker for LSCC prognosis and a potential target for therapeutic intervention. (Am J Pathol 2013, 182: 64-70; http://dx.doi.org/10.1016/j.ajpath.2012.08.042)

Laryngeal squamous cell carcinoma (LSCC) is one of the most aggressive neoplasms of the head and neck. Despite recent advances in oncological and surgical treatments, the prognosis of advanced LSCC remains poor. A recent study indicated overall 1- and 2-year survival rates of 56.4% and 26.5%, respectively, for LSCC patients without any treatment such as laryngectomy, radiotherapy, or chemotherapy. A meta-analysis indicated an overall 5-year survival rate of 64.2% for LSCC. A better understanding of the molecular mechanisms underlying LSCC carcinogenesis is therefore essential for selecting accurate predictive biomarkers and for developing effective therapeutic strategies.

A class of small noncoding RNAs (ncRNAs) called microRNAs (miRNAs) have been implicated in the progression, prognosis, and therapy of malignant tumors. Our previous study showed that miR-21 plays an important role in the invasion and progression of LSCC. Nevertheless, recent evidence suggested that other ncRNAs, such as long intergenic noncoding RNAs (lincRNAs) and the heterogeneous group of long noncoding RNAs (lnRNAs), also contribute to human disorders, including cancer. Homeobox (HOX) transcript antisense RNA (HOTAIR) is a lincRNA that is crucially involved in human neoplasia. HOTAIR is significantly overexpressed in breast and hepatocellular cancers. It remains unclear, however, whether HOTAIR plays an oncogenic role in LSCC. To address this issue, in the present study we first detected the level of HOTAIR in LSCC tissues by quantitative real-time RT-PCR (qPCR) and found that HOTAIR expression was correlated to the progression and prognosis of LSCC. Next, we investigated the biological functions of HOTAIR in LSCC cells and found that HOTAIR

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knockdown inhibited the malignant phenotypes of LSCC cells. Moreover, we found that HOTAIR modulated PTEN methylation, revealing a novel mechanism by which HOTAIR regulates tumorigenesis.

Materials and Methods

Patients and Samples

Included in the study were 72 patients with laryngeal cancer who underwent partial or total laryngectomy at the Department of Otorhinolaryngology of the Second Affiliated Hospital of Harbin Medical University between March 2005 and October 2006. The patients had not received any therapy before admission. After surgery, the matched specimens of LSCC and the corresponding adjacent non-neoplastic tissues obtained from patients were preserved in liquid nitrogen within 5 minutes of excision and then were transported frozen to the laboratory and stored at −80°C. The study was approved by the Ethics Committee of Harbin Medical University and informed consent was obtained.

Cell Culture and Virus Transduction

Hep-2 cells of human LSCC were kindly provided by the Harbin Medical University Laboratory of Cell Pathology. Cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (Gibco; Life Technologies, Carlsbad, CA) in a humidified incubator (37°C, 5% CO2). Hep-2 cells were plated in 24-well plates (2 × 10^4 cells/well) overnight. The lentiviruses were diluted in 0.2 mL [10^7 transduction units (TU)/mL] complete medium containing hexadimethrine bromide (Polybrene; 8 mg/mL) and were incubated with the cells for 1 hour at 37°C. Next, the cells were incubated with 0.3 mL fresh prepared Polybrene-Dulbecco’s modified Eagle’s medium for another 24 hours; the medium was then replaced with fresh Dulbecco’s modified Eagle’s medium and the cells were cultured for 48 hours.

qPCR

Total RNA was extracted from cancerous and noncancerous specimens and Hep-2 cells, and the expression level of HOTAIR was determined by qPCR, as described previously. The primers for HOTAIR were 5’'-GGAGAACACUUAAUAAGUTT-3’ (sense) and 5’'-ACUUAAUAAUGUGUUCCTCA-3’ (antisense), as reported previously. The recombinant lentivirus of HOTAIR siRNA and the control lentivirus (GFP lentivirus) were prepared and titered to 10^8 TU/mL.

Cell Invasion Assay

For invasion assay, modified Boyden chambers with filter inserts (pore size, 8 μm) coated with Matrigel (BD Biosciences, Bedford, MA) in 24-well dishes were used. Approximately 2.5 × 10^4 Hep-2 cells in 100 μL of complete RPMI 1640 medium were placed in the upper chamber, and 1 mL of complete RPMI 1640 medium was placed in the lower chamber. After 48 hours, cells were fixed in methanol for 15 minutes and then stained with 0.05% crystal violet in PBS for 15 minutes. Cells on the upper side of the filters were removed with cotton-tipped swabs. Cells that had invaded into the lower side of the filter were then stained with H&E and microscopically observed and counted in five fields of view at ×200 magnification. The invasive activity of cancer cells was expressed as the mean number of cells that invaded to the lower side of the filter. Results are expressed as the number of cells per field of view (mean ± SD). Experiments were repeated at least three times.

Apoptosis Assay

Hep-2 cells were washed twice with cold 10 mmol/L PBS and resuspended in 1× binding buffer (BD Biosciences, San Jose, CA) at a concentration of 1 × 10^6 cells/mL. Cells were stained with Annexin V/APC and propidium iodide, using an Annexin V apoptosis detection kit (KeyGen Biotech, Nanjing, China). Apoptotic cells were detected by flow cytometry. Hep-2 cells without any treatment were used as negative control, and the experiments were repeated at least three times. In addition, the morphological changes of apoptotic cells were detected by staining with the DNA-binding fluorochrome Hoechst 33258 (bisbenzimide), as described previously.

Xenografts in Mice

Sixteen BALB/c mice, age 5 to 6 weeks, were provided by Vital River Laboratories (Beijing, China). They were bred in aseptic conditions and kept at a constant humidity and temperature according to standard guidelines under a protocol approved by Harbin Medical University. All mice were injected subcutaneously in the dorsal scapula region with 100-μL suspension (1 × 10^6) of Hep-2 cells. The size of the tumor was measured twice a week with calipers, and the volume of tumor was determined using the simplified formula of a rotational ellipsoid (length × width^2 × 0.5). Once tumors reached approximately 0.5 to 0.6 cm^3, the mice received an injection into the tumor once a week for...
3 weeks. Mice in the experimental group (n = 8) were treated with 100 μL HOTAIR siRNA lentivirus; mice in the control group (n = 8) received an injection of 100 μL GFP lentivirus. Tumors were harvested 1 week after the end of treatment.

Methylation-Specific and Bisulfite Sequencing PCR Analyses

Promoter methylation of PTEN was determined by methylation-specific PCR and bisulfite sequencing PCR analyses. Genomic DNA was extracted from Hep-2 cells and subjected to bisulfite modification using an EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA) according to the manufacturer’s protocol. PCR was performed in a final volume of 25 μL containing 10 mmol/L Tris-Cl (pH 8.3), 50 mmol/L KCl, 1.25 mmol/L MgCl2, 100 μmol/L dNTP, 0.6 mmol/L of each primer, 1 unit Taq DNA polymerase, and bisulfite-modified DNA (25 ng). Primers for methylated PTEN were 5′-TTCGTTCCGTGCTGCTGATT-3′ (forward) and 5′-GCCGCTTAACTCTAACCGAACC-3′ (reverse), yielding a 206-bp product. Primers for unmethylated PTEN were 5′-GTGGTTGGTGG-AGGTAGTGTGT-3′ (forward) and 5′-ACCACCTAATCATTAAACCCACCAACA-3′ (reverse), yielding a 162-bp product. Primers for bisulfite sequencing PCR of PTEN were 5′-GAGGTTTTAGTTAAAGGTGTGATATG-3′ (forward) and 5′-CATACCCAATATAACTCATTAATAAACCTTAC-3′ (reverse). The amplification conditions were as follows: initial denaturation at 95°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 56°C ( unmethylated PTEN ) or 54°C ( methylated PTEN ) or 53°C ( bisulfite sequencing of PTEN gene) for 30 seconds, and 72°C for 60 seconds; and a final extension at 72°C for 10 minutes. PCR products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV illumination. For bisulfite sequencing, PCR products were directly cloned into TOPO TA vector and sequenced.

Statistical Analysis

Data are expressed as means ± SD of three independent experiments, each performed in triplicate. Differences between groups were assessed by unpaired, two-tailed Student’s t-test. Survival curves were estimated by using Kaplan–Meier method and were compared by using the log-rank test. Multivariate Cox proportional hazards analysis was performed with survival as the dependent variable. P < 0.05 was considered significant.

Results

HOTAIR Is Overexpressed in LSCC

qPCR analysis showed that HOTAIR levels were significantly higher (16-fold) in LSCC tumor tissues (T) than in adjacent non-neoplastic tissues (N): 3.308 ± 1.527 versus 0.205 ± 0.102, respectively (Figure 1). The T/N ratios of HOTAIR expression were statistically related with T grade, differentiation, neck nodal metastasis, and clinical stage of LSCC (Table 1 and Supplemental Figure S1). Tumors with grade T3 to T4, lymph node metastasis, poor differentiation, or advanced clinical stages expressed higher levels of HOTAIR.

Kaplan–Meier analysis showed that patients with high HOTAIR expression had significantly shorter overall survival than those with weak HOTAIR expression (P = 0.011) (Figure 2). Multivariate analysis showed that HOTAIR expression (P = 0.023; hazard ratio HR = 2.856, 95% CI = 1.154 to 7.071) and neck nodal metastasis (P = 0.015; HR = 2.873, 95% CI = 1.225 to 6.738) were independent prognostic factors of overall survival rate of LSCC patients. Taken together, these results suggest that HOTAIR plays an important role in the progression of LSCC.

HOTAIR Knockdown Inhibits the Invasion of Hep-2 Cells

To investigate the biological role of HOTAIR in LSCC progression, we selected Hep-2 cells as the experimental model. We used HOTAIR siRNA lentivirus to knock down HOTAIR expression in Hep-2 cells. More than 90% of Hep-2 cells expressed GFP at 72 hours after lentivirus transduction (Figure 3, A and B), indicating an efficient and stable transduction of the lentiviral vector. Furthermore, qPCR analysis showed that HOTAIR was expressed at a significantly lower level in Hep-2 cells transduced by HOTAIR siRNA lentivirus than in cells transduced by GFP lentivirus, indicating that HOTAIR siRNA decreased HOTAIR expression effectively (Figure 3C).

Next, a Boyden chamber was used to examine the effect of HOTAIR siRNA on the invasion of Hep-2 cells in vitro. The number of cells that invaded the Transwells was significantly reduced in HOTAIR siRNA lentivirus-transduced cells than in control lentivirus-transduced cells (Figure 3D).
HOTAIR Knockdown Induces Apoptosis of Hep-2 Cells

Flow cytometric analysis showed that the percentage of apoptotic cells was significantly increased in HOTAIR siRNA-transduced Hep-2 cells than in the cells transduced with GFP lentivirus (Figure 4, A–C). Furthermore, Hoechst 33258 staining showed that typical apoptotic morphological changes such as nuclear fragmentation, chromatin compaction, and condensation of cytoplasm were observed in HOTAIR siRNA-transduced Hep-2 cells (Figure 4, D–F). Both flow cytometric analysis and Hoechst 33258 staining suggested that HOTAIR exhibits antiapoptotic effects in Hep-2 cells.

HOTAIR Knockdown Suppresses LSCC Tumor Growth in Vivo

To provide in vivo evidence for the oncogenic role of HOTAIR in LSCC, we used a xenograft mice model. After the 16 mice were subcutaneously injected with Hep-2 cells, all of them developed detectable tumors. The growth of the LSCC xenograft was significantly inhibited in mice treated with HOTAIR siRNA lentivirus, compared with the control, as determined by qPCR analysis. Data are expressed as means ± SD. **P < 0.01. Original magnification, ×200.

Table 1  Relationship between HOTAIR Expression and Clinico-pathologic Parameters of LSCC

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (n = 72)</th>
<th>HOTAIR expression level*</th>
<th>P value</th>
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<td>Sex</td>
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<td></td>
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<tr>
<td>Male</td>
<td>50</td>
<td>15.41 ± 8.38</td>
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<tr>
<td>Female</td>
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<tr>
<td>&gt;59</td>
<td>36</td>
<td>16.69 ± 9.24</td>
<td>0.3029</td>
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<tr>
<td>≤59</td>
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<td>15.58 ± 10.80</td>
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<tr>
<td>T1-T2</td>
<td>44</td>
<td>13.98 ± 9.33</td>
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<tr>
<td>T3-T4</td>
<td>28</td>
<td>19.52 ± 7.35</td>
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<tr>
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<td>14.56 ± 8.49</td>
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</tr>
<tr>
<td>G2</td>
<td>20</td>
<td>20.22 ± 9.14</td>
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<td>Lymph node metastasis</td>
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<tr>
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<td>III–IV</td>
<td>33</td>
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*T/N expression ratio: expression in tumor tissue (T) versus adjacent nonneoplastic tissue (N).

HOTAIR Knockdown Inhibits the Invasion of Hep-2 Cells

Figure 3  HOTAIR siRNA inhibits the invasion of Hep-2 cells. A and B: Fluorescence (A) and light (B) micrographs of Hep-2 cells 72 hours after transduction with HOTAIR siRNA lentivirus. C: The relative expression level of HOTAIR in Hep-2 cells is significantly decreased by HOTAIR siRNA lentivirus, compared with the control, as determined by qPCR analysis. D: HOTAIR knockdown results in a significant reduction of Hep-2 cells invading the porous Transwells, compared with controls. Data are expressed as means ± SD. **P < 0.01. Original magnification, ×200.

Figure 2  Kaplan–Meier curves indicate significantly shorter 5-year overall survival for LSCC patients (n = 72) in whose tumors HOTAIR was highly expressed (46%; n = 33; lower curve), compared with weak expression (54%; n = 39; upper curve). P = 0.011.

Figure 4  HOTAIR lincRNA in LSCC

To provide in vivo evidence for the oncogenic role of HOTAIR in LSCC, we used a xenograft mice model. After the 16 mice were subcutaneously injected with Hep-2 cells, all of them developed detectable tumors. The growth of the LSCC xenograft was significantly inhibited in mice treated with HOTAIR siRNA lentivirus, compared with mice treated with GFP lentivirus (Figure 5). The average tumor weight in the HOTAIR siRNA-treated LSCC xenografts was significantly lower than in the control group: 1.113 ± 0.209 g versus 1.960 ± 0.584 g, respectively (P = 0.004).

HOTAIR Regulates PTEN Methylation in Hep-2 Cells

To investigate the potential mechanism by which HOTAIR contributes to malignant behaviors of Hep-2 cells, we focused on DNA methylation of PTEN, because lncRNAs are known to be involved in epigenetic regulation and because DNA methylation of PTEN has been confirmed in laryngeal cancer. PTEN promoter methylation was detected by promoter methylation analysis in control but not in HOTAIR siRNA-transduced Hep-2
cells. By contrast, unmethylated PTEN was detected in HOTAIR siRNA-transduced Hep-2 cells but not in control cells (Figure 6).

To further confirm that HOTAIR regulates PTEN methylation in Hep-2 cells, we performed bisulfite sequencing of cloned alleles over the region of −1120 to −1565 of the PTEN promoter. PTEN CpG islands were densely hypermethylated in control Hep-2 cells, but Hep-2 cells transduced with HOTAIR siRNA showed fewer methylated CpG dinucleotides (Figure 7). Taken together, these results suggest that HOTAIR participates in regulating PTEN methylation.

Discussion

HOTAIR is a lincRNA transcribed from the HOX locus; it regulates the chromatin methylation state of the HOXD locus through the recruitment of the polycomb repressive
complex (PRC2). Recent studies showed that the expression of HOTAIR is increased in several types of cancer and could be used to predict metastasis and death in primary breast tumors, independent of clinicopathologic factors such as tumor size, stage, and hormone receptor status.

In the present study, we examined the expression pattern of HOTAIR in LSCC tissues and investigated its clinical implications. qPCR analysis showed that HOTAIR was overexpressed in primary LSCC, compared with adjacent noncancerous tissues. Moreover, high HOTAIR expression levels were correlated with poor differentiation, lymph node metastasis, or advanced clinical stages of LSCC. These data suggest that HOTAIR promotes the malignant progression of LSCC. A recent multivariate analysis revealed HOTAIR as a powerful independent prognostic factor of hepatocellular carcinoma. In the present study, overexpression of HOTAIR had statistically significant association with decreased 5-year overall survival in LSCC. Cox multivariate analysis showed that HOTAIR expression was an independent prognostic factor of overall survival rate.

To further understand the biological function of HOTAIR in LSCC progression, both in vitro and in vivo assays were performed. Our in vitro data showed that siRNA-mediated knockdown of HOTAIR led to significant decrease of invasive ability of Hep-2 cells and significant increase of apoptosis. Furthermore, we injected Hep-2 cells into mice to make xenografts, which were treated with HOTAIR siRNA lentivirus. The average tumor weight was significantly lower in these mice, compared with control, suggesting that HOTAIR knockdown can effectively suppress the progression of LSCC in vivo. Taken together, these results indicate that high levels of HOTAIR promote the malignant phenotypes of LSCC cells, including invasiveness and resistance to apoptosis.

The tumor suppressor gene PTEN inhibits the phosphatidylinositol 3-kinase (PI3K) signaling pathway. PTEN is reported to be dysfunctional in the pathogenesis of a number of human malignancies, including laryngeal cancer, and aberrant methylation in tumor suppressor genes has been linked to cancer progression and outcome. In the present study, methylation-specific PCR analysis showed that promoter methylation of PTEN was correlated with the loss of PTEN expression in LSCC cells. siRNA-mediated knockdown of HOTAIR in Hep-2 cells significantly decreased the methylation of PTEN. Moreover, bisulfite sequencing showed that CpG methylation in PTEN promoter region was reduced after HOTAIR knockdown. These data suggest that HOTAIR suppresses PTEN expression via epigenetic modification, thus promoting tumorigenesis.

In summary, we report here for the first time that HOTAIR is overexpressed in LSCC tumor tissues and is associated with progression and prognosis of LSCC. Moreover, HOTAIR promotes invasiveness and resistance

![Figure 6](image6.png)

Figure 6  HOTAIR siRNA decreases PTEN methylation in Hep-2 cells. A: PTEN promoter methylation was detected in control Hep-2 cells (lanes 2 and 4), but not in HOTAIR siRNA-infected Hep-2 cells (lanes 1 and 3). B: Unmethylated PTEN was detected in the HOTAIR siRNA-infected Hep-2 cells (lanes 1 and 3), but not in control cells (lanes 2 and 4).

![Figure 7](image7.png)

Figure 7  Methylation of the human PTEN promoter in Hep-2 cells. A and B: Representative results of bisulfite sequencing for control Hep-2 cells (A) and Hep-2 cells infected with HOTAIR siRNA lentivirus (B). C and D: Methylation mapping of 23 CpG sites of the PTEN promoter region obtained from bisulfite sequencing in control Hep-2 cells (C) and Hep-2 cells infected with HOTAIR siRNA (D). Black circles, methylated; white circles, unmethylated.
to apoptosis in LSCC cells. These oncogenic effects of HOTAIR are related to promotion of PTEN methylation by HOTAIR. Taken together, these results suggest that HOTAIR could serve as a marker for LSCC prognosis and a potential target for therapeutic intervention.

Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2012.08.042.

References