The expression and function of long noncoding RNA IncRNA-ATB in papillary thyroid cancer

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Abstract. - OBJECTIVE: This study aimed to investigate the expression and clinical significances of long noncoding RNA-ATB (IncRNA-ATB) in papillary thyroid cancer (PTC), and to explore the roles of IncRNA-ATB in PTC cell proliferation and migration.

PATIENTS AND METHODS: The expression of IncRNA-ATB in 64 PTC tissues and paired adjacent noncancerous thyroid tissues was measured. The association between IncRNA-ATB expression and clinicopathological characteristics was analyzed by Pearson X2. The diagnostic value of IncRNA-ATB was evaluated by receiver operating characteristic curve (ROC) analyses. The effects of IncRNA-ATB on PTC cell proliferation were evaluated by Cell Counting Kit-8 assays and Ethynyl deoxyuridine incorporation assays. The effects of IncRNA-ATB on PTC cell migration were evaluated by transwell assays.

RESULTS: LncRNA-ATB is upregulated in PTC tissues compared with noncancerous tissues. IncRNA-ATB is also increased in PTC cell lines compared with normal thyroid follicular epithelial cell line. High-expression of IncRNA-ATB is associated with large tumor size and lymph node metastasis. ROC analyses revealed that IncRNA-ATB could sensitively discriminate PTCs from noncancerous tissues, as well as discriminating PTCs with lymph node metastasis from those without lymph node metastasis. Functional experiments showed that depletion of IncRNA-ATB significantly inhibits PTC cell proliferation and migration.

CONCLUSIONS: LncRNA-ATB is upregulated and functions as an oncogene in PTC. Furthermore, IncRNA-ATB may serve as a diagnostic biomarker and therapeutic target for PTC.

Key Words: Long noncoding RNA, IncRNA-ATB, Papillary thyroid cancer, Proliferation, Migration.

Introduction

Recently, the incidence rate of thyroid cancer is increasing markedly worldwide, making it the most common endocrine malignancy.3,4 Papillary thyroid cancer (PTC) is the main histologic type and accounts for more than 80% of all thyroid cancer.4,5 Although PTC is usually an indolent cancer with good prognosis and low mortality rate, some clinicopathological characteristics are known to be correlated with fatal outcomes, such as large primary tumor, lymph node metastasis, and distant metastasis.6-8 Therefore, it is essential to understand the molecular mechanisms of PTC, identify biomarkers for early diagnosis of PTC with high-risk of aggressiveness, and develop targeted therapy for PTC. Due to the great progresses in high-throughput sequencing technologies, it is estimated that 75% of the genome is actively transcribed, but only 2% of the geno-
me encodes protein. This implies that most of the transcriptome are noncoding RNAs. Among these noncoding RNAs, long noncoding RNAs (lncRNAs) represent a new type of RNA molecules with more than 200 nucleotides in length and limited protein-coding potential. Mounting evidence indicts that many lncRNAs have critical roles in various pathophysiological processes and the expressions of lncRNAs are frequently dysregulated in multiple diseases, including cancers. For example, lncRNA SAMMSON is upregulated in melanoma, and silencing SAMMSON drastically decreases melanoma cells viability. lncRNA SChLAP1 is overexpressed in prostate cancer and indicates poor outcomes of prostate cancer patients. Furthermore, SChLAP1 is critical for prostate cancer cells invasion and metastasis. LncRNA activated by transforming growth factor-β (lncRNA-ATB) is a recently identified lncRNA with dysregulated expression and critical roles in many cancers, including hepatocellular carcinoma, gastric cancer, breast cancer, etc. However, the expression, clinical significances and functions of lncRNA-ATB in PTC are still unknown. In this study, we measured the expression of lncRNA-ATB in PTC tissues and cell lines, analyzed its association with clinicopathological characteristics of PTC patients, and explored its diagnostic values for PTC and PTC with lymph node metastasis. Furthermore, we investigated the functions of this lncRNA in PTC cell proliferation and migration.

Patients and Methods

Patients and Samples
A total of 64 PTC tissues and paired adjacent noncancerous thyroid tissues were obtained from PTC patients who underwent radical surgical resections at Chinese PLA General Hospital (Beijing, China). None of the patients received radiotherapy or chemotherapy before surgery. All the tissues were confirmed by pathological examination in this hospital. These tissue samples were immediately frozen in liquid nitrogen after surgery and stored at -80°C until use. All these PTC patients signed written informed consent. This study was approved by the Ethical Committee of Chinese PLA General Hospital.

Cell Cultures
The human PTC cell lines IHH-4, TPC-1, K1 and HTH83, and normal thyroid follicular epithelial cell line Nthy-ori 3-1 were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). All these cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO2 atmosphere.

RNA Extraction and Real-time PCR
Total RNAs were isolated from tissues and cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. The first strand complementary DNA was synthesized using the M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. Real-time PCR was carried out using the SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, China) on StepOnePlus™ Real-time PCR Systems (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocols. LncRNA-ATB expressions in tissues and cells were normalized to β-actin using the comparative Ct method. The primer sequences used are as follows: for lncRNA-ATB, reverse transcription primer, 5’-ACACAGAATAAAATAAACAC-3’, Real-time PCR primer, 5’-TCTGGCTGAGGCTGGTGAC-3’ (forward) and 5’-ATCTCTTGCTGCTGGTGGAAGG-3’ (reverse); for β-actin, 5’-GGGAAATCGTGCGTGAATTAAG-3’ (forward) and 5’-TGTGTTGGTGCTACAGGTTTGT-3’ (reverse).

Small Interfering RNA (siRNA) Synthesis and Transfection
lncRNA-ATB specific and control siRNAs were synthesized by Invitrogen (Carlsbad, CA, USA). The siRNA sequences for lncRNA-ATB are: 5’-CTCTCTTGCTGCTGGTGAC-3’ (forward) and 5’-ATCTCTTGCTGCTGGTGGAAGG-3’ (reverse); for β-actin, 5’-GGGAAATCGTGCTGCTGGTGGAAGG-3’ (forward) and 5’-TGTGTTGGTGCTACAGGTTTGT-3’ (reverse).

Cell Proliferation Assays
Cell proliferation was assessed using Cell Counting Kit-8 (CCK-8) assays and Ethynyl deoxyuridine (EdU) incorporation assays. For CCK-8 assays, 4,000 cells/well were plated in 96-well plates. After culture for 0, 24, 48, and 72 h, cell viability was measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). The cell growth curves were plotted using the absorbance values at 450 nm at each time point. EdU incor-
poration assays were carried out using an EdU kit (Roche, Mannheim, Germany) following the manufacturer’s instructions.

**Cell Migration Assays**

Cell migration was assessed using transwell assays. Briefly, 50,000 cells suspended in serum-free Dulbecco’s Modified Eagle Medium (DMEM) with 1 μg/ml mitomycin C were seeded into the upper well of a 24-well poly-carbonate transwell filters (Millipore, Bedford, MA, USA). DMEM supplemented with 10% serum was added to the lower well. After incubation for 48 h, cells on the upper surface of filters were scraped off, and cells on the lower surface were fixed, stained and counted.

**Statistical Analysis**

All statistical analyses were performed with the GraphPad Prism Software (La Jolla, CA, USA). Wilcoxon signed-rank test, Pearson X2, ROC analysis and Student’s t-test were performed as indicated. *p*-values < 0.05 were considered as statistically significant.

### Table I. Association between lncRNA-ATB expression and clinicopathological characteristics of PTC patients.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>IncRNA-ATB</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>All cases</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Gender</td>
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<tr>
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<td>9</td>
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<tr>
<td>Female</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 45</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>≥ 45</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>Focality</td>
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<td></td>
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<tr>
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<td>17</td>
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<td>Multifocal</td>
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<td>15</td>
</tr>
<tr>
<td>Extrathyroidal extension</td>
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<td></td>
</tr>
<tr>
<td>Positive</td>
<td>8</td>
<td>14</td>
</tr>
<tr>
<td>Negative</td>
<td>24</td>
<td>18</td>
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<tr>
<td>Primary tumor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1-T2</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>T3-T4</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
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</tr>
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<td>N0</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>N1</td>
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<tr>
<td>III-IV</td>
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</table>

Median expression level of lncRNA-ATB was used as the cutoff. *p*-value was acquired by Pearson X2 test.

### Results

**LncRNA-ATB is Increased in PTC Tissues and Cell Lines**

To investigate the expression pattern and clinical significances of lncRNA-ATB in PTC, we first measured lncRNA-ATB expression in 64 PTC tissues and paired adjacent noncancerous thyroid tissues. As shown in Figure 1A, lncRNA-ATB was significantly increased in PTC tissues compared with noncancerous tissues (*p* < 0.0001 by Wilcoxon signed-rank test). Furthermore, our results also showed that lncRNA-ATB was significantly increased in PTC cell lines (IHH-4, TPC-1, K1 and HTH83) compared with normal thyroid follicular epithelial cell line (Nthy-ori 3-1) (Figure 1B). We next analyzed the association between lncRNA-ATB expression and clinicopathological characteristics of these 64 PTC patients. As shown in Table I, correlation regression analysis revealed that high expression of lncRNA-ATB was associated with large tumor size (*p* = 0.035) and lymph node metastasis (*p* = 0.021), implying that lncRNA-ATB may be involved in the growth and invasion of PTC.

**LncRNA-ATB Serves as a Diagnostic Biomarker for PTC**

We further investigated the diagnostic values of lncRNA-ATB for PTC. ROC analyses revealed that lncRNA-ATB could accurately discriminate PTC tissues from noncancerous tissues (Figure 2A). The area under the curve (AUC) was 0.9163 (95% CI: 0.8699-0.9627, *p* < 0.0001). The sensitivity and specificity values were 0.8281 and 0.8438, respectively. ROC analyses also revealed that lncRNA-ATB could accurately discriminate PTC tissues with lymph node metastasis from those without lymph node metastasis (Figure 2B), with an AUC of 0.8821 (95% CI: 0.7954-0.9687), a sensitivity of 0.8718, and a specificity of 0.76. These results demonstrated that lncRNA-ATB could serve as a promising biomarker for predicting PTC and lymph node metastasis.

**Silencing of lncRNA-ATB Inhibits PTC cells Proliferation**

To investigate the roles of lncRNA-ATB in PTC, we silenced lncRNA-ATB expression by transfecting lncRNA-ATB specific siRNAs into IHH-4 and HTH83 cells. The transfection of lncRNA-ATB specific siRNAs efficiently decreased lncRNA-ATB expression in both IHH-4 and HTH83 cells (Figure 3 A-B). CCK-8 assays
showed that silencing of lncRNA-ATB significantly decreased IHH-4 and HTH83 cells viability (Figure 3 C-D). Furthermore, EdU incorporation assays also revealed that silencing of lncRNA-ATB significantly inhibited IHH-4 and HTH83 cells proliferation (Figure 3 E-F).

**Silencing of IncRNA-ATB Inhibits PTC Cells Migration**

To investigate the roles of IncRNA-ATB in PTC cells migration, transwell assays were performed. As shown in Figure 4 A-B, silencing of IncRNA-ATB drastically inhibited IHH-4 and HTH83 cells migration. Collectively, these results revealed the important roles of IncRNA-ATB in PTC cells proliferation and migration.

**Discussion**

Although the overall prognosis of PTC is good, the PTC patients with lymph node metas-
In this study, we investigated the expression and clinical significances of lncRNA-ATB in PTC. Our results revealed that lncRNA-ATB is upregulated in PTC tissues and cell lines. High-expression of lncRNA-ATB correlates with large tumor size and lymph node metastasis. Furthermore, lncRNA-ATB could accurately discriminate PTC from noncancerous tissues, as well as PTC with lymph node metastasis from those without lymph node metastasis. Hence, our data suggest that lncRNA-ATB would be a potential biomarker for diagnosing PTC and predicting lymph node metastasis of PTC. Besides lncRNA-ATB, other lncRNAs have also been reported to be associated with PTC lymph node metastasis, including MEG3, NONHSAT037832 and LINC00271.

The combination of these lncRNAs with lncRNA-ATB would be more accurate in predicting metastasis of PTC, and these need further exploration. In addition to the diagnostic values of lncRNA-ATB in PTC, lncRNA-ATB also has critical roles in PTC cell proliferation and migration. Our results revealed...
that depletion of lncRNA-ATB drastically decreases PTC cell proliferation and migration, which implies that targeting inhibition of lncRNA-ATB would be a promising strategy for PTC therapy. Although several lncRNAs have been reported to regulate PTC cells proliferation and migration\textsuperscript{30-33}, our study identifies a new member of lncRNA with important biological roles in PTC and further verifies that lncRNA is a class of transcript critical in tumors. LncRNA-ATB has been reported to promote cancer metastasis via binding and negatively regulating miR-200 family, and then inducing epithelial-mesenchymal transition\textsuperscript{22}. LncRNA-ATB has also been reported to regulate cell cycle related protein and promote cell proliferation\textsuperscript{34}. Whether lncRNA-ATB promotes PTC cell proliferation and migration through the similar mechanisms, or whether other mechanisms contribute to the pro-proliferation and pro-migration effects of lncRNA-ATB in PTC, need further investigation.

**Conclusions**

We found that lncRNA-ATB is upregulated in PTC, whose upregulation associates with large tumor size and lymph node metastasis. Moreover, lncRNA-ATB could accurately discriminate PTC from noncancerous tissues, as well as PTC with lymph node metastasis from those without lymph node metastasis. Functionally, depletion of lncRNA-ATB drastically inhibits PTC cell proliferation and migration. We observed that lncRNA-ATB would be a promising diagnostic biomarker and therapeutic target for PTC.

**Conflict of interest**

The authors declare no conflicts of interest.

**References**


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IncRNA-ATB expression and function in PTC


