Upregulation of the long noncoding RNA LINC00538 contributes to the tumorigenesis in papillary thyroid cancer

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Abstract: The long noncoding RNAs (lncRNAs) have been demonstrated to be involved in various cancers. The recently identified lncRNA LINC00538 is a novel non-coding RNA which maps to a well-established cancer susceptibility locus. However, whether LINC00538 can play a role in papillary thyroid cancer (PTC) remains obscure. In current study, we identified a potential oncogenic role for LINC00538 in papillary thyroid cancer. We found that LINC00538 expression was significantly upregulated in tumorous tissues as well as cancer cell lines. Patients with higher LINC00538 expression displayed poor overall survival. Furthermore, knocking down LINC00538 levels can markedly inhibit colony formation, proliferation and migration in TPC1 and B-CPAP cells. In vivo implantation studies also revealed that reducing endogenous LINC00538 levels can attenuate xenograft tumor growth and showed decreased Ki-67 staining. LINC00538 knockdown can also inhibit epithelial-mesenchymal transition. Our findings suggest that LINC00538 may serve as an oncogene in papillary thyroid cancer and represent a potential diagnostic marker for pharmaceutical intervention.

Keywords: LINC00538, papillary thyroid cancer, EMT, IncRNA

Introduction

The thyroid carcinoma (TC) denotes a kind of endocrine malignancy which displays elevated incidence in recent years. The rate of incidence over the past decades has increased by over two fold [1]. The ever-increasing occurrence of thyroid cancer may possibly be ascribed to two coordinated processes: the diagnostic scrutiny and failure to successful recognition of specific thyroid carcinogens [2]. Despite effective treatment has been developed and relatively high five-year survival, the recurrence rates of TC is relatively high [3]. The TC is usually regarded as a disease with multiple environmental and genetic origins such as radiation, epigenetic causes or other uncertain factors [4]. The papillary thyroid cancer (PTC) represents the most common type of thyroid cancer which roughly contains over 80% cases [5]. Lymph node metastasis, significant tumor size and advanced tumor-node-metastasis (TNM) stages are often associated with unexpected prognosis. Especially, PTC patients with extrathyroidal extension (ETE) usually suffer from incomplete resection and high rate of recurrence [6]. Therefore, due to the complex nature of PTC development, identifying novel biomarkers for PTC patients demands extensive investigation.

The long non-coding RNAs (lncRNAs) denote a specific class of RNAs longer than 200 nucleotides in length [7, 8]. The lncRNAs play pivotal roles in various physiological processes such as angiogenesis, differentiation and cell growth [9-11]. In earlier studies, lncRNAs have been considered to mock transcripts with no obvious functions largely because they are not encoding sequences [12]. Recent studies have suggested that lncRNAs could act as either oncogenes or tumor suppressors and contribute to the tumorigenesis of multiple cancers including thyroid carcinoma [13, 14]. The lncRNA PVT1 has been reported to be positively correlated with thyroid cancer and this effect is mediated by recruiting EZH2 [15]. Kim et al. also found that upregulation of LOC100507661 can promote tumorigenesis of thyroid cancer although the
exact mechanisms remain unknown [16]. The IncRNA H19 can competitively binds miR-17-5p which function as a competitive endogenous RNA to modulate thyroid cancer progression [17]. Yang et al. recently used microarray strategies to identify altered expression of IncRNAs in thyroid cancer tissues and have provided critical insight into the regulatory network of IncRNAs [18]. Thus, IncRNAs can possibly be applied for thyroid cancer diagnosis and also behave like putative therapeutic targets.

The long non-coding RNA LINC00538, which is also named Yiya, is a 1.9 kb long intergenic RNA residing at chromosome 1q41 [19]. LINC00538 is uniformly distributed in both cytoplasm and nucleus and shows no significant homology beyond mammals [19]. LINC00538 was reported to be regulated during cell cycle and function as a modulator of G1/S transition [19]. However, the exact function of LINC00538 in PTC remains elusive.

In current study, we found that LINC00538 can behave like a candidate oncogenic IncRNA. It is frequently upregulated in PTC tissues compared with normal adjacent samples. Meanwhile, patients with higher LINC00538 expression display lower survival rates. LINC00538 knockdown can markedly decrease the colony formation, migration and proliferation of TPC1 and B-CPAP cells. LINC00538 knockdown can also decrease the xenograft tumor growth. We also found that the epithelial and mesenchymal markers are also consistently affected by altering LINC00538 expression suggesting that LINC00538 might be involved in epithelial-mesenchymal transition (EMT). Our data collectively suggest an oncogenic role of LINC00538 in PTC and may provide clues to potential diagnosis.

Materials and methods

Cell culture and human specimen collection

The PTC cell lines used in current research (TPC1, B-CPAP, IHH-4 and CG3) as well as the normal thyroid cell line Nthy-ori 3-1 were all purchased from Shanghai Institute of Cell Biology (Shanghai, China). The PTC cell lines were cultured in DMEM medium with 5% fetal bovine serum (FBS, TIANGEN, Shanghai, China) and maintained in incubator (5% CO₂ at 37°C). The cells displayed growth in monolayer and over 90% attachment were subject to passage.

Matched fresh PTC specimens and normal adjacent tissues were collected from 88 patients at Renmin Hospital of Wuhan University between May 2011 and September 2014. After surgical resection, tissues were immediately stored at -80°C until usage. None of the patients have received preoperative chemotherapy or radiotherapy. All patients have signed formal consent forms. The survival was calculated from the day of surgery to death or the last follow-up. The research for human samples were reviewed and formally approved by Ethics Committee of Renmin Hospital of Wuhan University.

LINC00538 knockdown

The sequences of specific siRNAs targeting LINC00538 and siRNA negative control (NC) were designed and synthesized by TIANGEN (Shanghai, China). For siRNA mediate transfection into TPC1 and B-CPAP cells, these cells were seeded into a 12-well plate with final density of 10⁵ cells/well and transfected with 100 nM LINC00538 specific siRNAs. All transfections were performed using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer’s instructions. Two days after the transfection, the cells were harvested for analysis.

Quantitative real-time RT-PCR

Total RNAs were isolated from both PTC cell lines (TPC1 and B-CPAP) and human samples with Trizol reagent (Invitrogen, Carlsbad, CA, USA). Totally, 2 ng of total RNAs in a final volume of 10 μl containing 5 mM dNTP Mix (TIANGEN, Shanghai, China) was used to generate complementary DNA (cDNA). The mixture was maintained in 70°C for 5 min and then a mixture composed of 5 × RT buffer, 50 U/μl reverse transcriptase, 100 U/μl RNase inhibitor was appended (TIANGEN, Shanghai, China). GAPDH was used as the control. Reactions were performed by the ABI PRISM® 7000 Sequence Detection System (Applied Biosystem, Foster City, USA) according to the manufacturer’s instructions. The expression of LINC00538 was calculated by the 2⁻ΔΔCt method. The experiments were performed by at least triplicates. The primer sequences were: LINC00538: sense: 5'-GAAGCTAGTTTACG-3'; anti-sense: 5'-CTTCTACGTAGTCGATGT-3'; GAPDH: sense: 5'-GTAAGGCGGCTTCCTAAGCTT-3'; anti-sense: 5'-ATGAGGCGGCTTCCTAAGCTT-3'.
Proliferation assay

The Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was used for proliferation assay. After treatment for 24 h, TPC1 and B-CPAP cells were re-suspended and seeded into a 6-well plate (10^5 cells/well) for 5 days. A total 15 ml MTT solutions were appended into the culture with final concentrations of 10 mg/ml. The crystalline formazan was resolved in 100 μl sodium dodecyl sulfate (SDS, 10%) solution for 24 h. Optical density at wavelength of 490 nm was monitored and each assay was repeated for triplicates.

Western blot

Totally, 10 μg proteins were extracted and separated by 10% SDS-PAGE. Then, the proteins were transferred into PVF membrane and incubated with anti-E-cadherin, N-cadherin, vimentin, N-cadherin, vimentin.

Table 1. Correlation between LINC00538 and clinicopathological features

<table>
<thead>
<tr>
<th>Clinicopathological features</th>
<th>No.</th>
<th>LINC00538 expression</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 55</td>
<td>48</td>
<td>High (n, %) 23 (47.9%)</td>
<td>0.415</td>
</tr>
<tr>
<td>≥ 55</td>
<td>40</td>
<td>Low (n, %) 25 (52.1%)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>36</td>
<td>High (n, %) 15 (41.7%)</td>
<td>0.139</td>
</tr>
<tr>
<td>Female</td>
<td>52</td>
<td>Low (n, %) 21 (58.3%)</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 2</td>
<td>39</td>
<td>High (n, %) 11 (28.2%)</td>
<td>0.001</td>
</tr>
<tr>
<td>≥ 2</td>
<td>49</td>
<td>Low (n, %) 28 (71.8%)</td>
<td></td>
</tr>
<tr>
<td>Metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>42</td>
<td>High (n, %) 14 (33.3%)</td>
<td>0.003</td>
</tr>
<tr>
<td>Present</td>
<td>46</td>
<td>Low (n, %) 28 (66.7%)</td>
<td></td>
</tr>
<tr>
<td>Clinical stages</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>35</td>
<td>High (n, %) 12 (34.3%)</td>
<td>0.014</td>
</tr>
<tr>
<td>III-IV</td>
<td>53</td>
<td>Low (n, %) 23 (65.7%)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Expression of LINC00538 in PTC tissues and cell lines. (A) Relative expression of LINC00538 in papillary thyroid cancer (PTC) and normal adjacent tissues (NATs). The association between relative expression of LINC00538 and tumor size (B) or metastasis (C). ***P < 0.001. (D) The expression of LINC00538 in PTC cell lines and normal human thyroid cell line Nthy-ori 3-1. **P < 0.01.

Colony formation assays

The colony formation assays were carried out partially according to a previous study [20]. Briefly, a total of 5000 B-CPAP or TPC1 cells transfected with either control siRNA or si-LINC00538 were added into a 12-well culture plate. After 12 days, the cells were washed using PBS and manipulated with 3.5% formaldehyde.

The migration assays were performed using the 12-well transwell chambers (8 μm pore size; BD Biosciences, San Jose, CA, USA). For migration assay, about 10^6 TPC1 and B-CPAP cells were suspended in 100 μl serum-free medium and placed into the top chambers. DMEM (200 μl) containing 10% FBS was added into bottom chambers. After incubation for 24 h at 37°C, cells not migrating into the pores were removed and cells on lower surfaces of the membrane were stained with crystal violet. The Leica microscope fluorescent microscope (DM-IRB, Leica, Germany) was used to show the results.

Western blot

Totally, 10 μg proteins were extracted and separated by 10% SDS-PAGE. Then, the proteins were transferred into PVF membrane and incubated with anti-E-cadherin, N-cadherin, vimen-
tin or GAPDH antibodies (Sigma, Shanghai, China) overnight at 4°C. After washing with TBST (3 times, 15 min/time), the samples were covered with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1.5 hours. Immunoblots were visualized with chemiluminescence film system (Amersham Pharmacia Biotechnology, Shanghai, China).

In vivo implantation and immunohistochemistry

The transfection of TPC1 cells was performed using lenti-virus transfection system. Initially, the TPC1 cells were maintained for 6 h and then cultured for additional 24 h. Then, cells were re-suspended and totally $5 \times 10^5$ cells were injected subcutaneously into null mice. 30 days later, mice were sacrificed and solid tumor weights were quantified. The nude mice (10 mice for each group, 5~6 weeks old with average weight 17.3 g) were purchased from the Model Animal Research Center (MARC, Nanjing, China). After retrieving antigens in sodium citrate buffer, tissue sections were covered with Ki-67 antibodies (TIANGEN, Shanghai, China). The immunostaining was carried out using primary anti-Ki-67 antibody (TIANGEN, Shanghai, China). Images were shown with 200 x magnification.

Statistical analysis

All statistical results were represented as mean ± SD. Statistical significance for two independent samples were determined by Mann-Whitney test (SPSS, version 16.0, Inc., Chicago, IL, USA) and the significance was identified if $P < 0.05$. Kaplan-Meier survival curve was evaluated using log-rank test. Fisher exact test was used to evaluate the correlation between LINC00538 and various clinicopathological features.

Results

The LINC00538 is upregulated in papillary thyroid cancer as well as cell lines

Since the lncRNA LINC00538 was identified recently [19] and no function has been reported especially in PTC, we next investigated the role of LINC00538 in PTC. We quantified the expression of LINC00538 in PTC tissues and paired normal tissues, the results showed that LINC00538 was significantly upregulated in PTC (Figure 1A). Correlation analysis between LINC00538 and clinicopathological features revealed that LINC00538 was significantly associated with tumor size, metastasis and Clinical stages (Table 1). However, the correlation was not significant for age and gender (Table 1). We also confirmed using qRT-PCR that the group with larger tumor size had higher LINC00538 expression (Figure 1B). Meanwhile, metastatic tumors were markedly associated higher LINC00538 expression (Figure 1C). In PTC cell lines, LINC00538 also displayed higher intrinsic levels (Figure 1D). These results suggested that LINC00538 expression was raised in PTC and may promote the tumor development. Since TPC1 and B-CPAP cells had relatively higher LINC00538 expression, these two cell lines were used for further study.

High LINC00538 expression dictates poor survival

We further determined whether LINC00538 may be correlated with overall survival. We drew the Kaplan-Meier plot and the results showed that higher LINC00538 expression may result in poor overall survival ($P = 0.002$, Figure 2). The lower branch may reach around 40% while the higher branch did not drop off 60% by the end of the follow-up. These results suggested that high LINC00538 level may lead to decreased survival.

Knockdown of LINC00538 may inhibit colony formation, proliferation and migration of PTC cell

The data above suggested that LINC00538 may promote PTC tumorigenesis. To further...
evaluate the biological functions of LINC00538, we knocked down endogenous LINC00538 levels in TPC1 and B-CPAP cells. The knockdown efficiency was verified (Figure 3A). We noticed that LINC00538 knockdown can severely attenuate the colony formation in TPC1 cells as well as B-CPAP cells (Figure 3B). We further evaluated the proliferative capacity of TPC1 and B-CPAP cells with or without si-LINC00538 transfection. The results showed that LINC00538 knockdown can significantly inhibit the proliferation of TPC1 and B-CPAP cells (Figure 3C and 3D). To further verify the effect of LINC00538 in vitro, we performed transwell migration assays. The results showed that si-LINC00538 substantially decreased the migration of TPC1 and B-CPAP cells (Figure 3E). Quantification results further confirmed the significant reduction in TPC cell line migration when cells were transfected with si-LINC00538 (Figure 3F). These results suggested that LINC00538 knockdown can effectively inhibit tumor progression of PTC and argued an oncogenic role for LINC00538.

LINC00538 exerts tumorigenic role in vivo

We also carried out in vivo implantation studies to verify the effect of LINC00538. TPC1 cells were either transfected with si-NC or si-LINC00538 for 48 h. The harvested TPC1 cells were then injected into the nude mice and the volume of solid tumors were evaluated. We found that knocking down LINC00538 levels can obviously decrease the xenograft tumor growth (Figure 4A). Meanwhile, we further found that si-LINC00538 also decreased the tumor weight (P < 0.001, Figure 4B). Ki-67 staining also displayed markedly reduced proliferation in LINC00538 knockdown group (Figure 4C). The fraction of positive staining can decrease by more than two fold (Figure 4D). Accumulating studies suggest that EMT is critically involved in the tumorigenesis of many cancer types. To explore whether EMT was affected by LINC00538 alteration, the expression of E-cadherin (epithelial maker), N-cadherin and Vimentin (mesenchymal markers) were quantified. We found that the expression of E-cadherin was substantially upregulated under the situation when LINC00538 was knocked down in TPC1 cells (Figure 4E). Consistently, N-cadherin and Vimentin expression was reduced with si-LINC00538 in TPC1 cells (Figure 4E, Nthy-ori 3-1 was used as normal controls). Quantifying the expression of these
markers in xenografts showed qualitatively similar results (Figure 4F). These results further confirmed that LINC00538 may enhance tumor development in vivo and might be possibly involved in EMT process.

Discussion

Recent advances in biological technology have greatly advanced our understanding about the patterns of IncRNAs [21]. A myriad of IncRNAs have been shown to play important roles in different malignant tumor types. Despite considerable progress in cancer related research, the potential molecular mechanisms of PTC development remain poorly understood.

Figure 4. LINC00538 potentiates tumor growth in vivo. A. The growth of solid tumors in nude mice injected with TPC1 cells either untreated or transfected with si-LINC00538. The tumor volume was measured using the formula length × width × height. B. At the end of the implantation, solid tumors were resected and the weight was measured. Bars denote the mean values. n = 10 for each. C. The Ki-67 staining for xenografts with si-NC or si-LINC00538 transfection. D. Quantification of the Ki-67 staining. E. Western blot analyses of E-cadherin, N-cadherin and Vimentin for TPC1 cells (the first two lanes) and Nthy-ori 3-1 cells (the last lane). The TPC1 cells were either untreated or transfected with si-LINC00538. F. Western blots of E-cadherin, N-cadherin and Vimentin for tumor xenografts.

Over 90% mammalian genomes denote non-coding sequences which do not encode proteins. The microRNA and IncRNAs represent the two principle types [22]. Tremendous efforts have been devoted to establishing associations between altered IncRNA expression and tumor incidence in recent years [12-14, 23, 24]. Owing to the complexity in tumor microenvironment, the IncRNA can play diverse roles in carcinogenesis in a tumor type specific manner. Since IncRNAs can also function as diagnostic or prognostic markers, determining the potential link between IncRNAs and various tumors has greatly challenged current researches [13, 23-25]. In current study, we identified an oncogenic role for LINC00538 in papillary thyroid cancer. We found that LINC00538 expression was dramatically elevated in PTC tissues as well as PTC cell lines. By obtaining the Kaplan-Meier curves, we found that patients with higher LINC00538 expression were predicted with relatively poor overall survival. Furthermore, si-RNA mediated LINC00538 knockdown can markedly inhibit colony formation, proliferation and migration in TPC1 and B-CPAP cells. In vivo implantation studies also exhibited that reducing endogenous LINC00538 levels can attenuate xenograft tumor growth. The xenograft resection displayed reduced Ki-67 staining in the group transfected with si-LINC00538. LINC00538 knockdown can also inhibit epithelial-mesenchymal transition with reduced N-cadherin, Vimentin expression and increased E-cadherin production. These results collectively argued that LINC00538 may possibly function as a tumorigenic IncRNA in papillary thyroid cancer.

The IncRNA LINC00538 was firstly identified in 2012 by Yang et al. by a negative selection
LINC00538 in PTC

The expression of LINC00538 is ubiquitously expressed in major tissues and genomic amplification in LINC00538 is observed in liver, esophageal, ovary and breast cancers [19]. The potential mechanism of LINC00538, however, remains largely elusive. Meanwhile, the role of LINC00538 has never been reported. Therefore, we have provided the first insight into the oncogenic role of LINC00538 in papillary thyroid cancer. The EMT might be implicated in the oncogenic role of LINC00538 as LINC00538 can regulate the expression of specific markers to favor EMT. Noticeably, LINC00538 is located upstream of the famous transcription factor Prospero-related homeobox 1 (PROX1) [19], which play important roles in cell fate decision and development. Whether there exists dynamic linkage between PROX1 and LINC00538 requires further investigation. Since LINC00538 may promote tumor development in various cancers, unraveling the intrinsic mechanisms of LINC00538 mediated tumor progression may create a fertile ground for future researches.

There are also some limitations in current study. The detailed signaling of LINC00538 mediated tumor promotion in PTC remains largely unknown. Furthermore, large scale screening studies are needed to identify other functional lncRNAs involved in PTC development. In addition, longer follow-up studies are required to consolidate our findings.

In conclusion, we have provided the first evidence that LINC00538 can promote papillary thyroid cancer progression by modulating EMT process. The oncogenic role of LINC00538 has been verified in current study using multiple strategies. Due to the significant effect on papillary thyroid cancer development, our data implied that LINC00538 may serve as a potential and novel prognostic biomarker. Therapeutics targeting for LINC00538 might be a promising strategy in papillary thyroid cancer and this effect should be evaluated in future.

Disclosure of conflict of interest

None.

Authors’ contribution

FS and SQS conceived the study. FS and SQS performed the experiments. FS and SQS analyzed the data. FS and SQS wrote the paper. All authors have read and approved the final version of the paper.

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