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Original Paper

The Long Non-Coding RNA ENST00000537266 and ENST00000426615 Influence Papillary Thyroid Cancer Cell Proliferation and Motility

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Key Words
LncRNA • Papillary thyroid cancer • Cell proliferation • Cell motility • Pathogenesis

Abstract
Background/Aims: Papillary thyroid cancer (PTC) is the most common histotype of Thyroid cancer (TC). Here, we detected the differentially expressed lncRNAs in tumor tissues and non-tumor tissues of PTC patients by lncRNA microarrays, and explored the function and molecular mechanisms of lncRNAs in the pathogenesis of PTC using a PTC cell line. ***Methods:*** CCK-8 assay, colony formation assay and EdU assay were used to detect the cell viability. Flow Cytometry was used to detect the cell cycle and apoptosis. Transwell and scratch assay were used to detect the cell motility. ***Results:*** CCK-8 assay, colony formation assay and EdU assay revealed that lncRNAs (ENST00000537266 and ENST00000426615) could inhibit cell proliferation. Cell cycle analysis showed that cell proportion was statistically significant increased in G1 phase and decreased in S phase and G2 phase in Si-266 transfected TPC-1 cells. In addition, a noteworthy increase of cell proportion in G1 phase accompanied by a decrease in S phase and unchanged G2 phase in Si-615 transfected TPC-1 cells were also observed. Meanwhile, transwell and scratch assay showed that ENST00000426615 could inhibit the cell motility while ENST00000537266 could not. ***Conclusion:*** Our results showed that lncRNAs (ENST00000426615 and ENST00000537266) might be important regulators of PTC cell proliferation and motility, which might provide new insight into the understanding of PTC pathogenesis.

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Introduction

Thyroid cancer (TC) is the most prevalent endocrine malignancy in the world, and its incidence has increased steadily over the past decades [1]. Papillary thyroid cancer (PTC), which is named after their histopathological structure, is the most common histotype of TC that accounts for approximately 80% of all TC in adults [2]. PTC is the fifth leading malignancy in women [3]. The treatment of PTC includes surgery, thyroid hormone, isotope therapy and radioiodine therapy. Although most of the PTC patients have good prognosis and long-term survival [4], it is still very hard to determine pathogenesis.

It is widely accepted that both genetic and environmental factors are important contributors to PTC, but the role of etiology is still not well elucidated [5]. Up to date, mitogen-activated protein kinase (MAPK) signaling pathway related genes including BRAF, RAS, RET have been identified to be associated with the pathogenesis of PTC [6]. Other genes, such as TERT and FoxP3, were also reported to be involved with PTC [7-9]. In addition, microRNAs (miR-21, miR-199a-3p, miR-146a) also have important impacts on the pathogenesis of PTC [10-12].

It is well known that long noncoding RNAs (lncRNAs) are more than 200 nucleotides in length and unable to be translated into proteins. Recently, increasing studies showed that lncRNAs, similar to miRNAs, played important roles in the development and progression of various cancer types [13-15]. Moreover, lncRNAs have been considered as biomarkers for cancer diagnosis and prognosis [16-18]. At present, a few lncRNAs were found to be important regulators of PTC. For instance, BRAF-activated lncRNA (BANCR) could increase PTC cell proliferation and activate autophagy [19]. And Papillary thyroid carcinoma susceptibility candidate 3 (PTCSC3) is a tumor suppressor [20]. However, the role of lncRNAs in PTC is less studied when compared with other cancers.

The aims of the present study were to detect the differentially expressed lncRNAs in tumor tissues and non-tumor tissues of PTC patients by lncRNA microarrays, and to explore the function and molecular mechanisms of lncRNAs in the pathogenesis of PTC.

Materials and Methods

Ethics statement and patient tissue samples

In this study, 22 patients (The Affiliated Jiangyin Hospital of Southeast University Medical College, JY) and 24 patients (Zhongda Hospital of Southeast University Medical College, NJ) with a definite pathological diagnosis of papillary thyroid cancer (PTC) participated in this study. Matched tumor and normal thyroid tissues were obtained from the same patient. All samples were collected with the informed consent of the patients and were immediately frozen in liquid nitrogen, then stored at −80°C. The study was approved by the Ethics Committee of the Southeast University Medical College. Meanwhile, this study was performed in accordance with the government policies and the Declaration of Helsinki.

RNA isolation, lncRNA microarrays, confirmation test of real-time quantitative (RT-PCR)

Total RNA was isolated from tissues and cells using the TRIZOL reagent (Invitrogen, CA, USA) according to the manufacturer's instructions, and the concentration of total RNA was determined by NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE). cDNA synthesis was performed with 1 μg of total RNA by the reverse transcription kit (Takara, Tokyo, Japan). The 4×180K SBC human lncRNA microarrays of 22 patients (JY) that contained about 63,431 lncRNAs were conducted by Shanghai Biotechnology Corporation (China). Slides were scanned by an Agilent Microarray Scanner (Cat#G2565CA, Agilent technologies, Santa Clara, CA, US). Raw data were extracted using Agilent Feature Extraction Software. LncRNA expression levels with more than 10 fold alteration were selected for further analysis. Confirmation test of RT-PCR was performed to determine the expression levels of lncRNAs in 22 patients (JY) and 24 patients (NJ). Primer sequences of lncRNAs are shown in Table 1.

Table 1. Sequences of lncRNAs

Gene		Sequences
ENST00000537266	Forward Primer	5'-TGAAAGTGCTATTGCCTAAAGTGAT-3'
	Reverse Primer	5'-TCCTCTTGGTGGAATGTGGGTGA-3'
ENST00000426615	Forward Primer	5'-TACACCCAGAGAGGGACGAATACAC-3'
	Reverse Primer	5'-TCAAGTTCAACAGCAGTCTTAGCCT-3'
ENST00000457989	Forward Primer	5'-ATGACATGATCGCAACTCCA-3'
	Reverse Primer	5'-GGCCATTGTTTTCAAGTTTCA-3'
ENST00000440673	Forward Primer	5'-TACGGAGGAACTTCAGAGGC-3'
	Reverse Primer	5'-AAGGCAATCAATACATCCAAAC-3'
Gapdh	Forward Primer	5'-GCACCGTCAAGGCTGAGAAC-3'
	Reverse Primer	5'-GGATCTCGCTCCTGGAAGATG-3'
Si-ENST00000537266	Sense	5'-GGAUGUAUCGGUCCUGUCU-3'
	Anti-sense	5'-AGACAGGACCGAUACAUCC-3'
Si-ENST00000426615	Sense	5'-GGACGAAUACACCUCUGAA-3'
	Anti-sense	5'-UUCAGAGGUGUAUUCGUCC-3'

Cell culture and transfection

Two papillary thyroid cancer cell lines (TPC-1, B-CPAP) were kindly provided by Stem Cell Bank, Chinese Academy of Sciences. TPC-1/B-CPAP cell line was maintained in 90% DMEM/RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/mL streptomycin at 37°C with 5% CO_2. Cells were passaged 1:3 once 90% confluence was reached on 10 cm dishes. All TC cell lines used in this study were reported previously [21]. siRNAs [Si-ENST00000537266 (Si-266), Si-ENST00000426615(Si-615), Si-NC] (Ribobio, Guangzhou, China) were used in transfection experiments. Primer sequences of Si-lncRNAs are shown in Table 1. TPC-1 and B-CPAP cells were cultured to 30 - 50% confluence, and the transfection was carried out using Lipofectamine 2000 (Invitrogen Corp, CA, USA).

Cell viability assay

TPC-1 cells were seeded at a density of 4×10^4 cells per well (100 μL) in a 96-well plate. Cell viability was evaluated by a cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Japan) after 0 h, 12 h, 24 h, 36 h, 48 h, 72 h transfection. The absorbance was determined by TECAN infinite M200 plate reader at 450 nm.

Colony formation assay

Colony formation assay was performed using crystal violet (Beyotime, China). About 2000 TPC-1 cells were seeded in a 10 cm dish. After transfection, growth medium was changed every day. Seven days later, cells were fixed with 75% ethanol for 30 min and stained with 0.2% crystal violet. Colonies (>50 cells/colony) were counted and photographed. Each experiment was performed in triplicate independently.

EdU assay

An EdU assay was performed using the Cell Light™ EdU kit (RiboBio, Cat. No. C10310). 8×10^3 TPC-1 cells were seeded in each well of a 96-well plate. After 48 h transfection, 50 μM EdU was added to the culture medium for an additional 2 hours. The cells were fixed with 4% formaldehyde for 30 minutes, and incubated by Glycine (2 mg/mL) for 10 minutes. Finally, the cells were permeabilized by 0.5% Triton X-100 for 20 minutes, and then staining liquid and hoechst 33342 were added. TPC-1 cell proliferation (the percentage of EdU positive cells) was measured by fluorescence microscope (Nikon Eclipse Ti Microscope, Japan). Assays were performed independently three times in triplicate.

Cell cycle analysis and apoptosis assay

After 48h transfection, cells were collected for cell cycle and apoptosis assay. Apoptosis assay was performed with Apoptosis Detection Kit (BD Pharmingen, San Diego, CA, USA). The cells were suspended by 100 μL binding buffer with 5 μL Annexin V and 5 μL propidium iodide, and incubated for 15 min protected

from light. Then, 400 μL binding buffer was added and the cells were resuspended. For cell cycle analysis, cells were fixed in 75% ethanol overnight at -20°C, then stained with propidium iodide (PI) for 30 min protected from light. The cells were analyzed by FACS Calibur Flow Cytometry (BD Biosciences, NJ, USA) to quantify the cell cycle or cell apoptosis. All experiments were performed independently three times in triplicate.

Cell migration assay

Cell migration was studied using 8 μm pores transwell chambers (Milllipore Corporation, Billerica, MA). 100 μL TPC-1 cells (2×10^5/mL) were resuspended in DMEM and seeded to the top chambers of each transwell. And 600 μL complete medium was added into the lower chambers. After 48h transfection, cells were fixed in 4% paraformaldehyde and stained with crystal violet. Then we removed the cells that remained in the top chamber by cotton swabs, and imaged by light microscope (Olympus, China). Assays were performed independently three times using triplicate wells.

Scratch wound assay

Scratch wound assay was carried out by measuring the area of the wounded surface at different time points using Biostation CT (Nikon, Japan). 1×10^5 cells were plated in each well of 6-well plates. After 48h transfection, a scratch was performed by a pipette tip. Images of the same fields were taken at different time points (0 h, 3 h, 6 h, 9 h, 12 h, 15 h, 18 h, 21 h, 24 h, 27 h, 30 h, 33 h, 36 h) after the scratch. Images of different time points formed animation in accordance with the timeline.

Data analysis

Values are expressed as means ± standard error of the mean (S.E.) for all experiments. Statistically significant differences between the tumor tissues and non-tumor tissues were determined by paired t test. Statistically significant differences between the siRNA treatments and the control were tested by one-way ANOVA, followed by Dunnett's multiple comparison test. Statistical analysis was performed using STATA9.2 and presented with GraphPAD prism 5 software. The statistical significance was set at $p < 0.05$.

Results

LncRNA expression profiles

Array hybridization of 22 patients (JY) was performed using the SBC 4×180K human lncRNA microarrays. We identified 777 lncRNAs that were expressed differently in PTC than in normal tissues, with 325 lncRNAs up-regulated and 452 lncRNAs down-regulated (data not shown).

Table 2. Ten candidate lncRNAs expressed > tenfold differently in PTC tissues than in normal tissues

Gene ID	Fold change	Lnc length	Lnc start	Lnc end	Chr
Uc001ssy	19.11566123	18999	66232298	66251296	chr12
Uc001gdd	18.03433474	105315	165446078	165551392	chr1
Uc009xsf	14.56790789	1662	81373514	81375175	chr10
ENST00000537266	14.22807816	3239	46714857	46718095	chr19
ENST00000457989	11.33053753	11374	40550041	40561414	chr12
NR_021485	11.14654427	20792	168527583	168548374	chr3
Uc001ndl	11.01011475	27968	46867977	46895944	chr11
ENST00000537356	10.92506153	3425	103357107	103360531	chrX
ENST00000440673	10.40367549	17391	18667739	18685129	chr22
ENST00000426615	10.37812563	1293	177042445	177043737	chr2

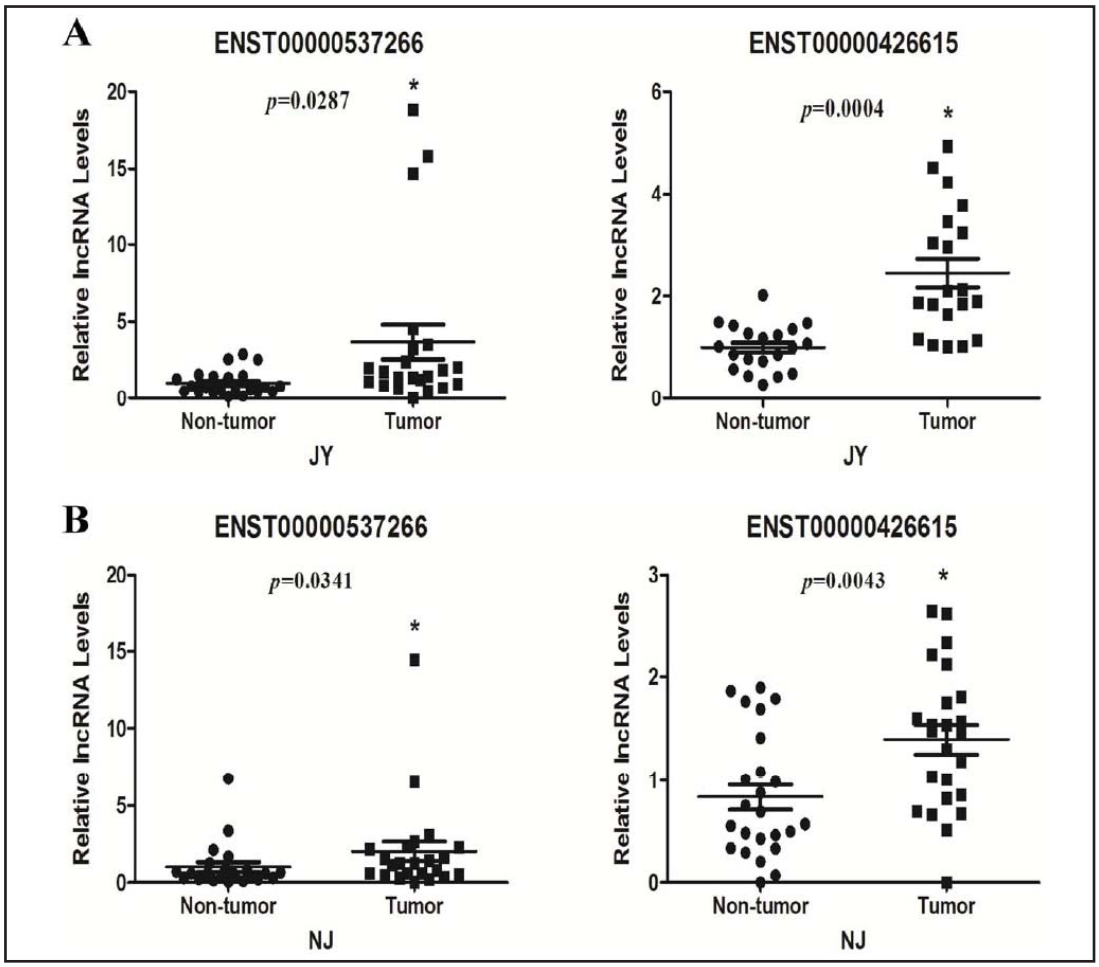

Fig. 1. The expression levels of ENS-266 and ENS-615 in PTC patients. (A) The expression levels of ENS-266 and ENS-615 in Tumor and Non-tumor tissues of PTC (JY, 22 patients) were detected by RT-PCR using a housekeeping gene GAPDH as an internal control. (B) The expression levels of ENS-266 and ENS-615 in Tumor and Non-tumor tissues of PTC (NJ, 24 patients) were detected by RT-PCR using a housekeeping gene GAPDH as an internal control. Cycle threshold values (Ct>30) were deleted. * indicates significant difference when the values were compared to that of the control ($p < 0.05$). All tests were performed independently in triplicate and presented as means ± S.E.

Validation of Microarray Data

LncRNA expression levels with more than 10 fold alteration were selected for further analysis (Table 2). To further choose more credible lncRNAs in the validation stage, ten lncRNAs were filtered by the database of lincRNA RNA-Seq reads expression abundances in the thyroid tissue in the UCSC (http://genome.ucsc.edu/). Four lncRNAs (ENST00000537266, ENST00000457989, ENST00000440673, ENST00000426615) overlapped the lincRNA RNA-Seq reads expression abundances in thyroid tissue were further validated by Real-time PCR in independent 46 paired papillary thyroid tissues. Consistent with the microarray data, RT-PCR showed that ENST00000537266 (ENS-266) and ENST00000426615 (ENS-615) were up-regulated compared with normal tissue in 22 patients (JY) and 24 patients (NJ) (Fig. 1), while the others were unchanged. The value of threshold cycle number (Ct) above 30 was deleted for quality control.

Effects of ENS-266/ENS-615 on PTC cell proliferation

We investigated the effects of Si-266/Si-615 on cell proliferation by three different assays (CCK-8 assay, Colony formation assay and EdU assay) in TPC-1 cells. CCK-8 assay

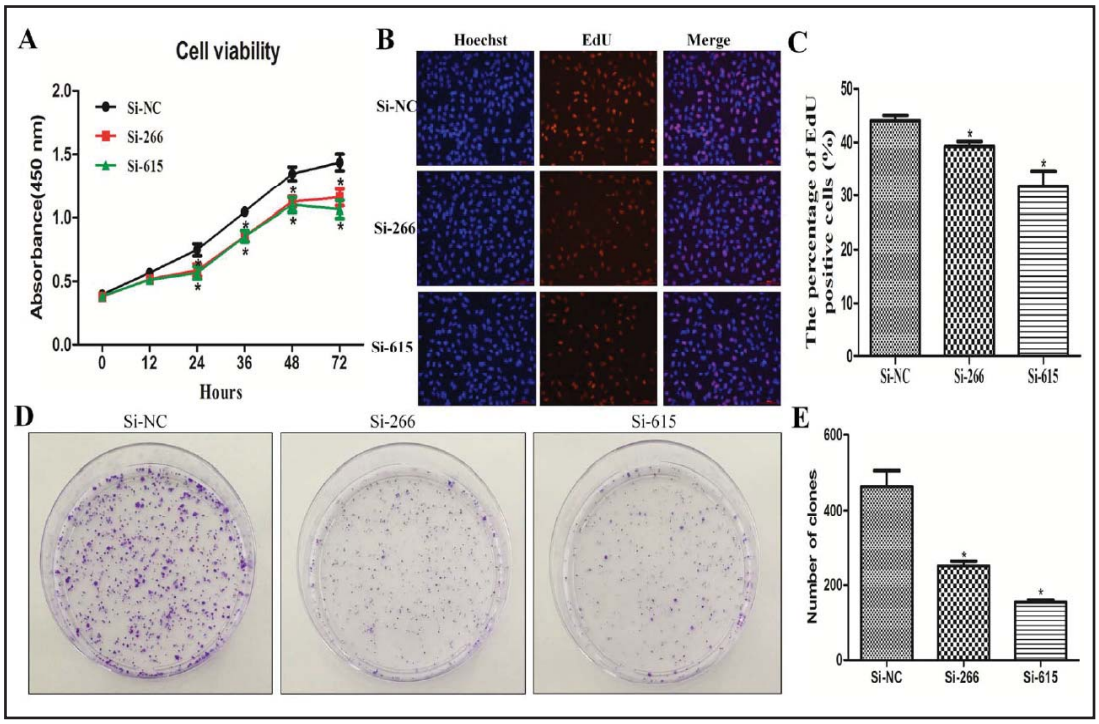

Fig. 2. Effects of ENS-266/ENS-615 on PTC cell proliferation. TPC-1 cells were transfected with Si-266/Si-615 or Si-NC. (A) CCK-8 assay was performed to determine the cell proliferation after 12 h, 24 h, 36 h, 48 h, 72 h transfection in Si-266/Si-615 transfected TPC-1 cells. (B) EdU assay in Si-NC/ Si-266/Si-615 TPC-1 cells. (Left) Hoechst images. (Middle) EdU images. (Right) Overlay of the Hoechst and EdU images. Images were acquired by Fluorescence microscopy with a 20x objective. The exposure time of Hoechest micrograph was 30 ms, and EdU is 100 ms under identical digital camera settings. (C) The EdU results were presented in histogram. (D) Colony-forming growth assay was performed to determine the proliferation of Si-266/Si-615/Si-NC transfected TPC-1 cells. The colonies were captured and counted. (E) The Colony-forming growth assay results were presented in histogram. * indicates significant difference when the values were compared to that of the control ($p < 0.05$). All tests were performed independently in triplicate and presented as means ± S.E.

revealed that cell proliferation was significantly impaired in Si-266/Si-615 transfected TPC-1 cells after 24 h, 36 h, 48 h, 72 h transfection (Fig. 2A). In addition, similar results were obtained by EdU assay. Hoechst staining nuclei in three groups was dense and contracted. Compared with Si-NC transfected TPC-1 cells, the percentage of EdU positive cells in Si-266/Si-615 transfected cells was decreased (Fig. 2B, 2C). Similarly, the results of Colony-formation assays revealed that number of colonies (>50 cells/colony) was significantly decreased in Si-266/Si-615 transfected TPC-1 cells when compared with Si-NC cells (Fig. 2D and 2E).

Effects of ENS-266/ENS-615 on PTC cell cycle and apoptosis

We further investigated the effects of ENS-266/ENS-615 on cell cycle and apoptosis by flow cytometery. The representative results of the cell apoptosis in Si-NC, Si-266 and Si-615 transfected TPC-1 cells were shown in Fig. 3A. We found no significant difference in cell apoptosis between Si-266/Si-615 groups and the control group (Fig. 3A and 3B). Cell cycle analysis showed that cell proportion was statistically significant increased in G1 phase and decreased in S phase and G2 phase in Si-266 transfected TPC-1 cells. Meanwhile, a noteworthy increase of cell proportion in G1 phase accompanied by a decrease in S phase and unchanged G2 phase in Si-615 transfected TPC-1 cells were also observed (Fig. 3C and 3D).

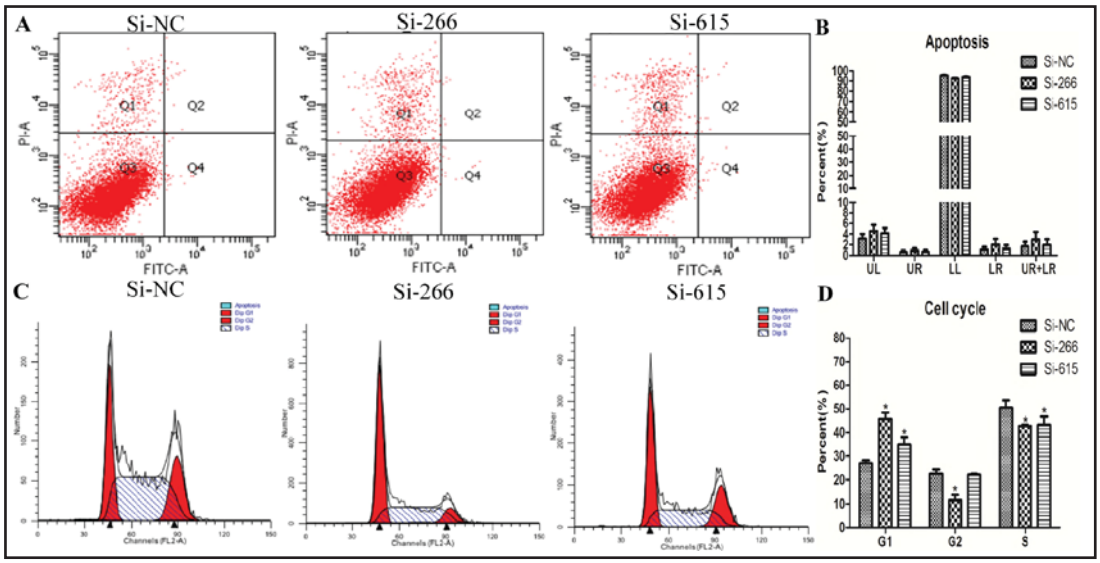

Fig. 3. Effects of ENS-266/ENS-615 on PTC cell cycle and apoptosis. TPC-1 cells were transfected with Si-NC/Si-266/Si-615 for 48 h. The cell cycle and apoptosis were analyzed by flow cytometry. (A) The flow cytometry images of apoptosis. (B) UL indicated dead cells, UR indicated late apoptotic cells, LL indicated live cells, LR indicated early apoptotic cells. The percentage of apoptotic cells was presented in histogram. (C) The flow cytometry images of cell cycle. (D) Results quantitated in cell cycle were shown as a percentage of total cells. * indicates significant difference when the values were compared to that of the control ($p < 0.05$). All tests were performed independently in triplicate and presented as means ± S.E.

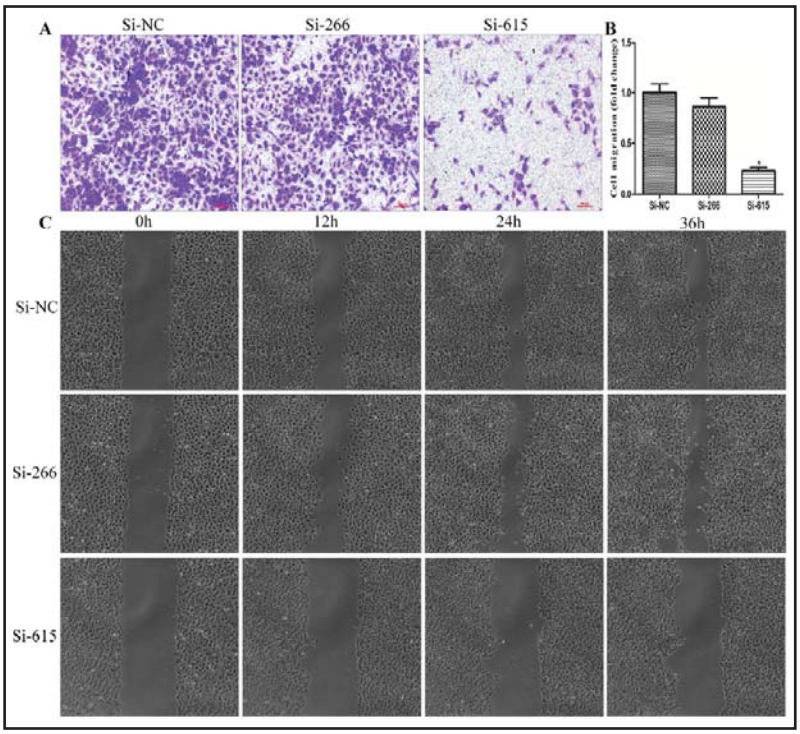

Fig. 4. Effects of ENS-266/ENS-615 on PTC cell motility. (A) Transwell assay was performed to determine the cell motility. The representative images of invasive cells at the lower chamber stained with crystal violet. (B) The quantifications of cell migration were presented as percentage migrated cell numbers. (C) A scratch test was detected by Biostation CT. Images of 0 h, 12 h, 24 h, 36 h were shown. * indicates significant difference when the values were compared to that of the control ($p < 0.05$). All tests were performed independently in triplicate and presented as means±S.E.

Effects of ENS-266/ENS-615 on PTC cell motility

To evaluate the effects of ENS-266/ENS-615 on PTC cell motility, two methods (Transwell and Scratch assay) were used. We down regulated ENS-266/ENS-615 by transfection with Si-266/Si-615. Transwell results revealed a significant decrease in cell migration in Si-615 transfected TPC-1 cells, while there was no significant difference in Si-266 transfected TPC-1

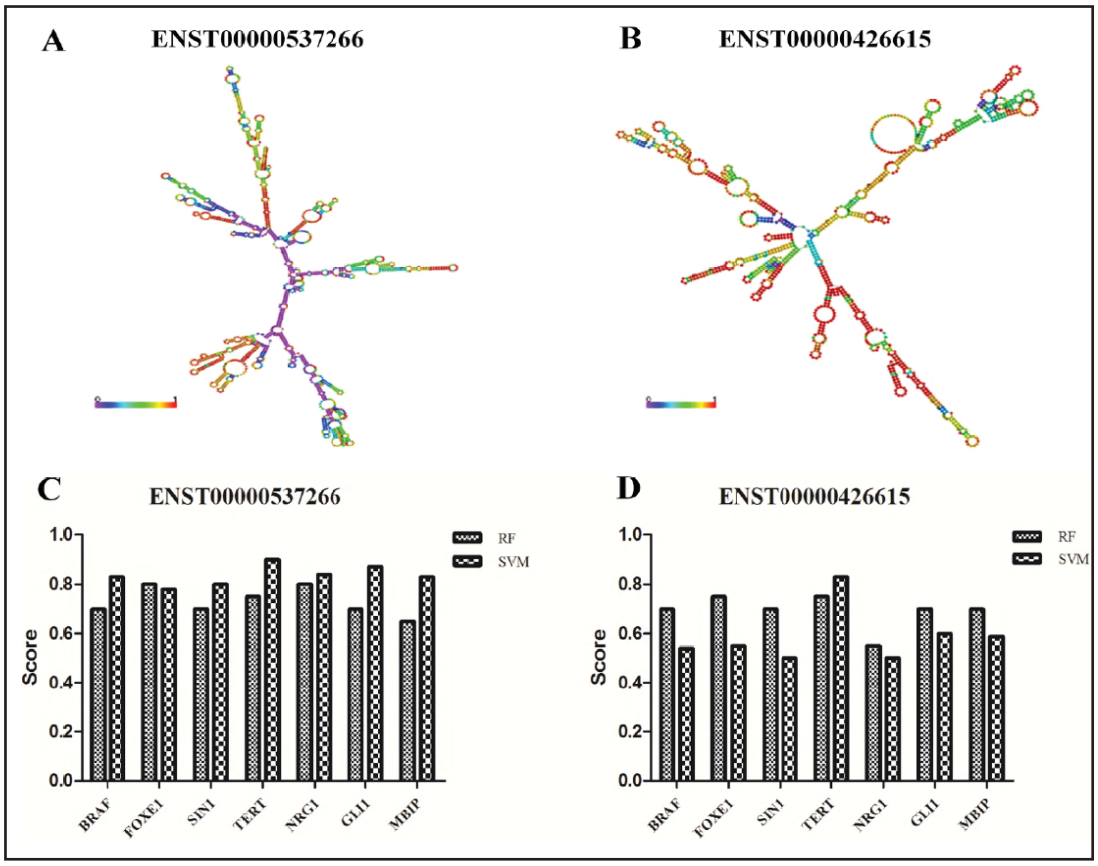

Fig. 5. (A and B) The second structure predicted by LNCipedia 3.0. (C and D) The scores of the interaction probability between lincRNAs and seven PTC associated genes (BRAF, FOXE1, SIN1, TERT, NRG1, GLI1, MBIP) predicted by RPIseq.

cells (Fig. 4A, 4B). Scratch assay showed that Si-NC transfected TPC-1 cells had fully healed the scratch, and Si-266 transfected TPC-1 cells had covered most of the scratch, whereas the Si-615 transfected TPC-1 cells had only covered a short distance after 36 h transfection (Fig. 4C). Taken together, these results showed that ENS-615 can suppress the PTC cell motility, while ENS-266 did not influence the PTC cell motility.

The structures of ENS-266 and ENS-615

The structures of lncRNAs (ENS-266 and ENS-615) were predicted with software LNCipedia 3.0 (http://www.lncipedia.org). Both of the lncRNAs have more than three stem-loop structures, which implied the existence of potential proteins or chromosomes binding sites. And thus these binding sites could be involved in the complex chromatin-modifying complexes and regulation of gene transcription [22] (Fig. 5A, 5B). Meanwhile, we used the RPISeq (http://pridb. gdcb.iastate.edu/RPISeq/) to predict whether some specific proteins, including BRAF, FOXE1, SIN1, TERT, NRG1, GLI1 and MBIP, are likely to interact with the lncRNAs. Predictions with probabilities > 0.5 were considered positive. We found that scores were more than 0.5 between the two lincRNAs and the proteins (Fig. 5C, 5D). It suggested that ENS-266 and ENS-615 might participate in the carcinogenesis.

Discussion

Increasing evidence has revealed that lncRNAs played critical roles in development and diseases, in particular human cancers [14, 18]. LncRNA can affect the cell proliferation, cell cycle and apoptosis, cell migration, and function as oncogenic and tumor suppressor

in tumorigenesis [10, 23]. Effective control of both cell growth and motility by lncRNAs is critical to the prevention of tumorigenesis, successful cancer therapy and cancer prognosis. Therefore, identification of cancer-related lncRNAs and clarification their effects and mechnisms may provide helpful data for PTC diagnosis and treatment. However, lncRNAs in PTC are still an emerging field, with only a handful of lncRNAs known to be involved in the pathogenesis of PTC [19, 20].

In the current study, we screened for two novel lncRNAs (ENS-266 and ENS-615), and found that they were up-regulated in PTC tissues. In addition, we identified the function of lncRNAs in TPC-1 cells by applying loss-of-function approaches. And our results illustrated that decreased lncRNA expression could influence the PTC cell proliferation, cell cycle and motility. Our results are of great significance for the following reasons. Firstly, this is the first study to demonstrate the lncRNA expression signature of PTC tissues. In addition, this study illustrated the critical roles of previously uncharacterized lncRNAs (ENS-266 and ENS-615) in PTC cell proliferation, cell cycle and motility, and our results indicated that ENS-266 and ENS-615 might function as tumor oncogenes.

Recent literature indicated that lncRNAs could control cell proliferation in cancer cells [24, 25]. Our study found that decreased lncRNAs (ENS-266 and ENS-615) expression could inhibit PTC cell proliferation through three different methods (CCK-8 assay, Colony formation assay and EdU assay), which was consistent with previous studies [26]. One mechanism through which lncRNAs regulate cell proliferation is via the modulation of cell cycle as previously reported in various cancer cells [27, 28]. Many previous sources have linked irregularities in the G1 phase or S phase to uncontrolled growth of tumors [28, 29]. In present study, decreased lncRNAs (ENS-266 and ENS-615) might decrease the cell proliferation by an increase in cells in G1 phase and a decrease in cells in S phase. As many cancers including skin and breast cancers have been prevented from proliferating by the arrest of G1 phase [30, 31], the cure for PTC might be focused on the G1 phase of the cell cycle.

In addition, a growing number of studies have demonstrated that lncRNAs could impact cell motility [32]. We discovered that decreased ENS-615 expression could inhibit PTC cell motility by two assays (Transwell and Scratch assay), which was similar to conditions found in other lncRNA function [26]. Transwell and Scratch assay are commonly used methods for the detection of cell motility. Two chamber transwell assay is prone to the analysis of chemotactic migration, while scratch assays can be used to study the collective and directional movement of populations of cells [33]. That means the decreased ENS-615 expression could inhibit the movement of both individual cells and populations cells. Thus, ENS-615 expression might be a potential biomarker of PTC prognosis.

Moreover, BRAF, FOXE1, SIN1, TERT, NRG1, GLI1 and MBIP were all PTC-specific proteins, which played important roles in the PTC [6, 8, 34-36]. RPISeq results showed that lncRNAs (ENS-266 and ENS-615) were likely to interact with these proteins. It suggested that ENS-266 and ENS-615 might participate in the carcinogenesis.

Overall, our work gives insights into the role of two novel lncRNAs (ENS-266 and ENS-615) in the pathogenesis of PTC. Clinically, these lncRNAs (ENS-266 and ENS-615) may be potential biomarkers due to their overexpression in PTC and functional roles in TPC-1 cells. Although the direct lncRNAs-related therapeutic has not yet been carried out, our research might provide a good basis for the knowledge of PTC.

Conclusion

Our results showed that ENS-615 could influence the PTC cell proliferation and motility while ENS-266 could only influence the PTC cell proliferation. These lncRNAs (ENS-266 and ENS-615) might be potential biomarkers for the pathogenesis of PTC.

Acknowledgments

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Disclosure Statement

The authors declare that they have no conflict of interest.

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