Long non-coding RNA HULC exerts oncogenic activity on papillary thyroid cancer in vitro and in vivo

Zhijia Yang, Guoqing Li, Chao Ding, Wencong Sun and Ji Zhang

ABSTRACT
Thyroid cancer is a frequently occurring malignancy in the human endocrine system. Papillary thyroid cancer (PTC) presents 70–80% of all thyroid cancer cases. Herein, we probed the possible oncogenic function of long non-coding RNA (lncRNA) highly up-regulated in liver cancer (HULC) in PTC. First, the HULC and microRNA-106a (miR-106a) expressions in PTC tissues and cells were tested. Plasmids or miRNAs transfections were done for altering HULC and miR-106a expressions. Then, cells viability and apoptosis, along with cell proliferative, migratory and invasive abilities, were tested, respectively. The PI3K/AKT and Wnt/β-catenin pathways activities were measured. Finally, the animal model of PTC was constructed and the tumour volumes and weights were gauged. We discovered that HULC and miR-106a had relative high expression levels in PTC tissues and cells. HULC overexpression enhanced TPC-1 cells viability and cell proliferative, migratory and invasive abilities. Silencing HULC induced TPC-1 cell apoptosis. miR-106a engaged in the oncogenic impacts of HULC. Moreover, HULC overexpression boosted PI3K/AKT and Wnt/β-catenin pathways activities via raising miR-106a expression. Besides, HULC overexpression enhanced the volumes and weights of PTC tumours. To sum up, HULC exhibited oncogenic function on PTC in vitro and in vivo.

Introduction
Thyroid cancer is a frequently occurring malignancy in the human endocrine system [1]. Papillary thyroid cancer (PTC) is a well-differentiated type of thyroid cancer that accounts for 70–80% of all thyroid cancer cases [2]. Epidemiological study reports that the incidence of thyroid cancer, particularly PTC, has noticeably raised in the last few years [3]. The molecular mechanism regarding the onset and development of PTC is very complex, which even includes epigenetic changes [4,5]. Efforts to understand the relationship between epigenetic changes and onset of PTC are considered to be useful for offering diagnostic biomarkers and therapeutic targets for PTC.

As regulatory RNA molecules taking part in epigenetic modification, long non-coding RNAs (lncRNAs) are discovered to attend to the modulation of lots of cell functions [6]. Aberrant expression of lncRNAs is reported to be happened in many human diseases, including cancers [7]. Highly up-regulated in liver cancer (HULC), a newly discovered lncRNA, has been found to exert oncogenic effects on hepatic cancer [8], breast cancer [9], gastric cancer [10], colorectal cancer [11], pancreatic cancer [12] and osteosarcoma [13]. Li et al. reported that HULC boosted epithelial-mesenchymal transition (EMT) of hepatocellular carcinoma [14]. Wang et al. reported that HULC conferred bladder cancer cell proliferation but suppressed cell apoptosis by regulating ZIC2 and PI3K/AKT pathway [15]. However, no literature can be found regarding the impacts of HULC on PTC.

Similarly to lncRNAs, miRNAs and another non-coding regulatory RNAs, also take part in the modulation of cells functions [16]. Many lncRNAs, including HULC, can present oncogenic impacts in cancer cells by regulating miRNAs levels [8,17]. miRNA-106a (miR-106a) was reported to be up-regulated and presented oncogenic activity in multiple cancer cells [18,19]. Shen et al. demonstrated that miR-106a enhanced the viability of PTC cells by modulating MEK/ERK pathway and inhibited apoptosis of PTC cells by modulating p38 MAPK pathway [20].

Herein, the potential oncogenic functions of HULC in PTC in vitro and in vivo were probed. Whether miR-106a took part in the oncogenic function of HULC was also explored.

Material and methods
Clinical specimens
From October 2016 to December 2017, 36 patients with PTC (10 male and 26 female, mean age 50.2 years) were recruited at Henan Provincial People’s Hospital (Zhengzhou, China).
Before surgery, all patients did not get any therapy. Tumour tissues and normal tissues were gathered in the process of surgery. Written informed consents were signed by every patient. Our experiment was approved by the Ethics Committee of our hospital.

**Cell culture**

Human thyroid follicular cell line Nthy-ori3-1 and human PTC cell lines, BCPAP and TPC-1, were supplied by American Type Culture Collection (ATCC, Manassas, VA); TPC-1 and BCPAP cells were grown in RPMI-1640 medium (Gibco, Carlsbad, CA), while Nthy-ori3-1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Carlsbad, CA) at 37°C with 5% CO₂. Both RPMI-1640 medium and DMEM were added 10% (v/v) foetal bovine serum (FBS, Gibco Carlsbad, CA).

**Plasmids or miRNAs transfection**

pcDNA3.1 plasmid (Addgene, Cambridge, MA) inserting with HULC sequence was applied to overexpress HULC and named as pc-HULC. The oligonucleotides sequence of sh-HULC was: 5’-GCCTTTACAAGGGAATGAAAGA-3’. The oligonucleotides sequence of sh-negative control (sh-NC) was: 5’-GGTACTGACGTGCTAATCCGTAG-3’. miR-106a inhibitor (5’-UCACAA-3’) and its NC (5’-UCACAA-3’) were supplied by Life Technologies Corporation (Carlsbad, CA). Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA) was conducted for plasmids or miRNAs transfection.

**qRT-PCR**

Total RNAs was separated with the help of TRIzol Purification kit (Invitrogen, Carlsbad, CA). cDNA was synthesised by SuperScript™ IV First-Strand Syntheses System (Invitrogen). Then, TaqMan™ Real-time PCR Mater Mix (Applied Biosystems, Foster City, CA) was applied to measure HULC and β-actin expressions. TaqMan™ OpenArray™ Real-Time PCR Master Mix (Applied Biosystems, Foster City, CA) was applied to measure miR-106a and U6 snRNA expressions. Sequences of primers were listed in Table 1. Data were analysed by 2⁻ΔΔCt method [21].

| Names | Sequences |
|-------|-----------|
| HULC  | Forward: 5’-ACCTCCGAACACTGTCCAAAATG-3’  | Reverse: 5’-TCTTGGCTTGCTCTTGCTG-3’ |
| β-actin| Forward: 5’-GAGAGGGGAATCTGCTGAG-3’     | Reverse: 5’-CATCTGCTGGAAGTTGAC-3’ |
| miR-106a| Forward: 5’-TGCCCAAGGAGTCTAACAGT-3’   | Reverse: 5’-GCTCGAGGTCCAGGAGG-3’ |
| U6 snRNA| Forward: 5’-CAGAATTCGTAGAGGAGTT-3’    | Reverse: 5’-TGGGTGTGAGGATTCG-3’ |

**Cell viability assay**

Cell viability was assessed by cell counting kit-8 (CCK-8) assay (Beyotime Biotechnology, Shanghai, China) as earlier reported [22]. Results were shown as the percentage of control.

**Cell apoptosis assay**

Cell apoptosis was tested by Guava Nexin assay (Guava Technologies, Hayward, CA) as previously described [23]. Guava EasyCyte flow cytometer (Guava Technologies, Hayward, CA) was utilised for recording results.

**Cell migration and invasion assay**

Cell migratory and invasive abilities were evaluated using a modified two-chamber transwell assay (Corning Incorporated, New York, NY) similarly with the earlier literature reported [24]. Results were shown as percentage of control.

**Western blotting**

Western blotting was done similarly with the previous reported [24]. All primary antibodies, including Cyclin D1 (ab226977), p53 (ab131442), p16 (ab151303), Pro-caspase 3 (ab32499), Cleaved-caspase 3 (ab2302), Pro-caspase 9 (ab138412), Cleaved-caspase 9 (ab2324), Matrix metalloprotein 9 (MMP-9, ab73734), Tissue inhibitor of metalloproteinases 1 (TIMP-1 and ab61224), TIMP-2 (ab1828), Vimentin (ab20346), p-Pi3K (ab182651), t-Pi3K (ab86714), p-AKT (ab183758), t-AKT (ab8805), Wnt3α (ab116222), β-catenin (ab32572) and β-actin (ab8226) were purchased from Abcam Biotechnology (Cambridge, MA).

**Animal experiment**

Twenty-one nude mice (8 male and 13 female, 8–9 weeks old, 20.2–24.6g body weight) were supplied by Beijing Huafukang Bioscience Co., Ltd. (Beijing, China) and randomly divided into control, pc-HULC and sh-HULC group (seven mice/group). For mice in pc-HULC or sh-HULC group, 1 × 10⁶ stably transfected pc-HULC (or sh-HULC) TPC-1 cells were subcutaneously injected into the right flank. The tumour volumes were gauged every week by formula long diameter (a)×short diameters (b)²/2. After 6 weeks, the mice were sacrificed by intraperitoneally injecting 2% pentobarbital sodium (40 mg/kg body weight, Sigma-Aldrich, St Louis, MO). Tumour samples were removed and weighed. All experiments using mice were approved by our hospital.

**Statistical analysis**

The data from three repeated experiments were shown as mean ± standard deviation (SD). Graphpad version 6.0 statistical software (Graphpad, San Diego, CA) was done for statistical analysis. p-values were computed by one-way ANOVA with Tukey’s post-hoc test. p < .05 was thought to be a significant difference.
Results

**HULC and miR-106a had relative high expression levels in PTC tissues and cells**

Figure 1(A,B) showed that both HULC and miR-106a had relatively high expression levels in PTC tumour tissues, in contrast with corresponding normal tissues ($p < .01$). Data in Figure 1(C,D) displayed that relative to human thyroid follicular Nthy-ori3-1 cells, both HULC and miR-106a had higher expression levels in human PTC TPC-1 and BCPAP cells ($p < .01$). These outcomes illustrated that HULC and miR-106a might present oncogenic function in PTC. TPC-1 cells were chosen for further experiments.

**HULC presented oncogenic function in TPC-1 cells**

Figure 2(A) showed that the HULC expression in TPC-1 cells was boosted by pc-HULC transfection and declined by sh-HULC transfection ($p < .05$). Figure 2(B) presented that pc-HULC transfection obviously enhanced TPC-1 cells viability ($p < .05$), while sh-HULC transfection notably lowered TPC-1 cells viability ($p < .05$). The results of Figure 2(C) displayed that the Cyclin D1 level was boosted ($p < .01$), while the p53 and p16 levels were reduced ($p < .05$) in TPC-1 cells by pc-HULC transfection. sh-HULC transfection had opposite impacts ($p < .05$). Moreover, Figure 2(D) indicated that TPC-1 cells apoptosis was potentiated by sh-HULC transfection ($p < .01$). The Cleaved-caspase 3 and Cleaved-caspase 9 levels were raised in TPC-1 cells by sh-HULC transfection (Figure 2(E)). Besides, data in Figure 3(A,B) presented that the TPC-1 cell migratory and invasive abilities were promoted by pc-HULC transfection ($p < .05$), but repressed by sh-HULC transfection ($p < .05$). Figure 3(C,D) displayed that the MMP-9 and Vimentin levels were boosted by pc-HULC transfection ($p < .01$) and declined by sh-HULC transfection ($p < .05$). On the contrary, the TIMP-1 and TIMP-2 levels were reduced by pc-HULC transfection ($p < .05$) and enhanced by sh-HULC transfection ($p < .01$). These above outcomes illustrated that HULC presented oncogenic functions in TPC-1 cells via promoting cell proliferative, migratory and invasive abilities.

**HULC overexpression raised miR-106a expression in TPC-1 cells**

Previous study proved that miR-106a enhanced the viability of PTC cells [20]. Moreover, HULC and miR-106a were both highly expressed in hepatocellular cancer [25]. Herein, data in Figure 4 displayed that, pc-HULC transfection remarkably raised the miR-106a expression in TPC-1 cells ($p < .05$), which hinted that miR-106a might attend to the functions HULC on TPC-1 cells.

**miR-106a attended to the functions of HULC overexpression on TPC-1 cells**

miR-106a inhibitor was applied to reduce miR-106a expression in TPC-1 cells (Figure 5(A), $p < .01$). Figure 5(B) displayed that the HULC overexpression-aroused TPC-1 increase of cells viability was mitigated by miR-106a inhibitor transfection ($p < .05$). The Cyclin D1 level was lowered, but the p53 and p16 levels were enhanced in pc-HULC + miR-106a inhibitor group (Figure 5(C), $p < .05$ or $p < .01$). Figure 6(A,B) showed...
that the HULC overexpression-aroused increases of TPC-1 cell migratory and invasive abilities were also weakened by miR-106a inhibitor transfection \( (p < .05) \). The MMP-9 and Vimentin levels were lowered, but the TIMP-1 and TIMP-2 levels were raised in pc-HULC + miR-106a inhibitor group (Figure 6(C,D), \( p < .05 \) or \( p < .01 \)). These above outcomes illustrated that miR-106a attended to the oncogenic functions of HULC on TPC-1 cells.

**HULC overexpression boosted PI3K/AKT and Wnt/β-catenin pathways activities in TPC-1 cells by raising miR-106a expression**

As displayed in Figure 7(A,B), pc-HULC single transfection notably boosted PI3K/AKT and Wnt/β-catenin pathways activities in TPC-1 cells through enhancing p/t-PI3K, p/t-AKT, Wnt3K and β-catenin expressions \( (p < .05 \) or \( p < .01 \)). However, pc-HULC transfection-aroused activation of these
two pathways was notably relieved by miR-106 inhibitor transfection (*p < .05). These outcomes illustrated that HULC overexpression boosted these two pathways activities in TPC-1 cells could be via raising miR-106a expression.

**HULC exerted oncogenic activity on PTC in vivo**

Figure 8(A) showed that the tumour volume in pc-HULC group was noticeably increased with a time-dependent...
manner after 3 weeks injection ($p < .05$, $p < .01$ or $p < .001$). In sh-HULC group, the tumour volume was distinctly decreased in a time-dependent manner after 3 weeks injection ($p < .05$ or $p < .01$). Figure 8(B) pointed out that by contrast with control group, the tumour weight was notably enhanced in pc-HULC group and noticeably reduced in sh-HULC group ($p < .01$). These outcomes illustrated that HULC also presented oncogenic function on PTC in vivo.

Discussion

As a key regulator of epigenetic modification, lncRNAs and miRNAs are reported to present oncogenic or tumour suppressive functions roles in multiple cancers, including PTC [26]. Herein, we discovered that both HULC and miR-106a had higher expression levels in PTC tissues and cells. HULC overexpression remarkably enhanced TPC-1 cell proliferative, migratory and invasive abilities, while HULC suppression obviously aroused cell apoptosis. Further results found that HULC overexpression raised the miR-106a expression in TPC-1 cells and miR-106a took part in the impacts of HULC on TPC-1 cells. Moreover, HULC overexpression boosted PI3K/AKT and Wnt/β-catenin pathways activities through raising miR-106 expression. Besides, HULC overexpression also enhanced the volume and weight of PTC tumours in vivo. HULC suppression had opposite effects.

In recent years, more and more efforts are made by researchers to probe the impacts lncRNAs and miRNAs in the onset and development of cancers [27]. HULC was demonstrated to exhibit oncogenic functions in a variety of cancers by promoting cell proliferation [8,11,28]. Herein, we discovered that HULC overexpression enhanced TPC-1 cell viability and proliferation, which further confirmed the oncogenic impact of HULC on PTC. Moreover, considering that many lncRNAs, such as thyroid carcinoma susceptibility candidate 3 (PTCSC3), bladder cancer-associated transcript 1 (BLACAT1),

![Figure 4.](image)

HULC overexpression raised the miR-106a expression in TPC-1 cells. TPC-1 cells underwent pc-HULC transfection, the miR-106a expression was detected. *$p < .05$.

![Figure 5.](image)

miR-106a attended to the function of HULC on TPC-1 cell viability and proliferation. (A) TPC-1 cells underwent miR-106a inhibitor transfection, the miR-106a expression was tested. TPC-1 cells underwent different transfection, (B) cell viability and (C) the Cyclin D1, p53 and p16 expressions were determined, respectively. *$p < .05$; **$p < .01$. 

![Figure 6.](image)
LOC100507661 and taurine up-regulated gene 1 (TUG1), were also associated with the pathogenesis of PTC [29–32], we could get conclusion that multiple lncRNAs collectively engaged in the development of PTC.

Tumour metastasis is a very complicated physiological process, which accompanied by the changing expression of multiple molecules in cancer cells and increasing cancer cell migratory and invasive abilities [5,31]. In this study, we discovered that the TPC-1 cell migratory and invasive abilities were boosted by HULC overexpression and declined by HULC suppression. MMP-9 and Vimentin, which take part in the process of cell migration and invasion, respectively [33], were both up-regulated by HULC overexpression and down-regulated by HULC suppression. Moreover, TIMP-1 and TIMP-2, two inhibitors of MMP-9 [34], were both down-regulated by HULC overexpression and up-regulated by HULC suppression. These findings confirmed that HULC presented oncogenic function in TPC-1 cells also by modulating cell metastasis.

Previous researches indicated that HULC exerted oncogenic impacts by modulating miRNAs expression [8,14]. miR-106a has been reported to present oncogenic function in a

Figure 6. miR-106a attended to the function of HULC on TPC-1 cell migration and invasion. TPC-1 cells underwent different transfection, (A and B) cell migratory and invasive abilities and (C and D) the MMP-9, TIMP-1, TIMP-2 and Vimentin expressions were measured, respectively. *p < .05; **p < .01.
number of cancer cells, including PTC cells [18,20]. In this research, we discovered that HULC overexpression remarkably raised the miR-106a expression in TPC-1 cells. Knockdown of miR-106a obviously weakened the oncogenic functions of HULC overexpression on TPC-1 cells, which illustrated that HULC presented oncogenic functions in PTC could be realised by up-regulating miR-106a expression. Braconi and Patel also reported that HULC and miR-106a were both up-regulated in hepatocellular cancer [25]. However, until now, no evidence has been reported to sustain the directly targeted relationship between HULC and miR-106a. Some reports provide evidence that lncRNAs may be the precursor of miRNAs and can be cleaved to generate pre-miRNAs [35,36]. Besides, some researchers provide a hypothesis that due to miRNAs genes are located throughout the genome and very small, it may be transcripted when other genes transcription, including lncRNAs [37,38]. Thus, we proposed that HULC influence the miR-106a expression in TPC-1 cells might be through serving as the precursor of miR-106a or enhancing the transcription of miR-106a. Further investigations are still needed to analyse the regulatory roles of HULC in miR-106a expression in the future. Furthermore, considering that there are also other miRNAs, such as miR-148a, miR-146b, miR-451a and miR-654-3p, that also be reported to take part in the regulation of PTC growth and metastasis [39–42]. Whether HULC can regulate these miRNAs expressions in PTC cells is not clear. If HULC also can regulate other miRNAs expressions in PTC cells, we can propose that there

Figure 7. HULC overexpression boosted PI3K/AKT and Wnt/β-catenin pathways activities through raising miR-106a expression. TPC-1 cells were underwent different transfection, (A and B) the p-PI3K, t-PI3K, p-AKT, t-AKT, Wnt3α and β-catenin expressions in TPC-1 cells were tested. *p < .05; **p < .01.

Figure 8. HULC presented oncogenic function on PTC in vivo. Animal model of PTC was established to assess the oncogenic function of HULC in vivo. (A) Tumour volume and (B) tumour weight were tested. *p<.05; **p<.01; ***p<.001.
might have a very intricate modulatory network regarding many lncRNAs and miRNAs in PTC cells. More investigations are still needed in the future.

Increasing numbers of reports provide evidence that the aberrant activation of PI3K/AKT and Wnt/β-catenin pathways took part in the onset of PTC [43,44]. Zhu et al. proved that HULC silencing inhibited the angiogenesis of human glioma via inactivating PI3K/AKT signalling pathway [45]. Li et al. presented that HULC overexpression activated Wnt/β-catenin signalling pathway in adipose-derived stem cells [46]. Herein, we found that HULC overexpression dramatically boosted PI3K/AKT and Wnt/β-catenin pathways activities in TPC-1 cells. Moreover, knockdown of miR-106a obviously weakened the HULC overexpression-aroused these two pathways activation. These outcomes illustrated that HULC might present oncogenic functions on TPC-1 cells via raising miR-106a expression and thereby boosting PI3K/AKT and Wnt/β-catenin pathways activities. Until now, there is no literature can be searched concerning the direct regulatory functions of miR-106 on PI3K/AKT and Wnt/beta-catenin pathways. However, bioinformatics analysis using TargetScanHuman version 7.1 (http://www.targetscan.org/vert_71/) found that phosphatase with tensin homology (PTEN), an upstream negative regulatory molecule of PI3K/AKT pathway, and secreted frizzled-related protein 4 (SFRP4), an upstream negative regulatory molecule of Wnt/beta-catenin pathway, were the target genes of miR-106. So, we proposed that miR-106 regulated PI3K/AKT and Wnt/beta-catenin pathways in TPC-1 cells might be through targeting PTEN and SFRP4. More investigations are demanded to verify our proposal.

Considering that an animal model of PTC is helpful for further verifying the oncogenic activity of HULC, we also established an animal model of PTC to further investigating the oncogenic activity of HULC in vivo. We discovered that HULC overexpression also enhanced the volume and weight of PTC tumours in vivo. On the contrary, HULC suppression reduced the volume and weight of PTC tumours in vivo. In conclusion, the oncogenic impact of HULC on PTC was verified in our experiments. In vitro, HULC overexpression enhanced TPC-1 cell proliferative, migratory and invasive abilities via raising miR-106a expression and thereby boosting PI3K/AKT and Wnt/β-catenin pathways activities.

Disclosure statement
No potential conflict of interest was reported by the authors.

ORCID
Zhijia Yang http://orcid.org/0000-0002-2986-0931
Guoqing Li http://orcid.org/0000-0002-2986-0931

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