Long non-coding RNA UCA1 exerts growth modulation by miR-15a in human thyroid cancer TPC-1 cells

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Abstract
Thyroid cancer is widely diagnosed as malignancy in endocrine system. This study attempted to validate UCA1 possessed modulatory function on cell proliferation and epithelial mesenchymal transition (EMT) in human thyroid cancer cell line TPC-1. Ectopic expression of UCA1 was induced in TPC-1 cells by transfection. CCK-8 assays were employed to value cell viability. Cell apoptosis analysis was conducted through flow cytometry. We found that overexpressed UCA1 strongly promoted cell proliferation. However, the knockdown of UCA1 suppressed cell proliferation and induced obvious cell apoptosis. Besides, cell EMT was promoted by overexpressed UCA1 and was inhibited by the knockdown of UCA1. Further study revealed that miR-15a level in TPC-1 cells was suppressed by overexpressed UCA1. Simultaneous overexpression of UCA1 and miR-15a partly alleviated UCA1-induced growth, identifying that miR-15a was a possible target of UCA1. At last, the Hippo and JNK signal pathways were activated by overexpressed UCA1 but were then weakened by the adding of miR-15a. In conclusion, our study revealed UCA1/miR-15a axis implicated in thyroid cancer cells EMT, exposing a novel mechanism of thyroid cancer progression.

Introduction
Thyroid cancer is generally diagnosed as the most common malignancy in endocrine system, with a rising incidence in several countries and regions. Histologically, thyroid cancer is normally divided into papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), medullary thyroid cancer (MTC) as well as anaplastic thyroid cancer (ATC). Although more than 90% of thyroid cancers are PTC with good prognosis, there are still 10–20% of thyroid cancer cases have the characteristics of local invasion, distant metastasis, drug resistance and recurrence, which contribute to low 10-year survival rate [1,2]. To date, its pathogenesis still remains unequivocal, albeit many genetic and behavioural genetic changes have been observed. Therefore, it would be the significance of molecular mechanism study, especially for developing new strategies to prevent, diagnose, and treat thyroid cancer.

Except genetic modification, other mechanisms, for instance, long non-coding RNA (lncRNA), have attracted more and more attention. LncRNAs are transcripts composed of more than 200 nucleotides (nt) which were regarded as “transcriptional noise” with no biological functions [3]. More and more studies have confirmed that lncRNAs implicates in the proliferation, apoptosis, invasion and metastasis in various cancer cells, including thyroid cancer [4]. For example, Li et al. revealed that lncRNA-PANDAR knockdown suppresses proliferation, disturbs cell cycle as well as promotes apoptosis in thyroid cancer cells [5]. Besides that, lncRNA BRAF-activated non-coding RNA emerges as a malignancy suppressor in PTC and overexpressed BANCR suppresses biological activities in cancer cells [6]. LncRNA urothelial cancer-associated 1 (UCA1) locates in chromosome 19p13.12. Its high expression has been observed in several kinds of cancers. For example, a recent study found UCA1 is overexpressed in pancreatic cancer and confirmed that UCA1 silence effectively inhibits the proliferative activities and accelerates apoptotic rate [7]. But until now, the exact roles of UCA1 in thyroid cancer have just been preliminary explored.

microRNAs (miRNAs) are a broad class of small (19–22 nt) ncRNAs and endogenously expressed to directly target 3’-untranslated region (3’-UTR) within mRNAs, thereby post-transcriptionally regulate the expression of target genes [8]. It is 30% of all protein expression that has been estimated to associate with miRNAs in humans [9]. For example, miRNA-148a was reported to inhibit lymphatic metastases of papillary thyroid cancer through STAT3 and PI3K/AKT signalling pathways [10]. In addition, miR-145 is regarded as a target of lncRNA-TUG1 and associates with tumour progression in thyroid cancer cells [11]. What is more, miR-15a-5p is a tumour suppressor in various kinds of cancers [12–14]. However, the effect of miR-15a in thyroid cancer is granted elusive.

In the present study, our team proposed investigating the biological function of UCA1 in thyroid cancer. Our study suggested that UCA1 knockdown moderated proliferation and...
facilitated apoptosis progress in human thyroid cancer TPC-1 cells. Furthermore, miR-15a was found as a target of UCA1 and mediated proliferation and epithelial mesenchymal transition (EMT). Further investigation also revealed that overexpressed UCA1 promoted the progression of thyroid cancer via suppressing miR-15a, with activating Hippo and JNK signal pathways. Our findings are supposed to pave the way for thyroid cancer treatment.

Materials and methods

Cell culture and treatment

Human thyroid carcinoma TPC-1 cells, from follicular carcinomas, were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium (American Type Culture Collection, Rockville, MD, USA) containing 10% (v/v) fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA), 100 units/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in a humidified atmosphere containing 5% CO₂ and 95% air at 37°C. To evaluate the EMT process, the cells were subjected to serum starvation for 12 h and then treated by 10 ng/mL TGFβ-1 for 24 h.

Transfection and generation of stably transfected cell lines

Full-length of UCA1 sequences were constructed in pcDNA™ 3.1/V5-His-TOPO (Invitrogen), and then it was transfected into TPC-1 cells (pc-UC1A1) with an empty control (pcDNA3.1). Lipofectamine 3000 reagent (Life Technologies Corporation, Carlsbad, CA, USA) was applied for transduction according to the user’s manual. Next, the cells were maintained in medium containing 0.5 mg/mL G418 (Sigma-Aldrich) and the stably transfected cell lines were selected. Small interfering RNA, directly targeting UCA1 RNA was applied to repress UCA1 expression in TPC-1 cells (si-UC1A1). si-UC1A1 and its scrambled negative control (si-NC) were both purchased from Life Technologies. miR-15a mimic and NC were both synthesized by Life Technologies.

Cell counting kit-8 (CCK-8) assay

The cells were maintained in a 96-well plate in a density of 5000 cells per well. The commercial CCK-8 (Dojindo Molecular Technologies, Gaithersburg, MD, USA) was used to assess cell viability. Briefly, the cells were incubated with CCK-8 solution for 1 h. Next, the absorbance was tested under 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Apoptosis assay

To examine apoptosis progress, Annexin V-FITC apoptosis detection kit (Abcam, Cambridge, MA, USA) was applied according to user’s instructions. Briefly, the cells were gently trypsinized by trypsin (Gibco) and directly grown on a coverslip. Then, the cells were incubated with Annexin V-FITC in the presence of 50 µg/mL RNase A (Sigma-Aldrich) for 1 h at room temperature in the dark. The cells were visualized under a FACScan (Beckman Coulter, Fullerton, CA, USA). Obtained data were analyzed with FlowJo software.

Quantitative real time-polymerase chain reaction (qRT-PCR)

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was applied to extract total RNA from TPC-1 cells with reference to manufacturer’s protocol. The One Step SYBR® PrimeScript® PLUS RT-RNA PCR Kit (TaKaRa Biotechnology, Kyoto, Japan) was applied to quantify UCA1 at RNA level. As for miR-15a, the Taqman MicroRNA Reverse Transcription Kit, in combination with Taqman Universal Master Mix II, was applied (Applied Biosystems, Foster City, CA, USA). β-Actin and U6 served as internal controls for UCA1 and miR-15a, respectively. ΔΔCt method was used to calculate relative RNA expression.

Immunoblotting

The cells were washed with cold phosphate buffered saline (PBS) (Sigma-Aldrich) and lysed with RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) in presence of protease inhibitors (Roche, Guangzhou, China). Total protein was quantified using BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA) and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad Bis-Tris Gel system (Bio-Rad). Next, separated proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA) which were blocked using 5% bovine serum albumin (BSA) (Thermo Fisher Scientific, Waltham, MA, USA) for 1 h and incubated overnight with the indicated primary antibodies at 4°C. Following wash, the membranes were incubated with secondary antibodies marked by horseradish peroxidase (HRP) for 1 h at room temperature. After rinsing, the membranes were transferred into the Bio-Rad ChemiDocTM XRS system and developed using Immobilon Western Chemiluminescent HRP Substrate (Millipore). The signals were captured and the intensity of the bands was cropped and processed using Image Lab™ Software (Bio-Rad).

Statistical analysis

All experiments were carried out at a minimum of three times. The results of multiple experiments were suggested as the mean ± SD (standard deviation). p Values were calculated using Student’s t-test or one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test using GraphPad Prism 6.0 software (GraphPad Software, California, USA). Difference was expected statistically significant as following indicated: p < .05, p < .01 or p < .001.
Results

**UCA1 overexpression enhanced cell viability while its knockdown suppressed cell viability and facilitated apoptosis progress**

UCA1 was overexpressed in pc-UCA1-transduced TPC-1 cells while down-regulated in si-UCA1-transduced TPC-1 cells compared with their corresponding control groups (Figure 1A,B). The results of CCK8 assay showed that cell viability was notably elevated in the pc-UCA1 group while was reduced in si-UCA1 group relative to the control group (Figure 1C,D). Besides, UCA1 overexpression-elicited accumulation of CyclinD1, CDK4 and CDK6 indicated that proliferative activity was promoted by UCA1 (Figure 1E,F), which further indicated by UCA1 silence-caused decrease of these three proteins (Figure 1G,H). In addition, cell apoptosis was examined through flow cytometry analysis and immunoblotting. As shown in Figure 1I,J, although there was no obvious difference in pc-UCA1 group in comparison with the control group, cell apoptosis rate was greatly increased by si-UCA1. Similar phenomenon was shown in Figure 1K,L; si-UCA1-caused cleavage of caspase-7/3/9 suggested that si-UCA1 induced obvious cell apoptosis compared with the control group. The abovementioned results implied that UCA1 acted as an oncogene and UCA1 silence blocked the progression of thyroid cancer cells.

Figure 1. Overexpressed UCA1 induces cell proliferation and knockdown of UCA1 suppresses cell proliferation and promotes cell apoptosis. One group of TPC-1 cells were transfected with pcDNA3.1 or pc-UCA1, with left untreated as the control. The other group was transfected with si-NC or si-UCA1 or left untreated as the control. (A and B) UCA1 in TPC-1 cells was evaluated through qRT-PCR. (C and D) CCK8 assay was used to examine cell viability. (E-H) The expression of CyclinD1, CDK4, CDK6 in TPC-1 cells was valued through immunoblotting. β-Actin was used as an internal control. (I and J) Apoptosis progress was examined through flow cytometry analysis. (K and L) Cell apoptosis-related proteins were examined through immunoblotting. β-Actin served as a reference. The bars showed means ± SD (n = 3). *p < .05 or **p < .01 compared with pcDNA3.1 group.
Overexpressed UCA1 promoted EMT in TPC-1 cells

We have known that EMT is closely associated with metastasis of cancer, thus it is essential to explore specific markers related to cell mobility. TGFβ-1 was used here as an EMT inducer to observe more obvious results. As shown in Figure 2, the level of EMT associated proteins was valued through immunoblotting. The results suggested that pc-UCA1 induced EMT by suppressing the level of E-cadherin and increasing Vimentin, Snail and ZEB1 (Figure 2A,B). On the contrary, si-UCA1 impeded EMT by elevating E-cadherin and down-regulating Vimentin, Snail and ZEB1 (Figure 2C,D). The results above showed that overexpressed UCA1 might promote the progression of thyroid cancer through inducing EMT in TPC-1 cells.

Overexpressed UCA1 promoted cell viability and EMT through down-regulating the level of miR-15a

The target of UCA1 in TPC-1 cells was explored. Our results presented that miR-15a was repressed by overexpressed UCA1, as shown in Figure 3A. miR-15a mimic was used in the following experiments to up-regulating the level of miR-15a (Figure 3B). Then we noticed that elevated cell viability by pc-UCA1 was then suppressed by co-transfection with miR-15a mimic (Figure 3C). Besides, the promoting effect of pc-UCA1 on cell proliferation was also blocked by the adding of miR-15a mimic (Figure 3D,E). In addition, enhanced EMT by the transfection of pc-UCA1 was also weakened by miR-15a mimic (Figure 3F,G). The results above indicated that overexpressed UCA1 facilitated cell proliferation and EMT through down-regulating the level of miR-15a.

Overexpressed UCA1 activated the Hippo and JNK signal pathways through down-regulating the level of miR-15a

The associated signal pathway was also investigated in our following experiments. The results showed that p/t-MST, p/t-YAP (Figure 4A,B), p/t-c-Jun, and p/t-JNK (Figure 4C,D) was increased by overexpressed UCA1 in TPC-1 cells, indicating the activation of the Hippo and JNK signal pathways. At the same time, increased p/t-MST, p/t-YAP, p/t-c-Jun and p/t-JNK was then suppressed when the TPC-1 cells were simultaneously transfected with miR-15a mimic. These above experiments indicated that overexpressed UCA1 activated the
Figure 3. Overexpressed UCA1 promoted cell proliferation and EMT through down-regulating the level of miR-15a. (A) TPC-1 cells were transfected with pcDNA3.1 or pc-UCA1 with left untreated as the control. The level of miR-15a was valued through qRT-PCR. (B) miR-15a mimic was used in the following experiments to up-regulate the level of miR-15a. The level of miR-15a was valued through qRT-PCR. (C) Apoptosis progress was examined through flow cytometry. (D and E) CyclinD1, CDK4, and CDK6 in TPC-1 cells were valued through immunoblotting. (F and G) E-cadherin, Vimentin, Snail and ZEB1 in TPC-1 cells were valued through immunoblotting. β-Actin was used as an internal control. The bars showed means ± SD (n = 3). *p < .05 or **p < .01 compared with pcDNA3.1 group; #p < .05 compared with pc-UCA1 group.
LncRNAs are normally accepted as a class of transcribed RNA molecules which are more than 200 nt long. Although these RNAs do not encode proteins, they regulate the accumulation of specific mRNAs. LncRNAs generally have the potential to regulate some important physiological processes at epigenetic, transcriptional and post-transcriptional levels [15]. At present, hundreds of aberrantly expressed lncRNAs have been found in various cancers and they have been regarded as drivers and biomarkers of cancers because of their high tissue specificity [16]. Similarly, there are also some lncRNAs which were found up or down-regulated in thyroid cancer and its adjacent tissues through gene chip and high-throughput sequencing technology, but the function and mechanism of most lncRNAs are still not clear. To further reveal the intrinsic relationship between lncRNAs and thyroid cancer, we consolidated that overexpressed UCA1 obviously induced cell proliferation. At the same time, we also made corresponding reverse validations. UCA1 knockdown through transfecting with specific si-UCA1 greatly suppressed cell proliferation and

**Discussion**

UCA1 is a potential new oncogene and prognostic factor in kinds of cancers. For example, UCA1 was regarded as an oncogenic lncRNA in bladder cancer, and up-regulated UCA1 is associated with enhanced cell invasion [17]. Besides, UCA1 was found to be up-regulated in colorectal cancer and UCA1 level is negatively correlated with survival time [18]. Similarly, the level of UCA1 was also high elevated in thyroid cancer. Lu et al. proposed that UCA1 is overexpressed in PTC tissues and thyroid cancer cells. In addition, overexpressed UCA1 promotes proliferation, with facilitating invasion, whereas inhibits apoptosis progress via Wnt pathway [19]. In accordance with the previous results, in our study, we also consolidated that overexpressed UCA1 obviously induced cell proliferation. At the same time, we also made corresponding reverse validations. UCA1 knockdown through transfecting with specific si-UCA1 greatly suppressed cell proliferation and
increased cell apoptosis rate. Thus, UCA1 holds great potential as a novel diagnostic and prognostic marker for thyroid cancer.

EMT is considered as the biological process of transforming epithelial cells into mesenchymal phenotype cells through specific procedures. EMT exhibits a significant role in embryonic development, chronic inflammation, tissue remodelling, cancer metastasis and various fibrosis diseases. In addition, epithelial cells lose cell polarity and junction with basement membrane and obtain higher migration and invasion, anti-apoptotic and extracellular matrix degrading ability and other mesenchymal phenotypes through EMT. EMT emerges as an important biological process for epithelial cell-derived malignant tumour cells to acquire migration and invasion. To address the molecular mechanism regulating the EMT process of malignant tumors, to clarify its pathological significance in the occurrence, development and metastasis of malignant tumors, and to explore the diagnostic methods based on the key molecules of EMT and the therapeutic methods targeting the key molecules of EMT are the key scientific issues in the study of the EMT mechanism of cancer metastasis. For example, down-regulated IncRNA TUG1 suppressed the progression of papillary thyroid cancer through inhibiting cell proliferation, migration/invasion and the reverse of EMT to MET [11]. In our study, we also observed that UCA1 overexpression induced obvious EMT through suppressing E-cadherin but elevating Vimentin, Snail and ZEB1 expression. As expect, the knockdown of UCA1 played the opposite role by suppressing EMT. Thus, we concluded that overexpressed UCA1 promoted the progression of thyroid cancer by inducing EMT.

Then, the underlying mechanisms by which UCA1 regulates cellular proliferation and EMT was further investigated. The cross-regulation between miRNAs and IncRNAs has attracted more and more attention in recent years, for instance, miRNA as trigger of IncRNA decay, IncRNAs as miRNA sponges/decoys, and IncRNAs as competitor with miRNAs for targeting mRNAs [20]. miR-15a usually acts as anti-oncogene in kinds of cancers. Just as Shi et al. reported that endogenous miR-15a-3p silence in osteosarcoma tumours is significantly ascribed to cancer patient’s poor clinical outcomes and low survival rate [21]. Besides that, IncRNA LINC00473 is overexpressed in colorectal cancer. Inhibition of LINC00473 restores the taxol-induced cytotoxicity, inhibits cell vitality, colony formation and induces apoptosis, impairs migration or invasion, but these effects could be abrogated by the inhibition of miR-15a, suggesting that miR-15a is a target of LINC00473 in colorectal cancer [22]. In support of this concept, we also found that the level of miR-15a was suppressed in TPC-1 cells transfected with pc-UCA1. Apart from this, the promoting effects of UCA1 overexpression on cell proliferation and EMT were both obviously weakened by the co-transfection with miR-15a mimic, implying that miR-15a also acted as a target of UCA1 in thyroid cancer.

The associated signal pathway was also investigated in our research. The Hippo signal pathway deregulation was reported to be closely associated with a broad range of human carcinomas [23]. The JNK signal pathway also regulates a wide spectrum of physiological processes, from inflammatory responses, morphogenesis, cell proliferation, differentiation to death. At the same time, the activation of JNKs has been demonstrated to be involved in cancer development and progression [24]. Additionally, Zhang et al. reported that UCA1 promotes migration and invasion through the Hippo signalling pathway [25]. The association between Hippo and JNK signal pathway has also been explored in previous studies. Ma et al. reported that activated Hippo signalling promotes cell invasion and EMT through JNK, as JNK signalling inactivation dramatically blocks Hippo pathway activation-induced matrix metalloproteinase 1 expression and cell invasion [26]. Similarly, in our study, we also found that the Hippo pathway and JNK pathway were both activated by overexpressed UCA1 and were then partly blocked by the adding of miR-15a mimic, identifying that UCA1 may act as an oncogene by suppressing the expression of miR-15a through activating the Hippo pathway and JNK pathway.

In conclusion, our study was the first to investigate the biological function of UCA1 on EMT and associated regulation mechanism in thyroid cancer. The results indicated that UCA1 was a novel potential oncogene, and it promoted the proliferation and EMT formation of thyroid cancer cells by targeting miR-15a through the Hippo pathway and JNK pathway. Our study implicates the potential application of UCA1 in the prognosis and treatment of thyroid cancer, providing more clues of efficient therapeutic agents.

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

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