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Original Article

Overexpression of lincRNA02471 promote cancer development though miR-758/HIPK3 signaling pathway in papillary thyroid cancer

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Abstract: Aims: In previous studies, numerous differential lncRNAs were identified via RNA-sequencing. In this dysregulated lncRNAs, lincRNA02471 attracted our attention due to its highest fold change. The aims of our study mainly focused on the function and mechanism of lincRNA02471 in papillary thyroid cancer. Materials and methods: Overexpression and knockdown vectors were constructed to investigate the function of lincRNA02471. Proliferation, apoptosis, invasion and EMT were performed to assess the function of lincRNA02471. Dual-luciferase reporter assay was performed to explore the relationship between lincRNA02471 and miR-758. Results: We found that lincRNA02471 was manifestly upregulated in papillary cancer tissues. Overexpression of lincRNA02471 significantly promoted the cell proliferation, invasion and inhibited cell apoptosis. Knockdown of lincRNA02471 inhibited the cancer development. We also found that lincRNA02471 negatively regulate miR-758 in papillary thyroid cancer. miR-758 can restore the effect of lincRNA02471. Besides, we identified that HIPK3 was the direct target of miR-758. Conclusion: We performed comprehensive study of lincRNA02471 and explore its function and mechanism in papillary thyroid cancer. lincRNA02471 can sponge miR-758 and positively regulate HIPK3 to promote papillary thyroid cancer development. Our study provides new target for clinical treatment and new clues for understanding the molecular mechanism of cancer development.

Keywords: lincRNA02471, papillary thyroid cancer, miR-758, HIPK3

Introduction

Thyroid cancer (TC) is a kind of malignant tumor originating from thyroid follicular cells or parafollicular cells [1]. In recent years, as one of the main types of TC, the global incidence of papillary thyroid cancer (PTC) has increased rapidly, accounting for more than 80% of all thyroid cancer, and has become the most common thyroid malignancy [2]. Currently, the main treatments for differentiated TC include surgical treatment, TSH suppression therapy and ^{131}I internal radiotherapy [3, 4]. Although the long-term prognosis of PTC after treatment is generally satisfactory, there are still some patients with recurrence and metastasis, seriously affecting the life quality even leading death [5]. Further in-depth study of its treatment methods and mechanisms is of great significance for the diagnosis and treatment of PTC.

PTC is a disease affected by many environmental and genetic factors. The main known factors include ionizing radiation exposure, obesity, genetic and epigenetic variations [6]. The Human Genome Project (HGP) shows that just 1% of genes can be transcribed into biologically functional RNA, while the remaining 99% genes do not have the function of directly coding proteins, and called non-coding RNA (nc RNA) [7]. Among non-coding RNAs, microRNAs has been extensively studied and has been proved to play an important role in PTC [8]. Recent studies have suggested that long non-coding RNAs (lncRNAs) also play a critical role in tumorigenesis and development [9]. For example, Zhou Q et al revealed that the expression of lncRNA PVT1 in thyroid cancer cells increased significantly. Silencing PVT1 significantly inhibited the proliferation and invasion of thyroid cancer cells. It also suggested that lncRNA PVT1 might

induce TC by regulating the expression of EZH2 and TSHR [10]. Nevertheless, up to now, the role and molecular mechanism of lncRNAs in thyroid cancer are still not fully clear.

We analyzed LncRNAs differentially expressed in PTC by microarray published previously. In this dysregulated lncRNAs, lincRNA02471 attracted our attention due to its highest fold change [11]. We validated the role of lincRNA02471 in PTC and further explored its possible molecular mechanism. Competitive binding and regulation of miRNAs is one of the main mechanisms by which lncRNA plays a biological role in many diseases [12, 13]. MiR-758, a major target-binding gene of lncRNA02471, predicted by software analysis, plays a role in regulating proliferation and invasion in many other cancer diseases [14, 15]. In addition, we further predicted and obtained the target binding gene HIPK3 of miRNA-758. We have conducted a series of experiments to prove the relationship between lncRNA02471-miR-758-HIPK3 and PTC and the specific role of lncRNA02471/miR-758/HIPK3 axis in PTC.

In conclusion, we will validate the role of lncRNA02471 and the potential molecular mechanism of lncRNA02471 in PTC, reveal the role of lncRNA02471/miR-758/HIPK3 axis in PTC, and provide clues and guidance for molecular therapy of PTC.

Materials and methods

Patients and thyroid tissues collection

PTC tissues and adjacent normal thyroid tissues were collected from 49 patients diagnosed with PTC. Normal kidney, spleen, heart, lung, brain and pancreas tissues were collected from patients who had undergone previous surgery. All the patients had no experience of chemotherapy or radiotherapy. All the studies were approved by the Ethical Committee of affiliated Suzhou Hospital of Nanjing Medical University and informed consent was obtained from all patients.

Cell culture and transfection

Human thyroid normal cell line, Nthy-ori 3-1, PTC cell line of BCPAP, KTC-1 and K1 were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's Modification of Eagle's medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Gibco) in 5% CO_2 at 37°C. lncRNA02471 overexpression and inhibition vector, miR-758 mimics, miR-758 inhibitor and controls were constructed by Shanghai Gene Pharma (Shanghai, China). 50 nM miR-758 mimic, miR-758 inhibitor or NC siRNAs with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) were transfected to K1 and KTC-1 cells according to the manufacturer's protocol.

Cell counting kit-8 (CCK-8) assay

CCK8 assay (Dojindo, Japan) was used to detect the proliferation of K1 and KTC-1 cells according to the manufacturer's protocol. All cells were transfected with lncRNA02471. After transfection, the cells were seeded ($3*10^5$ cells per well) into 96-well plates, and 10 μl CCK-8 reagent was added 0, 24, 48, 72 and 96 h later for 2 h, after which the OD 450 values were counted.

Transwell assay

K1 and KTC-1 cells were re-suspended in 100 μL serum-free medium and were plated in the top chamber of each insert (8-μm pore size, Corning, USA) with a Matrigel-coated membrane (BD Bioscience, San Jose, USA) for the transwell assay. Lower chambers of the inserts were filled with DMEM medium with 10% FBS. After 12 h of incubation, cells that invaded to the lower surface of the insert were fixed, stained, and counted under a light microscope.

Real-time polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from K1 and KTC-1 cells using TRIzol reagent (Thermo Fisher Scientific). The purity of RNA was determined by measuring the absorbance ratio of 260/280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific) Reverse transcription of RNA was carried out using a PrimeScript™ RT reagent Kit with gDNA eraser (RR047A; Takara, Tokyo, Japan), and cDNA was performed by qRT-PCR using SYBR® Premix Ex Taq™ (RR420A; Takara, Tokyo, Japan). The data were normalized to GAPDH levels and further analysed by the $2^{-\Delta\Delta CT}$ method. The primers used for qPCR are listed in **Table 1**.

Table 1. Primers used in this study

Primer	Sequences	Application
E-cadherin	F: TCCATTTCTTGGTCTACGCC	qPCR
	R: CACCTTCAGCCAACCTGTTT	
N-cadherin	F: GCGTCTGTAGAGGCTTCTGG	qPCR
	R: GCCACTTGCCACTTTTCCTG	
Vimentin	F: TGTCCAAATCGATGTGGATGTTTC	qPCR
	R: TTGTACCATTCTTCTGCCTCCTG	
miR-758	F: ACACTCCAGCTGGGAACGATG	qPCR
	R: CTCAACTGGTGTCGTGGAGTCGGCA	
HIPK3	F: ACATTGGAAGAGCATGAGGCAGAGA	qPCR
	R: CTGCTGAAAAGCATCACCACAACCA	
β-actin	F: CCACTGGCATCGTGATGG	qPCR
	R: GCGGATGTCCACGTCACACT	

Western blotting analysis

Total protein was isolated from K1 and KTC-1 cells and solubilized using RIPA lysis buffer containing proteinase inhibitor (Sigma, USA). Total protein concentrations were determined using the BCA assay (Pierce, Rock-ford, IL, USA). Equal amounts proteins of samples (30 ug) were separated electrophoretically by 10% SDS-PAGE and transferred to PVDF (polyvinylidene difluoride) membranes (Merk-Millipore, Billerica, MA, USA) for 90 mins. Following blocked for 2 h with 5% non-fat dry milk at room temperature, the membranes were indicated antibodies at 4°C overnight against E-cadherin (1:1000, Abcam, MA, USA), N-cadherin (1:1000, Abcam, MA, USA), Vimentin (1:1000, Abcam, MA, USA), HIPK3 (1:1000, Abcam, MA, USA) and GAPDH (1:1000, Abcam, MA, USA) at 4°C overnight. Immunopositive bands were analyzed by using a Fluor Chem M system (Protein Simple, San Jose, CA, USA).

Luciferase reporter assay

The 3'-UTR of lncRNA02471, with wild-type or mutant (mut) binding sites for miR-758, was amplified and cloned into the pGL3 vector (Promega, Madison, WI, USA) to generate the plasmid pGL3-wt-lncRNA02471-3'-UTR or pGL3-mut-lncRNA02471-3'-UTR. HEK 293 cells were used to perform the luciferase reported assay, and the Lipofectamine 2000 reagents was used to transfected the miR-758 mimics or inhibitor and the lncRNA02471 vector. Dual-Luciferase system was used to analyze the Luciferase activity according to the manufacture's protocol.

Statistical analysis

SPSS 22.0 was used to analyze all the values (means ± standard error of the mean (SEM). Statistical analyses were analyzed with Student's t test and X^2 test. $P<0.05$ was considered to have no statistical significance.

Results

Biological features of lincRNA02471

To investigate the biological features of lincRNA02471, we firstly detected the expression of lincRNA02471 in PTC cell lines, which was significantly upregulated in KTC1 and K1 cell lines (**Figure 1A**). Next, we detected the expression of lincRNA02471 in different organs. We collected mouse tissues and found that lincRNA02471 was highly expressed in thyroid, liver and pancreas (**Figure 1B**). To investigate the location of lincRNA02471, we performed nucles and cytoplasm separation experiment, in which Actin and U6 served as positive control. Our results showed that lincRNA02471 located in both apartment and mainly in cytoplasm (**Figure 1C**). Last, we collected PTC tissues and verified the expression of lincRNA02471, which was significantly upregulated in papillary thyroid cancer tissues compared with normal thyroid tissues (**Figure 1D**).

Overexpression functional analysis of lincRNA02471

To investigate the function of lincRNA02471, we constructed overexpression vector of lincRNA02471. The expression of lincRNA02471 was significantly upregulated in KTC1 cells and K1 cells, respectively (**Figure 2A**). Proliferation results showed that overexpression of lincRNA02471 significantly promoted cell proliferation through CCK-8 assay in KTC1 and K1 cells (**Figure 2B**, **2C**). In addition, transwell assay demonstrated that overexpression of lincRNA02471 manifestly increased the cell invasion and migration in two different cell lines (**Figure 2D**). Lastly, we detected the EMT effect. Overexpression of lincRNA02471 reduced the protein level of E-cadherin, whereas increased the protein level of N-cadherin and Vimentin in KTC1 and K1 cells (**Figure 2E**). Real-time PCR also showed same results. Thus, our results

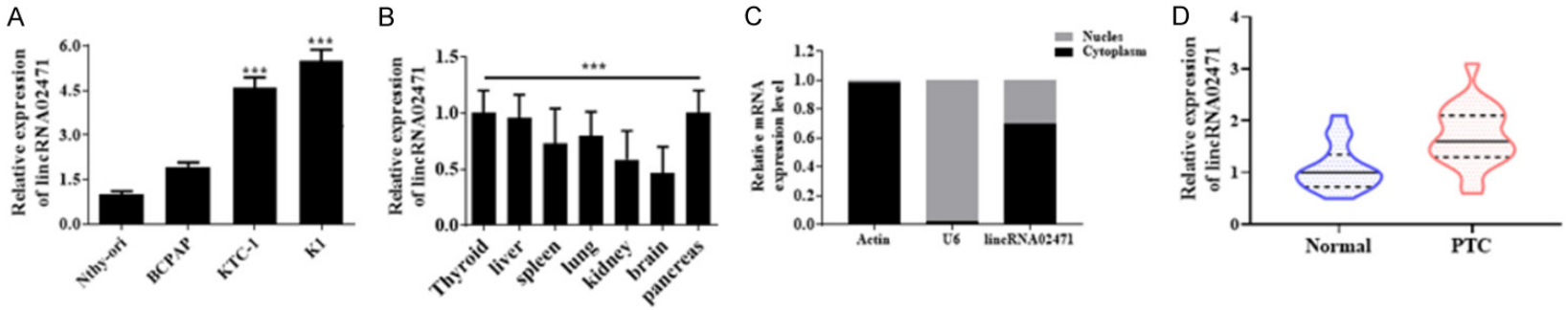

Figure 1. Biological features of lincRNA02471. A. The expression of lincRNA02471 was upregulated in PTC cell lines. B. The expression of lincRNA02471 in different mouse tissues were detected via real-time PCR. C. lincRNA02471 was located in cytoplasm. D. The expression of lincRNA02471 was upregulated in PTC tissues. Data represent the mean ± SD of 3 independent measurements. *P<0.05, **P<0.01, ***P<0.001.

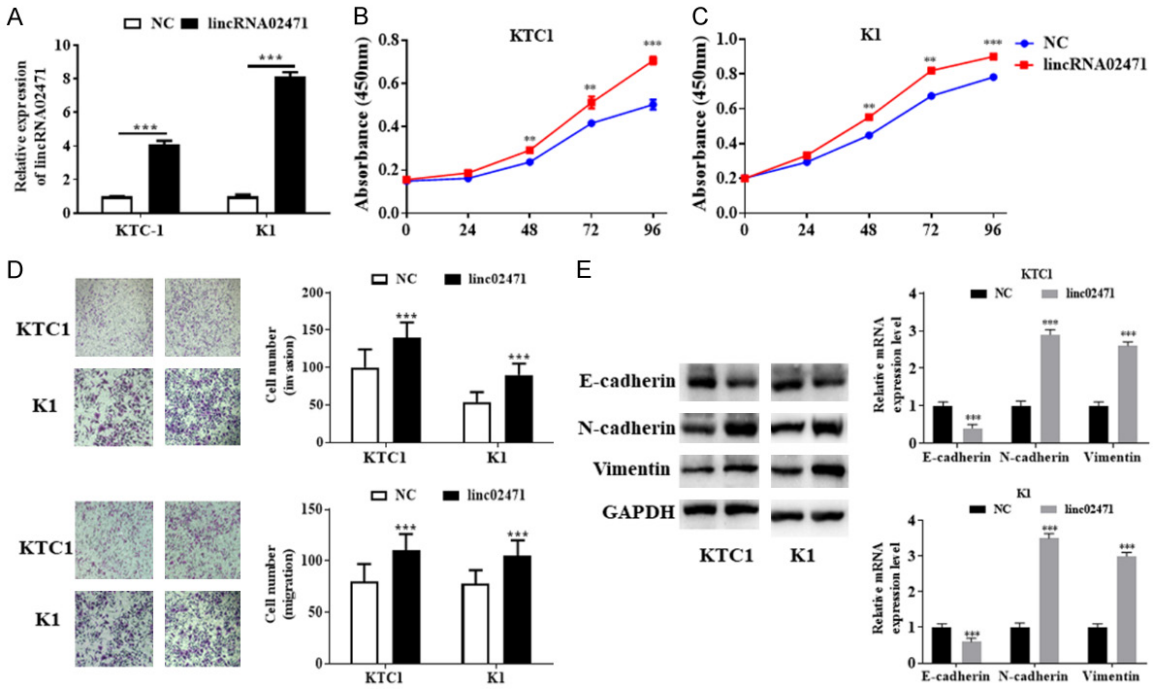

Figure 2. Overexpression functional analysis of lincRNA02471. A. Overexpression of lincRNA02471 was verified via real-time PCR. B. Proliferation was detected in KTC1 cells. C. Proliferation was measured in K1 cells. D. Invasion and migration were detected in two different PTC cell lines. E. EMT markers were analyzed in two different PTC cell lines through western blot and real-time PCR. Data represent the mean ± SD of 3 independent measurements. *P<0.05, **P<0.01, ***P<0.001.

showed that lincRNA02471 possessed function of promote-tumor development.

Knockdown functional analysis of lincRNA02471

To further prove the function of lincRNA02471, we constructed knockdown vector. The knockdown efficiency was verified using real-time PCR (**Figure 3A**). Knockdown of lincRNA02471 significantly inhibited the cell proliferation in KTC1 and K1 cells via CCK-8 assay (**Figure 3B**, **3C**). The number of invasion and migration was reduced after lincRNA02471 knockdown in 2 different cell lines, respectively (**Figure 3D**). In addition, E-cadherin was increased with treatment of lincRNA02471 knockdown, whereas N-cadherin and Vimentin was decreased via western blot and real-time PCR (**Figure 3E**). Thus, we concluded that knockdown of lincRNA02471 could inhibit the cancer progress.

miR-758 is the target of lincRNA02471

After detection of function of lincRNA02471, we consider to explore the mechanism involved in lincRNA02471. The mechanism of long non coding RNA can depend on its location. After we confirmed the location, we used bioinformatics software to predict the potential target of lincRNA02471. As shown in **Figure 4A**, miR-758 was predicted as the possible target of lincRNA02471. Next, we performed dual-luciferase reporter assay to examine whether miR-758 was the target of lincRNA02471. Our results showed that overexpression of miR-758 could reduce the relative activity of lincRNA02471 whereas inhibitor of miR-758 could increase the relative activity of lincRNA02471. However, no significant difference was detected in the mutant group (**Figure 4B**). Next, we wondered the expression of lincRNA02471 was changed after treatment of miR-758. Interestingly, the expression of lincRNA02471 was significantly reduced after overexpression of miR-758 and vice versa (**Figure 4C**). To date, there is no report about miR-758 in papillary thyroid cancer. We constructed miR-758 mimics vector and transfected KTC1 and K1 cells (**Figure 4D**). Our results showed that overexpression of miR-758 significantly inhibited the cell proliferation via CCK-8 (**Figure 4E**).

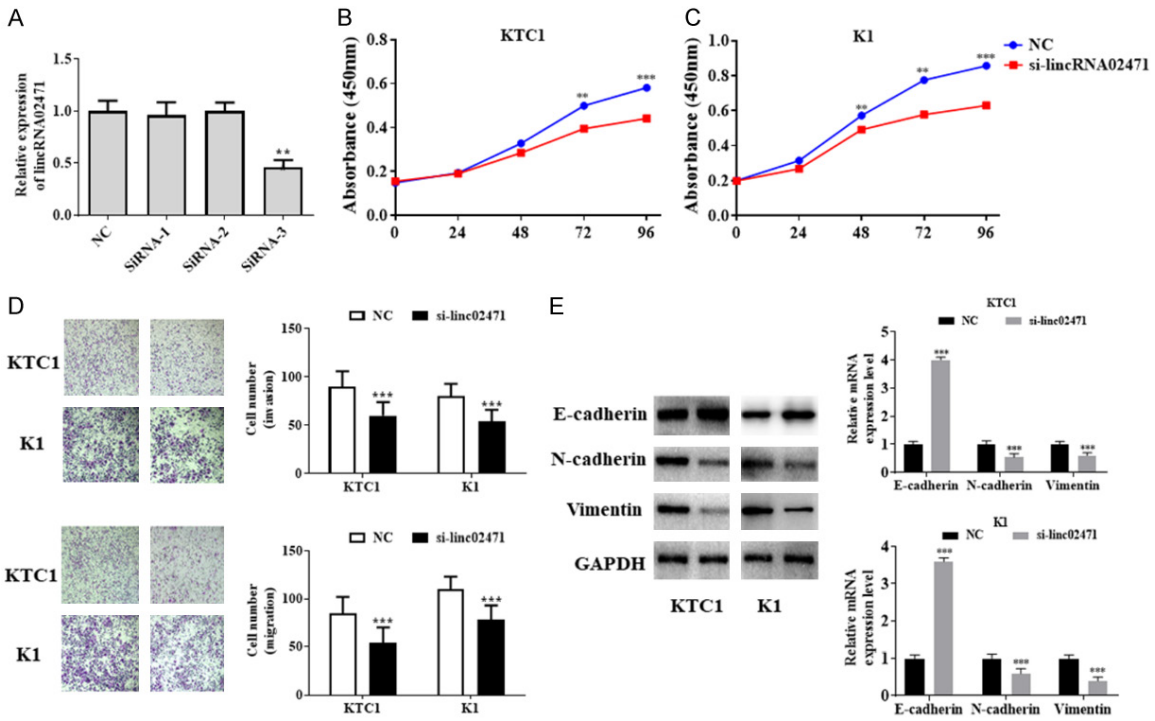

Figure 3. Knockdown functional analysis of lincRNA02471. A. Knockdown of lincRNA02471 was verified via real-time PCR. B. Proliferation was detected in KTC1 cells. C. Proliferation was measured in K1 cells. D. Invasion and migration were detected in two different PTC cell lines. E. EMT markers were analyzed in two different PTC cell lines through western blot and real-time PCR. Data represent the mean ± SD of 3 independent measurements. *P<0.05, **P<0.01, ***P<0.001.

LincRNA02471 function through negatively regulating miR-758

To prove miR-758 was the direct target of lincRNA02471, we performed rescue experiment. In KTC1 and K1 cells, we transfected lincRNA02471 knockdown vector and miR-758 overexpression vector at the same time and analyzed the function of lincRNA02471. Our results showed that overexpression of miR-758 could rescued the proliferation effect of lincRNA02471. Overexpression of miR-758 can reduce the proliferation rates compared with co-transfection group in KTC1 and K1 cells (**Figure 5A**, **5B**). We also detected the invasion and migration rates. Overexpression of miR-758 can restore the effect of lincRNA02471 (**Figure 5C**, **5D**). Moreover, we analyzed the EMT markers. Our results revealed that knockdown of lincRNA02471 could reduce the protein level of E-cadherin, and overexpression of miR-758 can partially restore the effect. Similar results could also be detected in K1 cells (**Figure 5E**). We also provided evidences detected by real-time PCR. Thus, our results suggested that lincRNA02471 function through negatively regulating miR-758.

HIPK3 was the target of miR-758

To further identify the possible target of miR-758, we used bioinformatics analysis to predict the possible target. Our results suggested that HIPK3 possessed 2 binding sites (**Figure 6A**). Dual-luciferase reporter assay demonstrated that the relative activity was reduced in wt group, whereas no changes detected in mutant group (**Figure 6B**). Similar results can be achieved in KTC1 cells (**Figure 6B**). Next, we detected the relationship between miR-758 and lincRNA02471. We found that overexpression of miR-758 could decrease the protein level of HIPK3 and vice versa in KTC1 cells (**Figure 6C**). Similar results could achieve in K1 cells (**Figure 6D**). Next, we detected the relationship between lincRNA02471 and HIPK3. We found that overexpression of lincRNA02471 could increase the protein level of HIPK3 in KTC1 and K1 cells (**Figure 6E**, **6F**). Knockdown of lincRNA02471 could reduce the expression

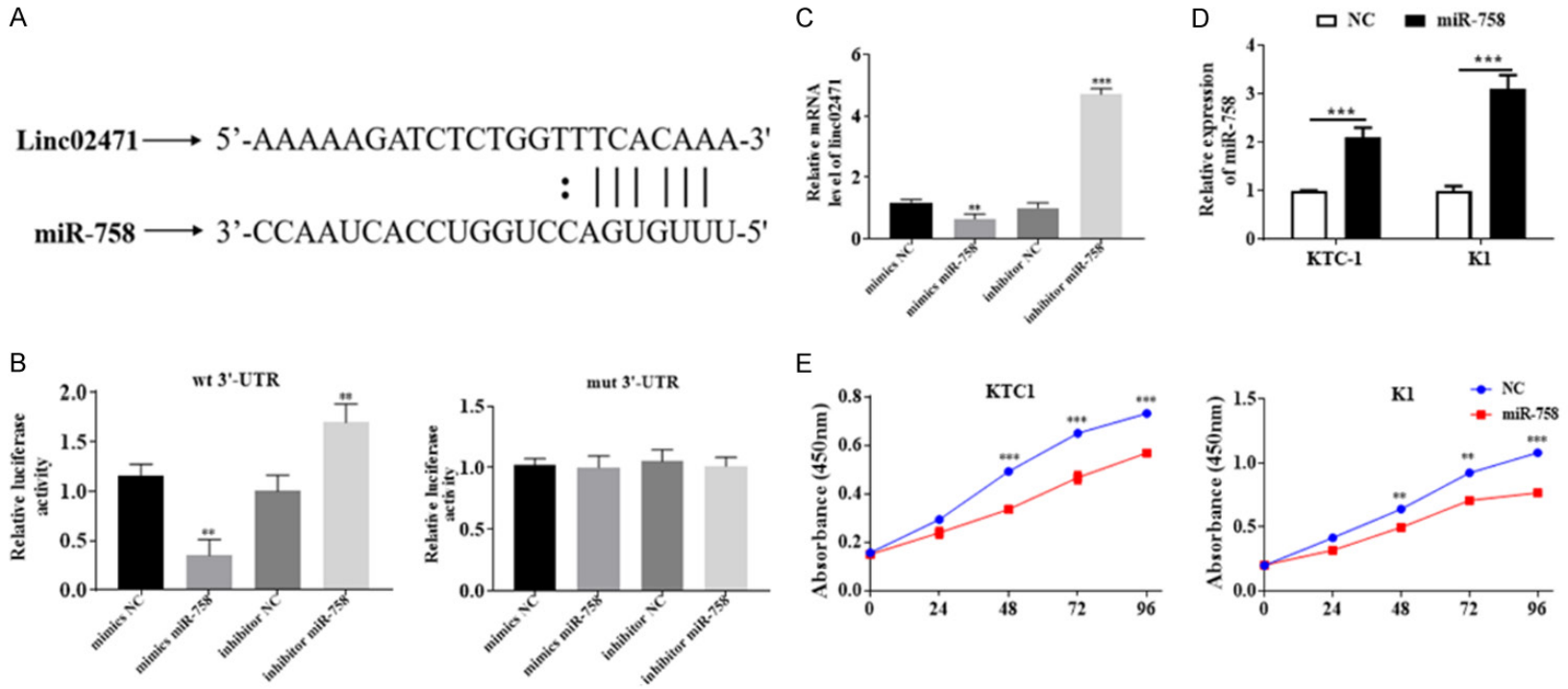

Figure 4. miR-758 is the target of lincRNA02471. A. The potential binding sequence was showed. B. Dual-luciferase reporter assay was performed. C. The expression of lincRNA02471 was detected in miR-758 overexpression and knockdown group. D. Overexpression of miR-758 was verified using real-time PCR. E. Proliferation rates were measured via CCK-8 in KTC1 and K1 cells. Data represent the mean ± SD of 3 independent measurements. *P<0.05, **P<0.01, ***P<0.001.

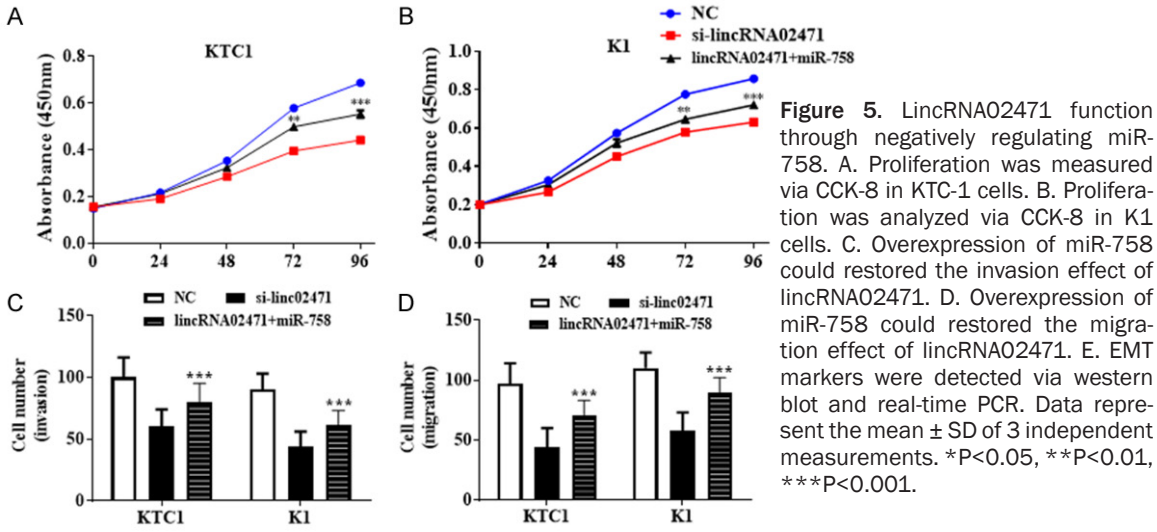

Figure 5. LincRNA02471 function through negatively regulating miR-758. A. Proliferation was measured via CCK-8 in KTC-1 cells. B. Proliferation was analyzed via CCK-8 in K1 cells. C. Overexpression of miR-758 could restored the invasion effect of lincRNA02471. D. Overexpression of miR-758 could restored the migration effect of lincRNA02471. E. EMT markers were detected via western blot and real-time PCR. Data represent the mean ± SD of 3 independent measurements. *P<0.05, **P<0.01, ***P<0.001.

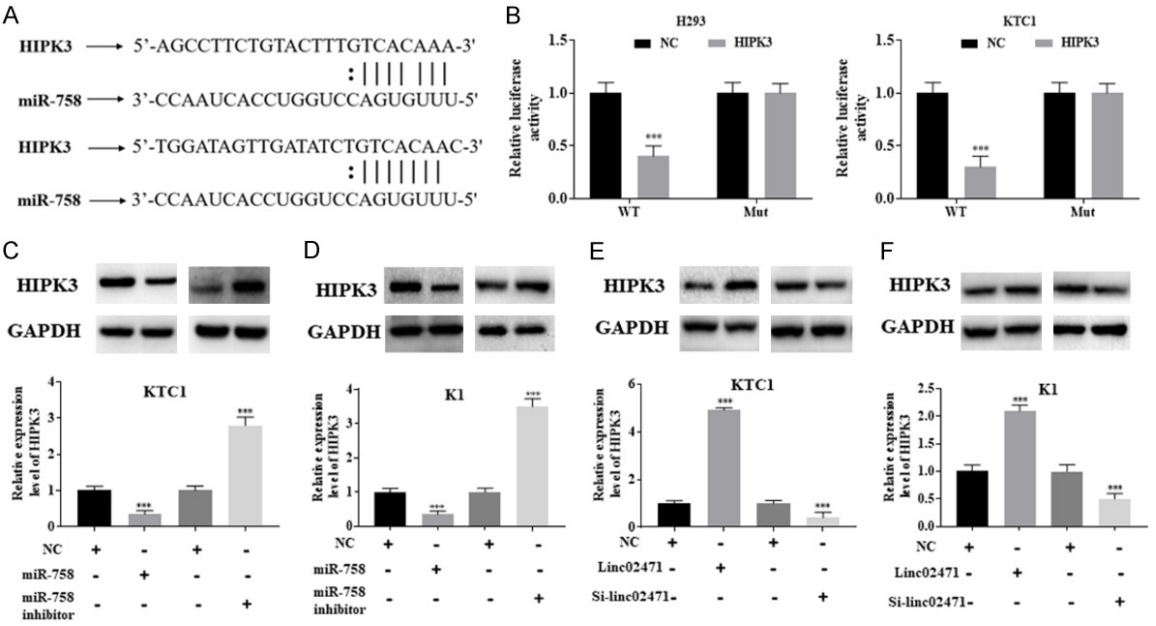

Figure 6. HIPK3 was the target of miR-758. A. The potential binding sequence between miR-758 AND HIPK3 was showed. B. Dual-luciferase reporter assay were performed in H293 and KTC1 cells. C. Overexpression of miR-758 could reduce expression of HIPK3 and vice versa in KTC1 cells via real-time PCR and western blot. D. Overexpression of miR-758 could reduce expression of HIPK3 and vice versa in K1 cells via real-time PCR and western blot. E. Overexpression of lincRNA02471 increased the expression of HIPK3 via real-time PCR and western blot in KTC1 cells. F. Overexpression of lincRNA02471 increased the expression of HIPK3 via real-time PCR and western blot in K1 cells. Data represent the mean ± SD of 3 independent measurements. *P<0.05, **P<0.01, ***P<0.001.

level of HIPK3, suggesting that lincRNA02471 could positively regulate HIPK3.

Discussion

In recent decades, as the most common malignant tumor of the endocrine system, the incidence of TC has been steadily increasing worldwide, and the growth rate in China is also ahead of many cancers [2]. PTC is particularly prevalent in women [16]. Although the current treatment has some effect, there are still some patients with recurrence and metastasis [17]. Therefore, it is particularly important to find out the accurate treatment methods and mechanisms of minimally invasive PTC patients, which can inhibit the occurrence, development and metastasis of the disease.

Precision medicine, a new medical concept and medical model, is based on genome sequencing technology, using huge biological information and large database information to search for altered genes, and taking them as the target for precise treatment of diseases, realizing individualized treatment of diseases and patients [18]. In recent years, researchers have found that there are still a large number of untranslated transcripts in this process, which are called ncRNA [19]. These ncRNAs play an important role in cell development, physiology and pathology, and participate in the occurrence and development of many diseases [20]. The function of lncRNA has been gradually recognized in recent years, and its study in PTC has just started. The role and mechanism of lncRNA in PTC have alsonot been fully understood.

In our study, we first verified the expression of lincRNA02471 was significantly upregulated in papillary thyroid cancer tissues compared with normal thyroid tissues. Overexpression of lincRNA02471 significantly promoted the cell proliferation, invasion and inhibited cell apoptosis in PTC cells. Knockdown of lincRNA02471 inhibited the cancer development. These data indicate that lincRNA02471 is mediated in the occurrence and development of PTC. It may have therapeutic effect on PTC by regulating the expression of lincRNA02471.

LncRNAs play a biological role in many diseases by targeting and regulating microRNAs, directly binding and modifying proteins and so on [21]. In our study, we predicted the potential binding gene miR-758 of lincRNA02471 through software analysis. We also found that lincRNA02471 negatively regulate miR-758 in papillary thyroid cancer. miR-758 can restore the effect of lincRNA02471. In addition, we also predicted and validated HIPK3 was the direct target of miR-758, and further demonstrated the role of lincRNA02471/miR-758/HIPK3 axis in PTC. MiR-758 has been proved to play an important role in regulating proliferation and invasion in many cancer diseases. In addition, the HIPK3 gene has been reported to regulate autophagy, proliferation and migration previously [22, 23]. Consistent with other findings, our study also confirmed the role of miR-758/HIPK3 axis in PTC.

In conclusion, we performed comprehensive study of lincRNA02471 and explore its function and mechanism in PTC. lincRNA02471 can sponge miR-758 and positively regulate HIPK3 to regulate PTC development. Long non coding RNA has shown great potential and ability to predict or served as an biomarker in PTC and other diseases [24]. Our study provides new target for clinical treatment and new clues for understanding the molecular mechanism of cancer development.

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Disclosure of conflict of interest

None.

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