Received: 15 March 2021 | Revised: 14 May 2021 | Accepted: 17 May 2021

DOI: 10.1002/lio2.593

ORIGINAL RESEARCH

Laryngoscope Investigative Otolaryngology

Long non-coding RNA HOTAIR/microRNA-761 sponge regulates PPME1 and further influences cell biological functions in thyroid carcinoma

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Funding information
Shanghai Pujiang Program, Grant/Award Number: 18PJD004; Shanghai Municipal Jiading District Science and Technology Commission Foundation, Grant/Award Number: JDKW-2018-W14; Shanghai Municipal Jiading District Fifth Medical Key Subject Program, Grant/Award Number: 2020-JDYXZDXK-06

Abstract

Background: Most well-differentiated thyroid carcinomas display good therapeutic outcomes, but there are still some patients who are not sensitive to the general treatments lose their treatment opportunities. Thus, it is important to understand the molecular mechanisms that cause thyroid carcinoma, so as to find effective diagnostic and therapeutic targets.

Aim of the study: To explore the role of homeobox transcript antisense RNA (HOTAIR) in thyroid carcinoma through protein phosphatase methylesterase 1 (PPME1) by sponging microRNA 761 (miR-761).

Methods: The regulation network amongst HOTAIR, miR-761 and PPME1 was predicted by online sources. RT-PCR was conducted to evaluate the expression of HOTAIR and miR-761 in tumor tissues. Clinical data was collected and analyzed by Chi-square test. Cell apoptosis and proliferation was evaluated using three types of cancer cells (HTh-7, CAL-62, BCPAP) after treated with si-HOTAIR and miR-761inhibitor. The binding site among HOTAIR, miR-761 and PPME1 was verified by dual luciferase reporter assay. PPME1 expression was measured after HOTAIR and miR-761 were suppressed by western blot. Survival time was measured in nude mice using log-rank test.

Results: HOTAIR was expressed to a significantly greater extent than miR-761 in thyroid tumor tissues ($P < .001$). miR-761 and PPME1 were negatively correlated (coef $= -1.91$, $P < .001$). HOTAIR competitively binds to miR-761 and miR-761 directly targets PPME1. HOTAIR was highly correlated with TNM ($\chi^2 = 5.797$, $P = .016$), tumor size ($\chi^2 = 7.955$, $P = .005$) and lymphatic metastasis ($\chi^2 = 6.0$, $P = .014$). HOTAIR promoted cell proliferation and inhibited cell apoptosis, whereas miR-761 did not. HOTAIR elevated and miR-761 suppressed PPME1 expression. HOTAIR expression appears to affect the survival time in vivo.

Conclusion: HOTAIR regulated thyroid cancer cells by binding to miR-761 through PPME1.

KEYWORDS
HOTAIR, miR-761, PPME1, thyroid cancer

1 | INTRODUCTION

Thyroid carcinoma is a common cancer disease in the head and neck region. The incidence rate is increasing about 4% every year.[1] Although most of the patients have the well differentiated papillary thyroid carcinoma (PTC), the biological behavior is still variable, from slowly developing indolent micro-carcinoma to aggressive cancer that can metastasize and cause death.[2]

Recent commonly accepted effective treatments for thyroid carcinoma are surgical treatment, iodine radiotherapy and TSH suppressive therapy. Most well-differentiated thyroid carcinomas display good therapeutic outcomes, but there are still some patients who are not sensitive to the general treatments still lose their treatment opportunities.[3] Thus, it is important to understand the molecular mechanisms that cause thyroid carcinoma, so as to find effective diagnostic and therapeutic targets.[4]

PPME1 is a protein phosphatase 2A (PP2A)-specific methylesterase that mediates the demethylation and inactivation of PP2A. The reversible methylation of PP2A occurs at the carboxyl group of the carboxy-terminal leucine 309 residue of PP2A (PP2Ac Leu 309) and is catalyzed by an s-adenosylmethionine-dependent leucine carboxyl methytransferase and PPME1.[5,6] In human astrocytic glioma patients, PPME1 was found to be increased and correlated with malignant progression and extracellular regulated protein kinases (Erk) pathway activity.[7] PPME1 was also determined in lung, gastric and colorectal cancer as an important therapeutic target.[8,9] Interestingly, PPME1 was reported to relate to thyroid cancer after low-dose radiation exposure.[10] And PPME1 has been verified as a targeted domain of miRNA-195 and may be regulated by miRNA-195.[11] As is known to us, miRNAs serve as oncogenes or tumor suppressors in various cancers.[12] Moreover, miR-761 may bind directly to PPME1, as predicted by Starbase (database for RNA research). Also, evidence indicates that long non-coding RNAs (lncRNAs) can affect cancer initiation and progression by regulating miRNAs.[13] In addition, lncRNAs are involved in many cancer biological processes, such as cell proliferation, apoptosis and tumorigenesis.[14,15] HOTAIR was found to promote tumorigenesis of breast and laryngeal cancer.[16,17] Meanwhile, HOTAIR was verified to promote the thyroid cancer cell growth, invasion, and migration through miR-17-5p or miR-1.[18,19] Furthermore, HOTAIR has been predicted by Starbase and Mammalian NcRNA-Disease Repository (MNDR, database for ncRNA research) v2.0 to bind with miR-761 as competing endogenous RNAs (ceRNA) and to act as a thyroid cancer oncogene via promoting tumorigenic properties of thyroid cancer cells. These findings led to a hypothesis that HOTAIR participated in the development of thyroid cancer by serving as a ceRNA of miR-761.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

The study was approved by Institution Review Board of Jiading Central Hospital Affiliated to Shanghai University of Medicine & Health Sciences. All clinical specimens were collected after informed consents were signed by patients. All animal experiments were approved by the Animal Care and Use Committee of Jiading Central Hospital Affiliated to Shanghai University of Medicine & Health Sciences.

2.2 | Patient collection

We collected the patients with thyroid cancer from January 2017 to January 2019 in Jiading Central Hospital. The demographic data of the patients were collected, such as age, sex, TNM stage, histological type and lymphatic metastases.

2.3 | Cell treatment

The thyroid cancer cell lines (HTh-7, Human Thyroid Cancer Cells; CAL-62, Thyroid Anaplastic Carcinoma Cells; and BCPAP, Papillary Thyroid Carcinoma Cells) were provided by Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cell lines were cultured in minimum Eagle's medium (MEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in 5% CO_2 and finally sub-cultured. Following HOTAIR and miR-761 treatment, thyroid cancer cells were treated with si-HOTAIR and si-miR-761.

2.4 | RNA isolation and quantitation

Total RNA was obtained. Table 1 shows the designed and synthesized primers. Quantitative reverse transcription-PCR (qRT-PCR) (SYBR-Green assay kits, Life Science, UK) was used to quantify HOTAIR and miR-761 levels. U6 (small RNA) or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as an internal control for miR-761 and HOTAIR. The $2 - \Delta\Delta Ct$ formula was used.

2.5 | Western blot analysis

Thyroid cancer tissues and cells were suspended in a homogenate and total protein was isolated by adding with lysis buffer. The protein was then separated by electrophoresis and transferred to a nitrocellulose membrane, which was sealed overnight with 5% skim milk at 4°C.

TABLE 1 Primer sequence for RT-qPCR

Target genes[a]	Forward primer	Reverse primer
miR-761	5′-TGCTTAAGAATACGCGTAGGTC-3′	5′-CCAGTGCGTGTCGTGG-3′
HOTAIR	5′-CCCTAGCCTTTGGAAGCTCT-3′	5′-GGGTCCCACTGCATAATCAC-3′
U6	5′-CTCCTGGCTTTCGGCAGC-3′	5′-ATTAGCTTGCTGACGCAGAT-3′
GAPDH	5′-AGGTCGGAGTCAACGGATTT-3′	5′-TGACAAGCTTCCCGTTCTCA-3′

[a]miR-761, microRNA-761; HOTAIR, homeobox transcript antisense RNA; U6 snRNA, U6 small nuclear RNA; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

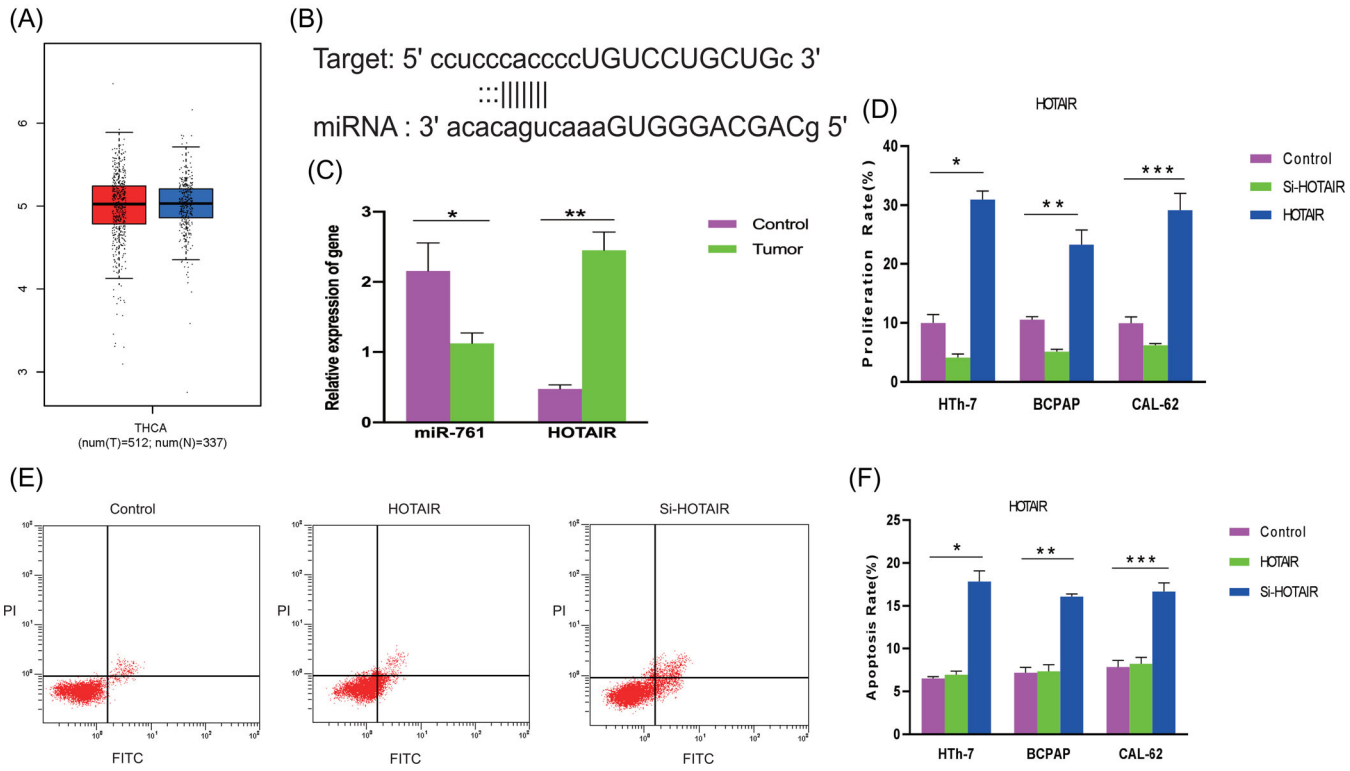

FIGURE 1 HOTAIR is upregulated in thyroid cancer tissue, and promoted cell proliferation and inhibited cell apoptosis. A, TCGA database revealed that PPME1 was more highly expressed in thyroid cancer tissue than in control tissue. B, Starbase online resource showed a binding site between HOTAIR and miR-761. C, RT-PCR showed HOTAIR was highly expressed and miR-761 lowly expressed in thyroid cancer tissue ($P < .05$, $n = 50$). D, HOTAIR promoted the proliferation of three types of thyroid cancer cells and si-HOTAIR inhibited ($P < .05$). E,F, Si-HOTAIR promoted the apoptosis of three types of thyroid cancer cells and HOTAIR inhibited ($P < .05$). Each test was repeated three times

The membrane was incubated with rabbit anti human polyclonal antibody (1:500, ab86409) against PPME1 overnight. Next, anti-horseradish peroxidase labeled Goat anti rabbit IgG (1:100, ab109489, Abcam Inc, Cambridge, Massachusetts) was added to the membrane and soaked at 37°C for 1 hour. After immersion in the solution for imaging in electro-chemi-luminescence, the relative level of protein was analyzed.

2.6 | Dual luciferase reporter gene assay

We synthesized PPME1 and HOTAIR 3′UTR gene fragments and introduced to the pMIR-reporter. Then, we designed the mutation sites. A target fragment was inserted into reporter plasmids (pGL3 basic). The reporter plasmids PPME1 and HOTAIR (Wt/Mut) were transfected with an miR-761 mimic to HTh-7 and BCPAP cells. A luciferase assay kit (ab228530) was used to measure the luciferase activity.

2.7 | EdU (5-ethynyl-2′-deoxyuridine) staining for cell proliferation

We prepared appropriate 50 μm EdU medium. Cells were then cultured with this medium for 24 hours. After that, cells were fixed with polyformaldehyde and cultured in 5% glycine for 5 minutes.

After treatment with 0.5% Triton X-100, anti EdU antibody was added and stained with Hoechst 33342.

2.8 | Flow cytometry

Cells were adjusted to a concentration of 1×10^6/mL, fixed with 75% ice cold ethanol for 1 hour at 4°C, treated with RNase A (250-500 μg/mL) at 37°C for 30 minutes, and stained with malondiimide (PI) in the dark at 4°C for 30 minutes. Then cells were suspended in annexin-V-fluorescein isothiocyanate (FITC)/propidine Iodide (PI) solution (1:2:50) for 15 minutes using APOAF-20TST (Sigma). After adding with hydroxyethyl piperazineethanesulfonic acid (HEPES) buffer, FITC and PI fluorescence were detected at 488 nm.

2.9 | Xenograft tumor in nude mice

Nude mice (4-6 weeks old) from the Animal Experimental Center of Shanghai University of Medicine & Health Sciences were raised at SPF level lab. The HTh-7 thyroid cancer cells with lower or higher HOTAIR expression were placed into cell suspension (1×10^8/mL) and then inoculated into axilla of nude mice. The time of death was recorded for each mouse.

2.10 | Statistical analysis

Graphpad 7.0 and STATA 14.0 were used for data analysis. Means ± SD were used and compared by *t* test or one-way ANOVA. Overall survival time of nude mice was calculated using the Kaplan-Meier curve and log-rank test. Categorical variables were assessed by the Chi-square test. Statistical significance was determined as $P < .05$. The relationship between miR-761 and PPME1 was assessed by linear correlation.

3 | RESULTS

3.1 | High HOTAIR expression in thyroid cancer

TCGA database revealed that PPME1 was expressed to a greater extent in thyroid cancer tissue than in control tissue (Figure 1A). miR-761 was predicted to target PPME1 by an online source (Starbase) (Figure 1B). We collected one hundred thyroid cancer and normal tissues. RT-PCR showed HOTAIR was expressed to a significantly greater extent in thyroid cancer tissue than in control tissue ($P < .05$) (Figure 1C).

3.2 | HOTAIR silencing inhibits thyroid cancer cell proliferation and promotes apoptosis

Compared to normal control, HOTAIR treatment showed an elevated cell growth rate in all three types of cancer cells ($P < .05$), whereas treatment with si-HOTAIR presented a decreased growth rate ($P < .05$) (Figure 1D). In apoptosis, we observed opposite trends ($P < .05$) (Figure 1E,F).

TABLE 2 Mutifactors associated with HOTAIR expression

	HOTAIR		χ^2	*P*-value
	Low	High		
Sex				
Male	33	31	0.174	.677
Female	17	19		
Age				
≥45	38	34	0.679	.410
<45	17	21		
Tumor size				
≥5	21	35	7.955	.005
<5	29	15		
TNM				
I-II	29	17	5.797	.016
III	21	33		
Pathological type				
Papillary	25	27	0.170	.680
Follicular	16	9		
Medullary	4	5		
Undifferentiated	5	9		
Lymphatic metastases				
Positive	14	26	6.000	.014
Negative	36	24		

3.3 | HOTAIR affects the thyroid tumor staging

In the clinical data, we collected information about 100 patients and divided them into HOTAIR high- and low- expression groups. HOTAIR was highly correlated with TNM stages ($\chi^2 = 5.797$, $P = .016$), tumor size ($\chi^2 = 7.955$, $P = .005$), and lymphatic metastasis ($\chi^2 = 6.0$, $P = .014$) (Table 2). Thus, we concluded that HOTAIR may affect the tumor staging.

3.4 | HOTAIR competitively binds to miR-761

The binding site of HOTAIR and miR-761 was predicted by the website Starbase. The dual luciferase reporter assay revealed that, in contrast to controls, luciferase activity in the HOTAIR-Wt decreased ($P < .05$), but that of HOTAIR-Mut remained almost the same ($P > .05$) (Figure 2A-C). However, RT-PCR showed miR-761 was expressed only slightly in thyroid cancer tissue ($P < .05$) (Figure 1C), whereas HOTAIR was expressed to a significantly greater extent ($P < .05$) (Figure 1C). Furthermore, when miR-761 was inhibited, the expression of HOTAIR in HOTAIR-Wt was elevated (Figure 2B,C). In western blot analysis, we found that miR-761 expression was decreased when treated with HOTAIR and increased when treated with si-HOTAIR (Figure 2D). Compared to normal controls,

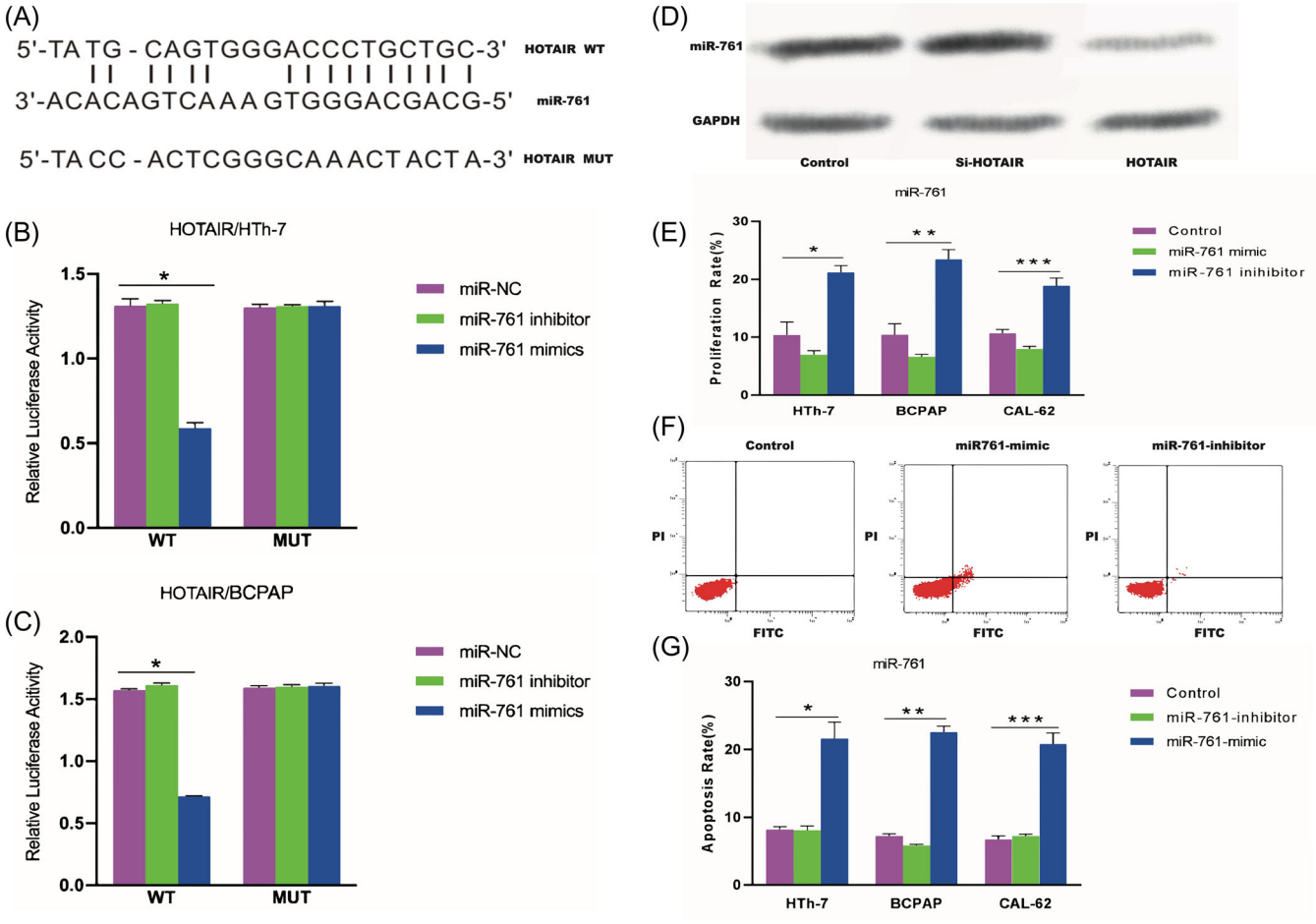

FIGURE 2 HOTAIR sponges miR-761. A, The binding site between HOTAIR and miR-761 predicted by bioinformatics website. B,C, The luciferase activity in each group detected by dual luciferase reporter gene assay for two types of thyroid cancer cells. D, In western blot analysis, when treated with HOTIAR, the miR-761 expression was decreased. When treated with si-HOTAIR, the expression was elevated. E, Compared to normal control, miR-761 mimic showed an decreased cell growth rate in all the three types of cancer cells ($P < .05$), while treated with miR-761 in opposite trends ($P < .05$). F,G, miR-761 mimic could promote the apoptosis of three types of thyroid cancer cells and miR-761 inhibitor decreased ($P < .05$). Each test was repeated three times

the miR-761 mimic showed a decreased cell growth rate in all three types of cancer cells ($P < .05$), whereas treatment with the miR-761 inhibitor presented an increased growth rate ($P < .05$) (Figure 2E). In apoptosis, we observed opposite trends ($P < .05$) (Figure 2F,G).

3.5 | miR-761 targets PPEM1 directly

Analyses from the bioinformatics website Starbase revealed the presence of a specific binding region between 3′UTR of PPME1 and miR-761 sequences.

The dual luciferase reporter assay revealed that, in contrast to normal control, luciferase activity in PPME1-Wt decreased ($P < .05$), but that of PPME1-Mut remained almost the same ($P > .05$) (Figure 3A-C). Furthermore, when miR-761 was inhibited, the expression of PPME1 in PPME1-Wt was elevated (Figure 3B, C). In western blot analysis, the miR-761 mimic group exhibited lower PPME1 expression and the miR-761 inhibitor group exhibited higher PPME1 expression (Figure 3D). miR-761 and PPME1 were negatively correlated after analyzing by linear regression (coef $= -1.91$, $P < .001$) (Figure 3E).

3.6 | miR-761 targets PPME1 to inhibit Erk signaling pathway activation

In western blot analysis, miR-761 mimic and si-PPME1 decreased the expression of PPME1 and p-Erk/ERK. miR-761 inhibitor elevated the expression of PPME1 and p-Erk/ERK. miR-761 inhibitor+ si-PPME1 did not change the expression of PPME1 and p-Erk/ERK (Figure 4A).

3.7 | Relationship between PPME1 and HOTAIR

We hypothesized that HOTAIR binding to miR-761 regulates PPME1 expression. To determine whether a relationship exists between these

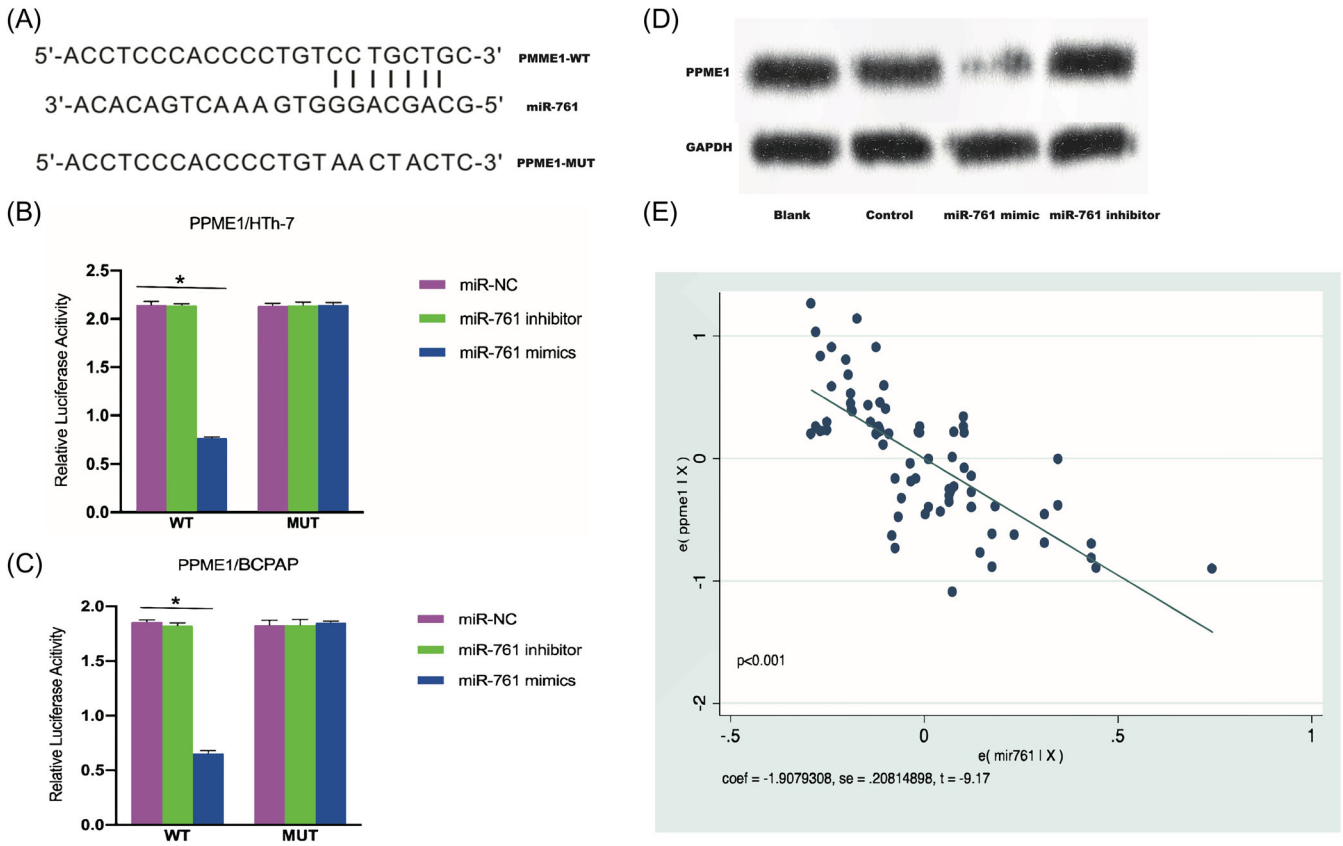

FIGURE 3 miR-761 targets PPME1. A, Verification of the targeting relationship between miR-761 and PPME1. B,C, The luciferase activity in each group detected by a dual luciferase reporter gene assay for two types of thyroid cancer cells. D, In western blot analysis, miR-761 mimic group exhibited lower PPME1 expression and miR-761 inhibitor group exhibited higher PPME1 expression. E, miR-761 and PPME1 were negatively correlated after analyzing by linear regression (coef = −1.91, $P < .001$, n = 50)

two genes, western blot analysis was conducted. PPME1 level was increased after treatment with HOTAIR but decreased after treatment with by Si-HOTAIR (Figure 4B).

3.8 | The expression of HOTAIR affects the survival time in vivo

We used the lower and higher expression of HOTAIR thyroid cancer cells to xeno-graft tumors in nude mice. After an extended follow up, we found that the group with lower expression of HOTAIR experienced longer survival time ($P < .001$) (Figure 4C).

4 | DISCUSSION

Thyroid cancer is defined as a tumor with better prognosis. But different types or stages of cancer may cause different outcome of the diseases. Several studies had verified that dys-regulation of lncRNAs could causes important changes in tumor development and metastasis.[20,21] In addition, HOTAIR was reported to be correlated with cell apoptosis, proliferation, invasion and metastasis.[18,19] Thus, we investigated the role of HOTAIR in thyroid cancer. The findings revealed that silencing HOTAIR up-regulates miR-761, which down-regulates PPME1, thus inhibiting cell proliferation and promoting cell apoptosis of thyroid cancer.

HOTAIR was expressed to a great extent in thyroid cancer tissue. By silencing HOTAIR, the proliferation of thyroid cancer cells was inhibited and the apoptosis was elevated. Over-expression of HOTAIR had the opposite effect. LncRNAs are involved in many cellular processes, such as cell proliferation, migration and invasion.[22] In our vivo study, we found thyroid cancer cells that have high expression of HOTAIR lead to increased mortality of nude mice. Thus, HOTAIR affects the prognosis of thyroid cancer. Through data collected from the thyroid cancer patients in this study, we found that HOTAIR is an independent predictive factor for thyroid tumor staging.

Moreover, we found that HOTAIR binds competitively to miR-761 as a ceRNA. LncRNAs could negatively regulate miRNAs by serving as ceRNAs of miRNA.[23] In recent studies, HOTAIR was shown to bind with miR-17-5p and miR-1 to regulate the biological function of thyroid.[18,19]

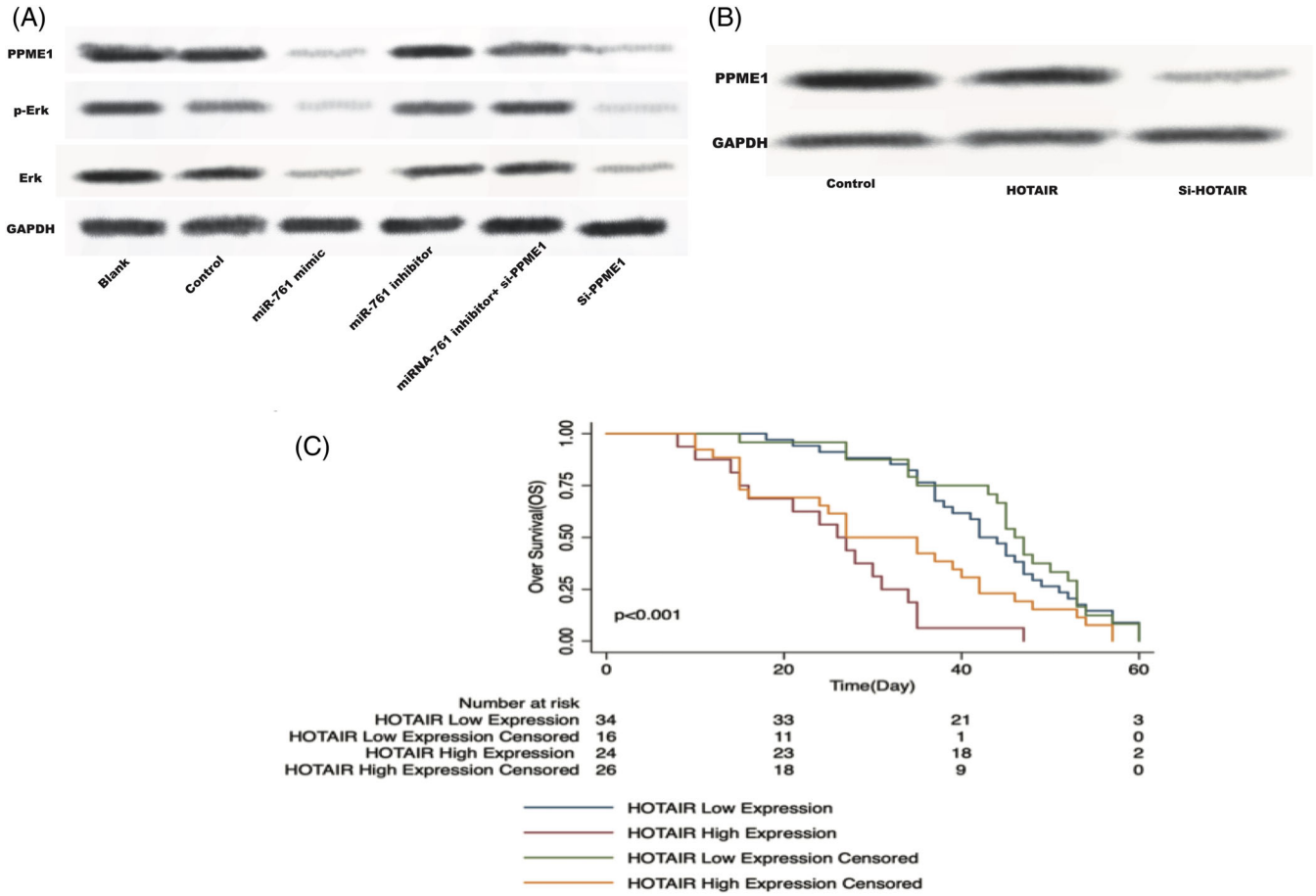

FIGURE 4 miR-761 inhibits the activation of the Erk signaling pathway by targeting PPME1. A, The grey value of PPME1, p-Erk and Erk. GAPDH served as an internal control. B, PPME1 protein in each group by western blot analysis treated by HOTAIR. C, After extended follow up, we found that the group with lower expression of HOTAIR experienced longer survival times ($P < .001$, n = 50)

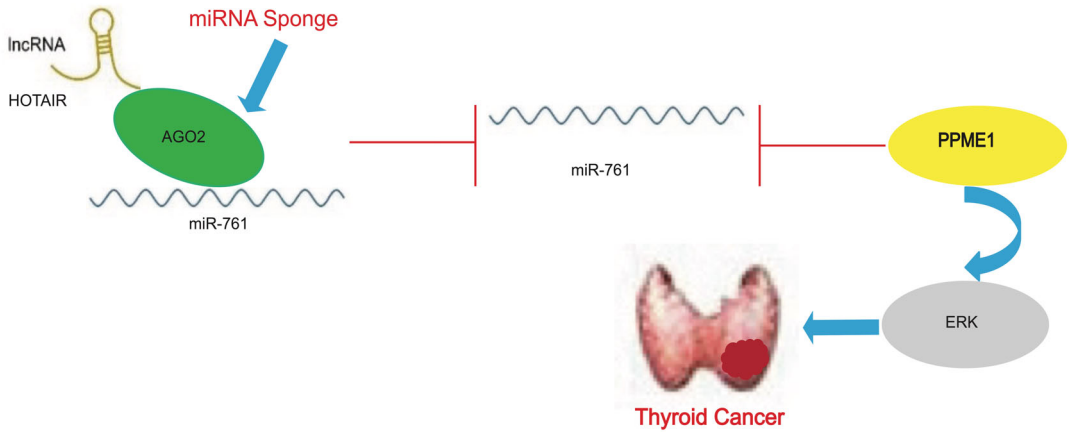

FIGURE 5 HOTAIR competitively binds to miR-761, thereby promoting PPME1 expression, activating Erk signaling pathway

Furthermore, we found that miR-761 targeted PPME1 to inhibit the proliferation and promote the apoptosis of thyroid cancer through the Erk pathway. We also found that miR-761 and PPME1 were negatively correlated after analyzing by linear regression. miR-761 may suppress osteosarcomas and affect their biological function, such as proliferation, apoptosis, invasion and metastasis.[24] Consistent with our results, Lv et al highlighted the tumor- suppressing abilities of miR-761. Further, PPME1 is a target of miR-761, and miR-761 could down-regulates PPME1 expression. From the results of our study, miR-761 inhibited tumor cell

proliferation and promoted apoptosis in thyroid cancer via targeting PPME1. PPME1 was found to be increased and correlated with malignant progression and Erk pathway activity.[7] We also verified in this study that miR-761 could targets PPME1 to inhibit Erk signaling pathway activation. The Erk signaling pathway is important in cancer tumorigenesis and development.[25]

5 | CONCLUSION

Silencing HOTAIR inhibits biological functions of thyroid cancer via PPME1 down-regulation by binding competitively to miR-761. HOTAIR competitively binds to miR-761, thereby stimulating PPME1 expression, activating the Erk signaling pathway (Figure 5). Thus, HOTAIR silencing can serve as a therapeutic target for thyroid cancer. From the clinical data, we found that HOTAIR is an independent predictive factor for thyroid tumor staging.

ACKNOWLEDGMENT

This study is supported by Shanghai Pujiang Program (18PJD004), Shanghai Municipal Jiading District Science and Technology Commission Foundation(JDKW-2018-W14) and Shanghai Municipal Jiading District Fifth Medical Key Subject Program(2020-JDYXZDXK-06).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Runsheng Guo, Yong Ning, Ye Ma, and Qianhuang Lin did all the experiments and statistics. Na Shen and Peidong Shi designed the experiments and wrote the manuscript.

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How to cite this article: Guo R, Ning Y, Ma Y, Lin Q, Shen N, Shi P. Long non-coding RNA HOTAIR/microRNA-761 sponge regulates PPME1 and further influences cell biological functions in thyroid carcinoma. *Laryngoscope Investigative Otolaryngology*. 2021;6(3):438–445. https://doi.org/10.1002/lio2.593