



LncRNA ABCC6P1 promotes proliferation and migration of papillary thyroid cancer cells via Wnt/ β -catenin signaling pathway

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Background: LncRNAs play an important regulatory function in the occurrence and progression of papillary thyroid cancer (PTC). This study aimed to investigate the role and mechanism of ATP binding cassette subfamily C member 6 pseudogene 1 (ABCC6P1) in PTC.

Methods: Cancerous and paracancer normal thyroid tissues were collected from 18 patients with PTC, who were operated at the Second Affiliated Hospital of Harbin Medical University. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to investigate the levels of ABCC6P1. Cell proliferation was evaluated using Cell Counting Kit-8 (CCK-8) and colony formation assays. Wound healing and Transwell invasion assays were performed to examine cell migratory and invasive ability. Western blotting analysis was used to detect the expression levels of EMT-related markers and Wnt/ β -catenin signaling pathway-related proteins.

Results: The expression of ABCC6P1 was upregulated in PTC tissues and cells. ABCC6P1 silencing could significantly suppress the proliferation, colony formation ability, migratory and invasive ability in PTC cells. Moreover, knockdown of ABCC6P1 induced cell cycle arrest at G0/G1 phase and inhibited epithelial-mesenchymal transition (EMT) process of PTC cells by increasing the E-cadherin expression, but downregulating N-cadherin and vimentin expression. In addition, knockdown of ABCC6P1 caused a significant decrease in levels of Wnt/ β -catenin signaling pathway members (including β -catenin, c-myc, and cyclin D1) in PTC cells.

Conclusions: Our study confirms that ABCC6P1 exerts an oncogenic activity in PTC which may be mediated by the Wnt/ β -catenin pathway, suggesting that ABCC6P1 may be a promising therapeutic target for PTC.

Keywords: RNA; long noncoding; thyroid neoplasm; Wnt signaling pathway; cell proliferation

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Introduction

Papillary thyroid cancer (PTC) is the most common endocrine cancer, accounting for 3.4% of all cancers diagnosed each year (1). Surgery, chemotherapy, and

radioactive iodine treatment are the most commonly used medical treatment options. However, these treatments appear to have little impact on the prognosis of recurrent PTC (2). The challenge in the diagnosis and treatment of

PTC is to identify patients at high risk of radioactive iodine refractory disease and to prevent overtreatment. Therefore, the identification of novel biomarkers and dissection of molecular mechanisms are pivotal to improve the therapeutic options for patients and the overall management of PTC.

Thyroid cancer is a complex disease that is influenced by the environment and genes. The main known factors include ionizing radiation exposure, obesity, and genetic and epigenetic variations (3). The incidence of thyroid cancer varies greatly among different populations and races in the world, suggesting that the occurrence of thyroid cancer is related to genetic inheritance and social customs (4), but there are still a lot of problems to be solved about its pathogenic factors and pathogenesis. Currently, genes found to be associated with thyroid cancer include BRAF and RAS, RET/PTC, MAPK, P13K/Akt, PAX8, PPAR 1, TP53, etc. (5). However, these genes are not enough to fully explain the pathophysiological processes such as occurrence, invasion and metastasis of thyroid cancer, and no breakthrough progress has been made in the clinical diagnosis and treatment of thyroid cancer.

lncRNAs are non-coding transcripts that are more than 200 nucleotides long and engage in numerous cellular processes, chromosome X inactivation, genomic imprinting, maintenance of pluripotency, and the formation of different organs through regulating chromatin, transcription and translation (6,7). Numerous studies have shown that lncRNAs exert a very diverse role in the process of tumorigenesis and development, and participate in proliferation, apoptosis, migration and angiogenesis (8-11). In PTC, several lncRNAs, including HOTAIR, NEA1, UCA1, and LINC00460, have been identified as tumor promoters and can be used as potential biomarkers for early diagnosis or potential therapeutic targets (12-15). Nevertheless, some lncRNAs, such as SLC26A4-AS1, PAR5, RPL34-AS1, and DLX6-AS1, exhibit tumor suppressive abilities (16-19). These findings indicate that lncRNAs can regulate almost all processes related to cancer initiation and progression. Chromosome 16 locates lncRNA ABCC6P1 which, along with its parent ABCC6, shares near-identical promoter sequences and similar tissue-specific expression profiles (20). In addition, ABCC6P1 is transcriptionally active and can regulate ABCC6 at the transcriptional level (21). However, the function and mechanism of ABCC6P1 have not been reported in the field of human cancer. We discovered that ABCC6P1 was upregulated in thyroid cancer through online database

analysis.

Here, we for the first time revealed the oncogenic role of ABCC6P1 in PTC. We investigated the expression of lncRNA ABCC6P1 in PTC tissues and cell lines using qRT-PCR method and the effects of ABCC6P1 on cellular function in PTC were analyzed in vitro. Moreover, the regulatory mechanisms related to ABCC6P1 regulating PTC progression were also further investigated. We present the following article in accordance with the MDAR checklist (available at <http://dx.doi.org/10.21037/atm-21-505>).

Methods

Tissue samples

Eighteen patients with PTC who received surgical treatment in the Second Affiliated Hospital of Harbin Medical University from July 2017 to May 2018 were recruited. The 18 patients included 8 males and 10 females, with a mean age of 58 ± 18.2 years. The following patients were included as samples: patients diagnosed with PTC by B-ultrasound and histopathology; No iodine-131 endoradiotherapy, thyrotropin suppressive therapy or conventional radiotherapy were received before enrollment. Patients with normal heart, lung, liver, kidney and other vital organs. Patients with the following conditions were excluded: Patients who received TSH suppressive therapy and iodine-131 radiotherapy prior to surgery were excluded; patients with incomplete clinical or pathological data, such as lack of pathological diagnosis report or lymph node metastasis report; patients with other malignant tumors; patients with other malignant tumors metastatic to the thyroid; patients with a serious systemic infection or other serious systemic disease. The study was authorized by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University and conformed to Helsinki Declaration (as revised in 2013). All patients signed written informed consent before their participation in the study.

qRT-PCR

Total RNAs of tissues or cells were collected using TRIzol Reagent (Thermo Fisher Scientific, USA). High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, USA) was used for reverse transcription. qRT-PCR was carried out with a Platinum SYBR Green qPCR SuperMix-UDG kit (Thermo Fisher Scientific, USA). The relative expression of ABCC6P1 was calculated using the

Table 1 Sequences of primers and shRNAs used in the study

Primers	Sequences
ABCC6P1	
Forward primer	5'-CGGGGTACCCTGGGACCCACGACGACAGAAGGCG-3'
Reverse primer	5'-CGCGGATCCACAAAATTTATTAGAAAGTTACAT-3'
GAPDH	
Forward primer	5'-GGACCTGACCTGCCGTCTAG-3'
Reverse primer	5'-GTAGCCCAGGATGCCCTTGA-3'
ABCC6P1 shRNA	
Sense	5'-GATCCGGCAAGACCCAAAGCTTCCCCAGAGATCCCTCGAGGGATCTCTGGGGAAGCTTTGGGTCTTGCCTTTTTGA-3'
Anti-sense	5'-AGCTTCAAAAAGGCAAGACCCAAAGCTTCCCCAGAGATCCCTCGAGGGATCTCTGGGGAAGCTTTGGGTCTTGC CG-3'

$2^{-\Delta\Delta Ct}$ method. The sequences of the primers are summarized in *Table 1*.

Western blot

TPC-1 and B-CPAP cells were lysed in RIPA buffer. Equal total protein of cell lysates from each condition were separated by 15% SDS-PAGE and then immunoblotting. The primary antibodies were as the following: anti-E-cadherin (Cat No. 60335, Proteintech, China); anti-N-cadherin (Cat No. 66219, Proteintech, China); anti-Vimentin (Cat No. 60330, Proteintech, China); anti-Beta-Actin (Cat No. 66009, Proteintech, China); anti-beta-catenin (Cat No. 66379, Proteintech, China); anti-cyclin D1 (Cat No. 66186, Proteintech, China); anti-c-myc (Cat No. 67447, Proteintech, China); anti-GAPDH (Cat No. 60004, Proteintech, China).

Cell culture

All cells and mediums used in this study were commercially acquired from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Human thyroid follicular epithelial cells Nthy-ori-3-1 and human PTC cell lines K1, TPC-1, 8305C, and B-CPAP were used in this study. The Nthy-ori-3-1 cells were cultured in CM2-1 medium, 8305C cells in EMEM medium, and K1, TPC-1 and B-CPAP cells in DMEM medium.

Cell transfection

The shRNA vectors targeting ABCC6P1 (sh-ABCC6P1) and corresponding negative control (NC) were purchased from GenePharma (Shanghai, China). Transfection was performed with Lipofectamine 3000 (Thermo Fisher Scientific, USA).

Cell Counting Kit-8 (CCK8)

We next plated 3000 transfected B-CPAP and TPC-1 cells in 96-well plates. At a designated time point, 10 μ L CCK8 solutions (Beyotime Institute of Biotechnology, China) were added to each well and after incubation for 4 hours, OD values at 450 nm were observed using a microplate reader (Bio-Rad, USA).

Colony formation assay

After transfection with sh-ABCC6P1 or empty vector, B-CPAP and TPC-1 cells were plated in 6-well plates at a concentration of 500 cells/well. After incubation for two weeks, cells were treated with 10% neutral buffered formalin, followed by staining with 0.1% crystal violet solution. The plates were then washed, and cell colonies were counted using an inverted microscope.

Wound healing

After designated treatment, B-CPAP and TPC-1 cells were

seeded in six-well plates at a concentration of 2×10^5 cells/well and grown to a monolayer. A cell monolayer was then made with a 200- μ L pipette tip and washed three times to remove floating cells. Finally, after treatment for 0 and 24 h, cell images were photographed with a phase-contrast microscope (Zeiss, Primovert, Axiocam) and migration was quantified with Image J Software.

Transwell invasion assay

Before invasion assay, the upper chamber of a 24-well Transwell chamber (8 μ m) was precoated with Matrigel (Corning, Life Sciences, USA). Cell suspension were then prepared with 200 μ L serum-free DMEM and added to the upper cavity, while 500 μ L DMEM with 20% serum was loaded to the lower cavity. After cultivation for 48 hours, the cells on the lower surface were stained with crystal violet. Finally, the migrated cells were analyzed with a microscope.

Statistical analysis

All experiments were conducted in three independent replicates, and statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA). The data were analyzed by student *t*-test or one-way ANOVA and $P < 0.05$ was considered statistically significant.

Results

LncRNA ABCC6P1 was highly expressed in PTC tissues and cell lines

The expression of ABCC6P1 in PTC tissues was firstly analyzed through GEPIA dataset (<http://gepia.cancer-pku.cn/>) (22), and the results showed it was upregulated in thyroid cancer tissues compared with normal tissues ($P < 0.05$, *Figure 1A*). We next examined the levels of ABCC6P1 in 18 paired samples of PTC tissues and matched normal thyroid tissues using qRT-PCR assay. As expected, the expression of ABCC6P1 in PTC tissues was dramatically higher than that in corresponding normal tissues ($P < 0.01$, *Figure 1B*). Furthermore, high levels of ABCC6P1 were associated with TNM stage III tumors ($P < 0.05$, *Figure 1C*), but not with lymph node metastasis (*Figure 1D*). Additionally, high levels of ABCC6P1 were also found in PTC cells compared with Nthy-ori-3-1 cells (*Figure 1E*). As B-CPAP and TPC-1 cells expressed the first and second levels highest of ABCC6P1

levels, these were selected for further experiments.

Knockdown of ABCC6P1 suppresses PTC cell proliferation

To explore the role of ABCC6P1, B-CPAP and TPC-1 cells were transfected with sh-ABCC6P1 or NC. As presented in *Figure 2A*, the endogenous expression of ABCC6P1 was markedly reduced in B-CPAP and TPC-1 cells following transfection with the ABCC6P1 shRNA plasmid and CCK-8 assay demonstrated that ABCC6P1 silencing obviously suppressed the growth of B-CPAP and TPC-1 cells (*Figure 2B,C*). Moreover, colony formation assay indicated that the colony formation ability of PTC cells was dramatically suppressed by ABCC6P1 silencing (*Figure 2D*). These data suggest that ABCC6P1 knockdown restrains the proliferation of PTC cells.

Knockdown of ABCC6P1 inhibits PTC cell migration and invasion

Migration and invasion are two important biological features of malignancies which contribute to tumor progression. Therefore, wound healing and Transwell invasion assays were conducted to examine the effect of silencing ABCC6P1 on PTC cell migratory and invasive capacities. As displayed in *Figure 3A,B*, wound healing assay revealed that knockdown of ABCC6P1 significantly inhibited the migratory capacity of B-CPAP and TPC-1 cells. In addition, Transwell invasion assay revealed the invasive ability in sh-ABCC6P1 groups was significantly reduced compared with that in NC groups in B-CPAP and TPC-1 cells (*Figure 3C,D*).

ABCC6P1 influences cell cycle in PTC cells

We further examined whether knockdown of ABCC6P1 alters the cell cycle and found knockdown of ABCC6P1 moderately induced cell cycle arrest at G0/G1 phase and reduced the proportion of cells at S phase in B-CPAP and TPC-1 cells (*Figure 4*). These results indicate that ABCC6P1 regulates tumor cell proliferation by altering the cell cycle in PTC.

Knockdown of ABCC6P1 alters the EMT process

We further examined the effect of ABCC6P1 on EMT-related markers. Western blot analysis showed ABCC6P1 knockdown increased the expression of epithelial marker

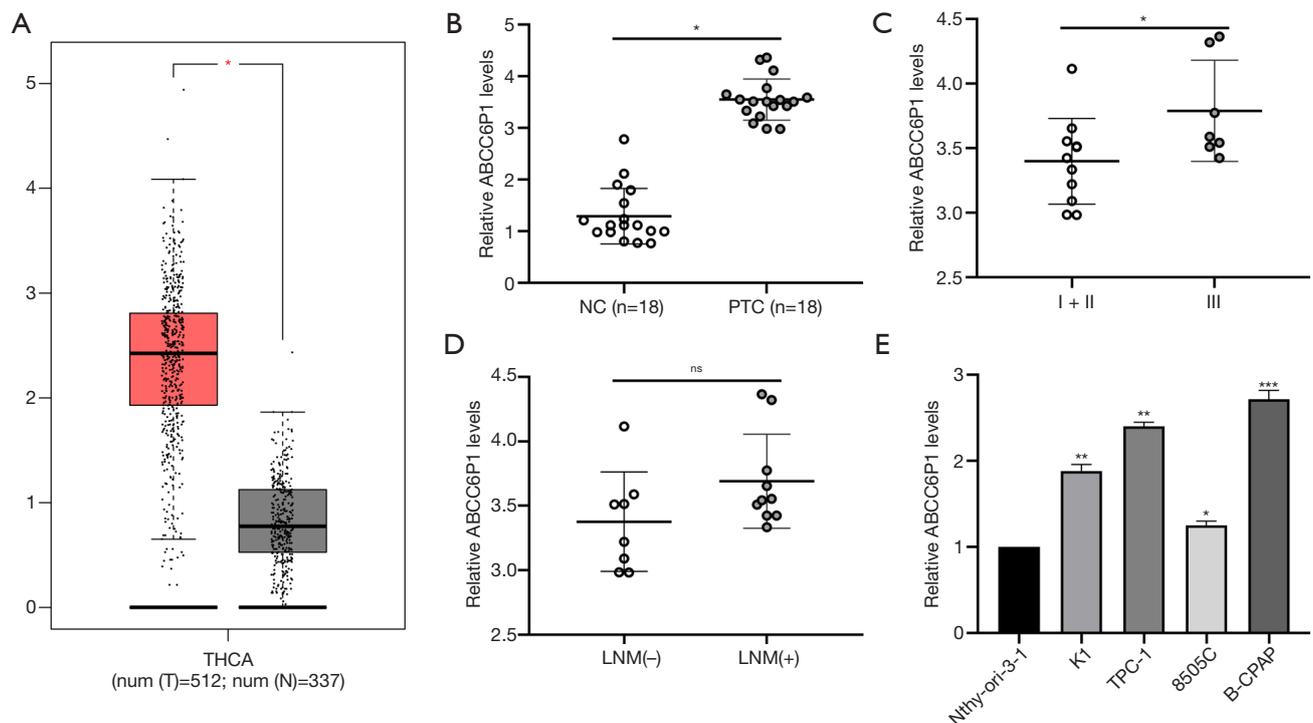


Figure 1 ABCC6P1 is highly expressed in PTC. (A) The expression of ABCC6P1 was analyzed between THCA tissues and normal tissues using the GEPIA database; (B) the expression of ABCC6P1 in PTC tissues (n=18) and matched normal tissues (n=18) were examined using qRT-PCR; (C) the expression of ABCC6P1 in TNM stage III (n=7) and TNM stage I + II (n=11); (D) the expression of ABCC6P1 in LNM-positive tissues (n=10) and LNM-negative tissues (n=8); (E) the expression of ABCC6P1 in PTC cell lines (K1, TPC-1, 8505C, and B-CPAP) and normal human thyroid Nthy-ori-3-1 cell line. ns, no significance; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. THCA, thyroid carcinoma; PTC, papillary thyroid cancer; LNM, lymph node metastasis.

E-cadherin and decreased the expression of mesenchymal markers (N-cadherin and Vimentin) in B-CPAP and TPC-1 cells (Figure 5A). These results indicate that ABCC6P1 could alter the EMT process to regulate cell migration and invasion in PTC.

Knockdown of ABCC6P1 inactivates Wnt/ β -catenin signaling pathway

The Wnt/ β -catenin signaling pathway is a conserved signaling axis involved in various physiological processes such as proliferation, differentiation, apoptosis, migration, and invasion. Increasing evidence shows dysregulation of the Wnt/ β -catenin cascade is associated with the development and progression of PTC (23). To investigate whether the Wnt/ β -catenin signaling pathway is involved in the regulation of ABCC6P1 in PTC, Western blot was employed to examine the expression of related proteins, including β -catenin, cyclin D1, and c-myc. As shown in

Figure 5B, ABCC6P1 knockdown obviously suppressed the expression of β -catenin, cyclin D1 and c-myc in B-CPAP and TPC-1 cells.

ABCC6P1 promotes PTC cell function by Wnt/ β -catenin signaling pathway

To further investigate whether ABCC6P1 promotes the malignant behavior of PTC cells through activating the Wnt/ β -catenin pathway, B-CPAP and TPC-1 cells were treated with LiCl, which is an agonist of Wnt/ β -catenin pathway. The results of CCK-8 and colony formation assays showed that LiCl treatment restored the inhibitory effect of ABCC6P1 silencing on proliferation in B-CPAP and TPC-1 cells (Figure 6A,B). Furthermore, wound healing and Transwell assays demonstrated that the inhibition of the migratory and invasive ability induced by ABCC6P1 knockdown in PTC cells was restored by LiCl (Figure 6C,D). Together, these data suggest ABCC6P1

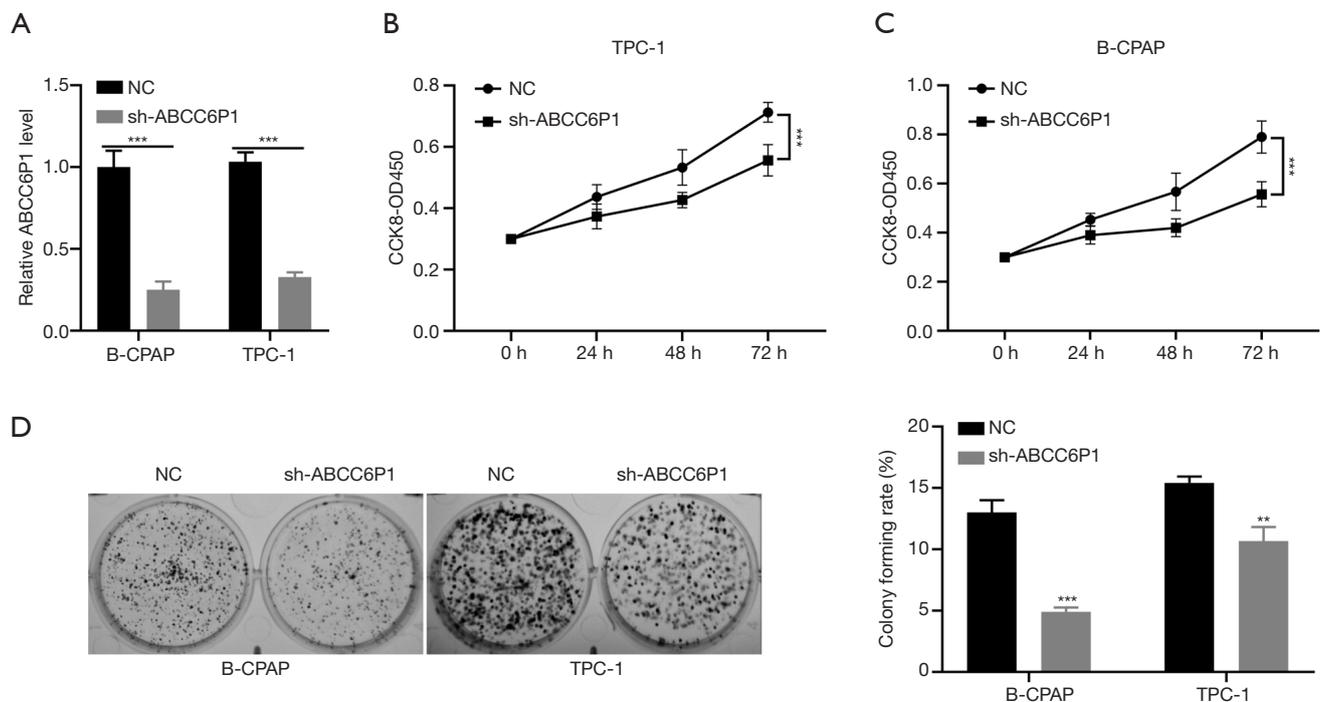


Figure 2 Knockdown of ABCC6P1 suppresses the proliferation of PTC cells. (A) qRT-PCR was used to detect the expression of ABCC6P1 in B-CPAP and TPC-1 cells transfected with sh-ABCC6P1 or empty vector; (B,C) CCK8 assay demonstrated that ABCC6P1 silencing inhibited the proliferation in B-CPAP and TPC-1 cells; (D) colony formation assay revealed that ABCC6P1 knockdown reduced colony forming rate of PTC cells. **, $P < 0.01$; ***, $P < 0.001$. PTC, papillary thyroid cancer.

activates the Wnt/ β -catenin pathway, which in turn promotes proliferation, migration and invasion of PTC cells.

Discussion

Thyroid cancer is a common malignancy of the endocrine system and PTC accounts for 70–80% of all thyroid cancer cases (24). The identified risk factors for thyroid cancer include ionizing radiation and excess iodine consumption (25,26). In addition, accumulating evidence indicates lncRNAs play a key role in the occurrence and development of PTC. Therefore, research into the role and mechanism of cancer-related lncRNAs may support new perspectives for the pathogenesis of PTC and facilitate its diagnosis and treatment.

A growing body of evidence supports the view that lncRNAs play a crucial regulatory role in the tumorigenesis and progression of PTC (27,28). Wen *et al.* have shown lncRNA SET-binding factor 2-antisense RNA1 promotes PTC cell proliferation and cycle, and inhibits cell apoptosis

in a ceRNA manner (29). In addition, lncRNA HOTAIR has been shown to enhance the viability and metastasis of PTC cells via modulating epithelial-mesenchymal transition (EMT) mediated by the Wnt/catenin pathway (30). Here, we found that a new lncRNA ABCC6P1 was upregulated in PTC tissues and cells and high levels of ABCC6P1 was associated with TNM stage III. Besides, we also analyzed the correlation between ABCC6P1 and pathological factors with the chi-square test, but no significance was found (Table S1), which may be due to the small sample size. Further, ABCC6P1 silencing suppressed cell proliferation, migratory, and invasive ability in PTC cells, indicating the important role of ABCC6P1 in PTC. Cell cycle is a complex series of events involved in cell division and replication and uncontrolled cell cycle is a major feature of cancer cells (31). Here, we found that knockdown of ABCC6P1 could lead to cell cycle arrest, which may explain the inhibition of cell proliferation. Additionally, ABCC6P1 also altered EMT process in PTC. EMT is one of the important mechanisms of tumor progression, which often occurs in PTC (32). EMT confers the ability of tumor cells to metastasize and

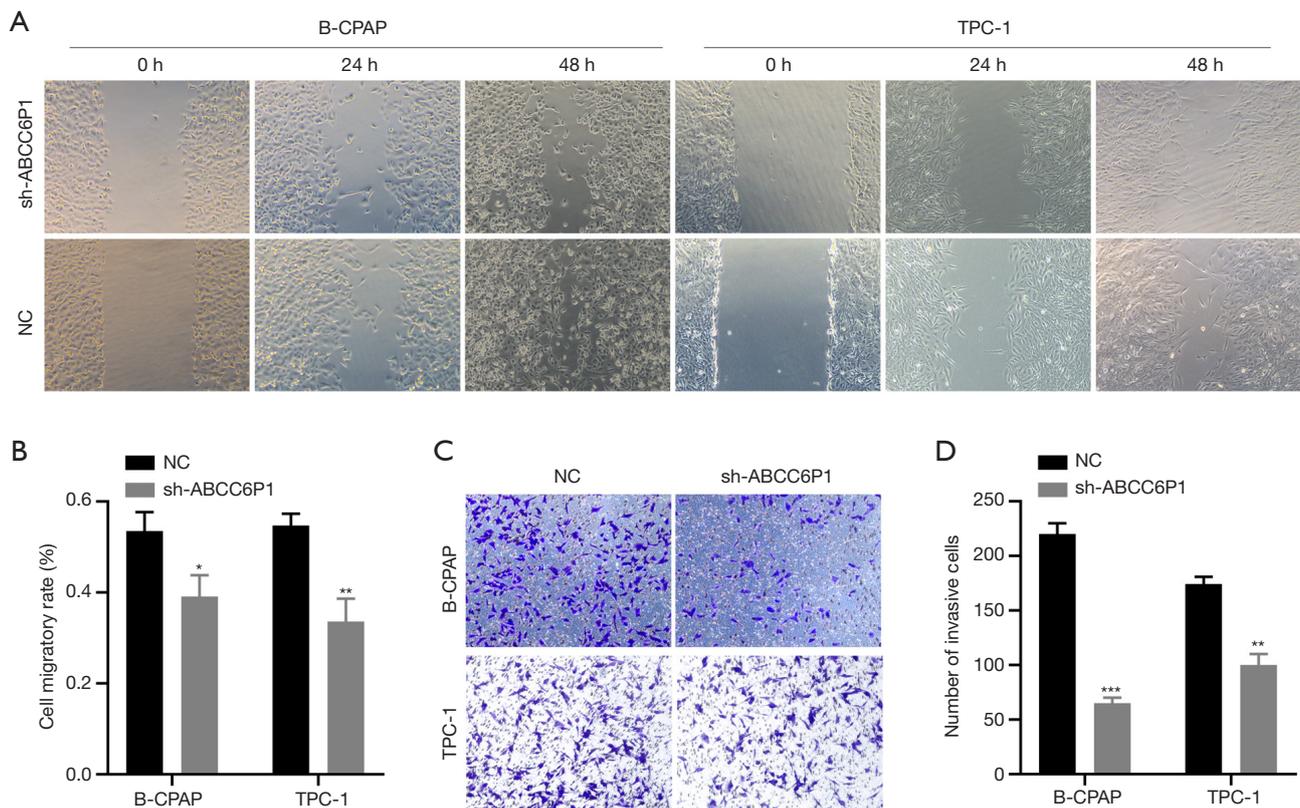


Figure 3 Knockdown of ABCC6P1 restrains the migratory and invasive activity of PTC cells. (A,B) Wound healing assay showed that ABCC6P1 knockdown restrained the migratory ability of B-CPAP and TPC-1 cells (magnification: 100 \times); (C,D) Transwell invasion assay (magnification $\times 100$, crystal violet staining) demonstrated that ABCC6P1 knockdown weakened the invasive ability of B-CPAP and TPC-1. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. PTC, papillary thyroid cancer.

invade, and is associated with various metastatic traits such as tumor-initiation, therapeutic resistance, immune escape and anchoring independent growth (33). At the molecular level, EMT is characterized by loss of the epithelial cell surface marker E-cadherin and the acquisition of interstitial markers such as Vimentin and N-cadherin. In this study, we found that knockdown of ABCC6P1 caused increased E-cadherin and decreased Vimentin and N-cadherin, suggesting ABCC6P1 promotes EMT process, thereby conferring the migratory and invasive ability of PTC cells.

Wnt/ β -catenin signaling pathway participates with cell development and differentiation and aberrant activation is responsible for the development of pathologies such as cancer. Activated Wnt signaling drives β -catenin to enter the cell nuclei, resulting in transcription of downstream target genes, including c-myc and cyclin D1, to promote the differentiation and proliferation of tumor cells. Zhang *et al.* (34) revealed that activation of the Wnt/ β -catenin

pathway causes a significant increase of cyclin D1 levels, which is linked to lymph node metastases in PTC. Zhou *et al.* showed that knockdown of TRIM44 could inhibit the proliferation and invasion of PTC cells by inhibiting the Wnt/ β -catenin pathway (35), and down-regulation of SOX9 inhibited the EMT process through inactivating the Wnt/ β -catenin signaling pathway in PTC (24). Similar with these studies, we revealed that knockdown of ABCC6P1 impaired the activity of β -catenin and inactivated downstream effector molecules cyclin D1 and c-myc, whereas LiCl, an agonist of Wnt/ β -catenin pathway, restored inhibitory effect of ABCC6P1 silencing on malignant behavior. These results confirm that ABCC6P1 activates Wnt/ β -catenin pathway, thereby promoting tumor progression in PTC.

Conclusions

In this study, we found ABCC6P1 was highly expressed

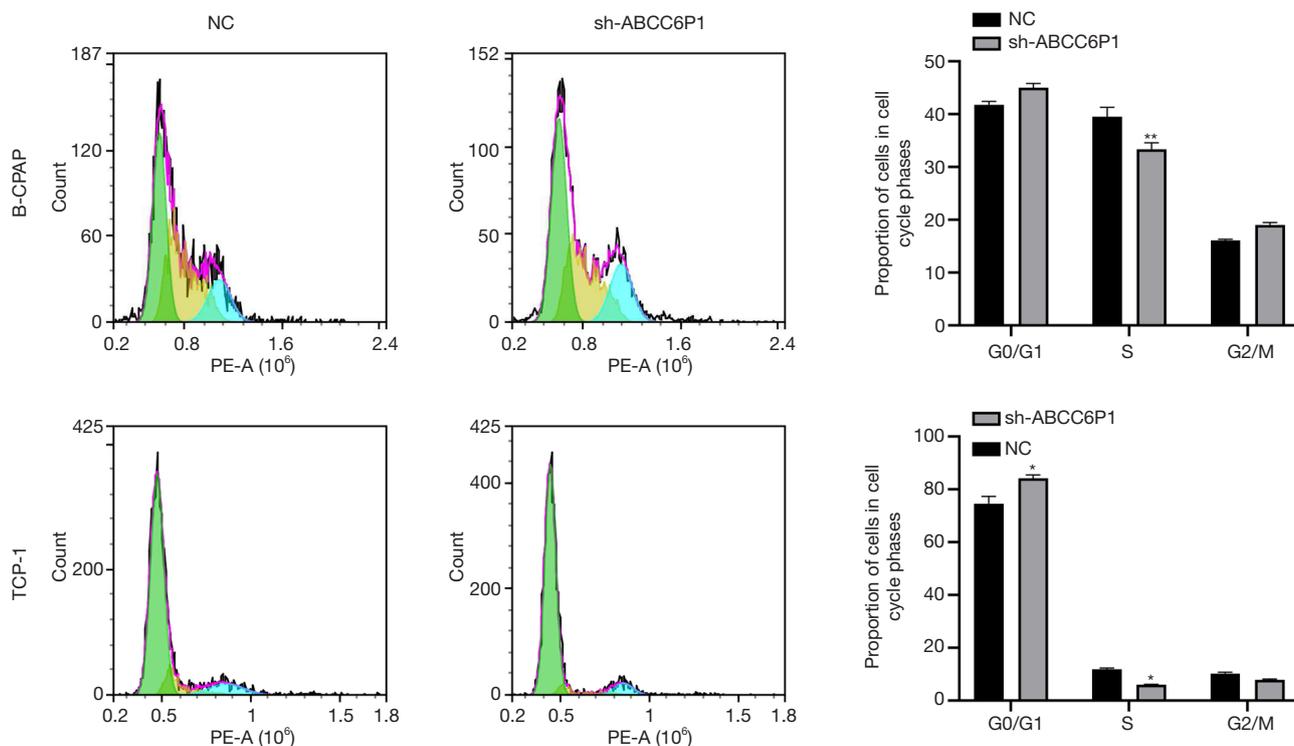


Figure 4 ABCC6P1 influences cell cycle in PTC cells. Flow cytometry analysis for cell cycle in PTC cells after sh-ABCC6P1 transfection. *, $P < 0.05$; **, $P < 0.01$. PTC, papillary thyroid cancer.

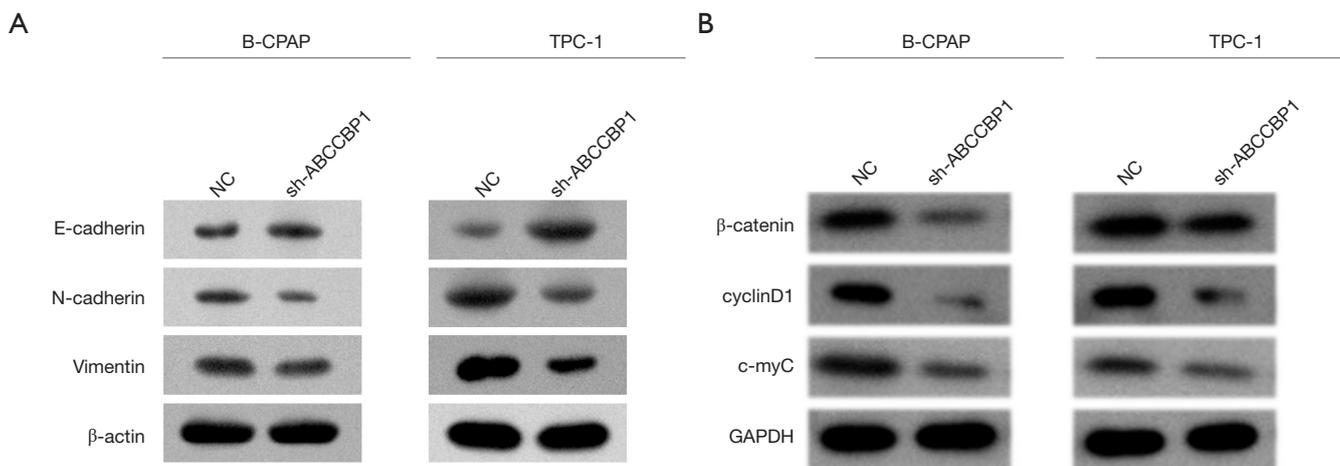


Figure 5 Knockdown of ABCC6P1 regulates EMT and Wnt/ β -catenin signaling pathway. (A) Western blot analysis showed that ABCC6P1 knockdown caused increase of E-cadherin and decrease of N-cadherin and Vimentin; (B) Western blot analysis showed that ABCC6P1 knockdown suppressed the expression of β -catenin, cyclin D1 and c-myc in B-CPAP and TPC-1 cells.

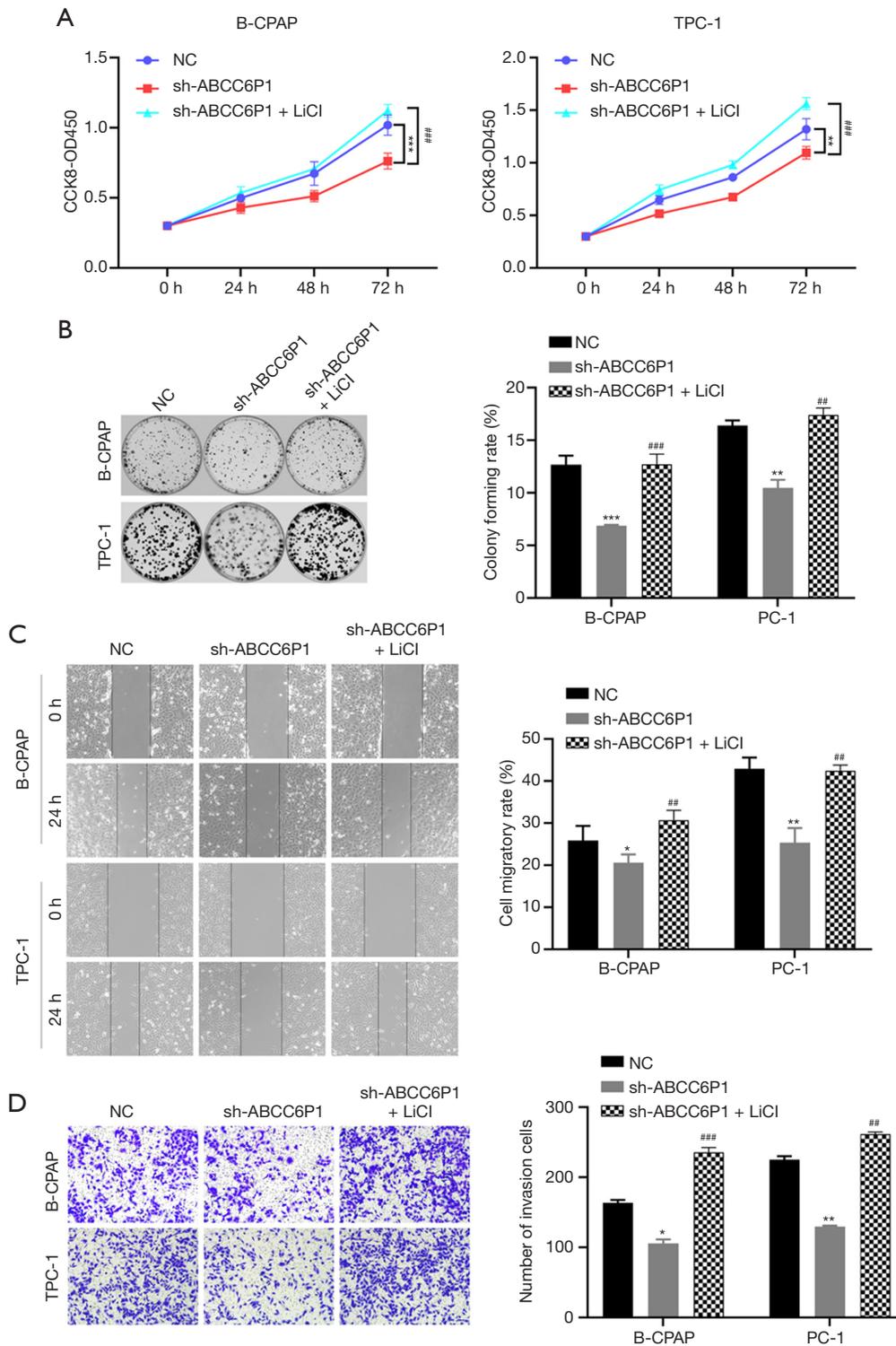


Figure 6 ABCC6P1 promotes PTC cell function by Wnt/ β -catenin signaling pathway. (A,B,C,D) CCK-8 assay, colony formation, wound healing and Transwell invasion assays (magnification $\times 100$, crystal violet staining) displayed that LiCl, as a Wnt activator, reversed the inhibitory effect of ABCC6P1 knockdown on the malignant behavior of PTC cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ vs. NC group; ##, $P < 0.01$; ###, $P < 0.001$ vs. sh-ABCC6P1 groups. PTC, papillary thyroid cancer.

in PTC tissues and knockdown of ABCC6P1 by RNA interference technique could inhibit cell proliferation, migration and invasion through the Wnt/ β -catenin signaling pathway. These data indicate that blockade of ABCC6P1 may be a potential therapeutic option for PTC.

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Footnote

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Data Sharing Statement: Available at <http://dx.doi.org/10.21037/atm-21-505>

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University and was conducted in accordance with Helsinki Declaration (as revised in 2013). All participants signed written informed consent prior to their participation in the study.

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Table S1 Correlation analysis between ABCC6P1 and pathological factors

Characteristics	N	Relative ABCC6P1 expression		P value
		High	Low	
Age (years)				
<55	9	4	5	0.99
≥55	9	5	4	
Gender				
Male	8	5	4	0.81
Female	10	5	5	
TNM staging				
I-II	11	4	7	0.78
III-IV	7	3	4	
Extrathyroidal extension				
Present	9	5	4	0.64
Absent	9	4	5	
Multifocality				
Present	5	3	2	0.60
Absent	13	6	7	
Lymph node metastasis				
Present	10	6	4	0.67
Absent	8	4	4	
Distant metastasis				
Present	2	2	0	0.47
Absent	16	7	9	