ORIGINAL ARTICLE

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LncRNA MCM3AP-AS1 promotes proliferation and invasion through regulating miR-211-5p/SPARC axis in papillary thyroid cancer

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Abstract

Background Long non-coding RNAs (lncRNAs) are an emerging class of regulators in cancer. A lncRNA, MCM3AP-AS1, has been demonstrated as a versatile mediator in many cancers, except papillary thyroid cancer. The aim of this study is to investigate the role and mechanism of MCM3AP-AS1 in papillary thyroid cancer.

Methods Quantitative real-time PCR was used to assess the level of MCM3AP-AS1 and miR-211-5p in papillary thyroid cancer tissues and cells. Western blot was used to detect E-cadherin and secreted protein acidic and cysteine rich (SPARC) protein levels. CCK-8, scratch wound assay, and transwell assay were used to evaluate papillary thyroid cancer cell proliferation, migration, and invasion, respectively. BLAST alignment and luciferase assay were used to explore the interaction among MCM3AP-AS1, mi/r-211, and SPARC.

Results In papillary thyroid cancer, MCM3AP-AS1 was upregulated, while miR-211 was downregulated. MCM3AP-AS1 overexpression promoted papillary thyroid cancer proliferation, migration, and invasion. Further, MCM3AP-AS1 was shown to be negatively correlated with miR-211-5p. We next validated that miR-211-5p overexpression could reverse the promoting role of MCM3AP-AS1 in papillary thyroid cancer, whereby SPARC plays an important regulating role. In vivo, we confirmed the anti-tumor role of MCM3AP-AS1 silencing and the close relation among MCM3AP-AS1, miR-211-5p, and SPARC.

Conclusions MCM3AP-AS1 promotes papillary thyroid cancer by regulating the MCM3AP-AS1/miR-211-5p/SPARC axis, which could potentially be a therapeutic target in papillary thyroid cancer.

Keywords Papillary thyroid cancer · Therapeutic target · MCM3AP-AS1

List of abbreviations

PTC	Papillary thyroid cancer
lncRNA	Long non-coding RNA
miRNAs	microRNAs
GECs	glioma-associated endothelial cells
GBM	Glioblastoma
GFP	green fluorescent protein

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Introduction

Papillary thyroid cancer (PTC) is the most widespread type of thyroid cancer, which accounts for ~3.4% of all new tumor cases [1]. There are about 56,870, new thyroid cancer cases in 2017 with an estimated 2010 deaths according to The U. S. National Cancer Institute [2]. The annual estimated number thyroid cancer cases in China is 90,000, with an estimated 6800 deaths [3]. However, there is still a gap to develop effective therapies to overcome this complicated disease. Treatment of PTC is a challenge in clinics. Although surgical resection is still the most effective strategy to treat PTC, re-operation of the thyroid gland could be a waste of medical resources and can increase the risk of severe complications, such as permanent hypoparathyroidism or bilateral recurrent laryngeal nerve damage [1]. Increasing studies have been devoted to discovering the molecular mechanism of PTC progression [4]. It was shown that gene therapy has been shown a promising strategy in

treating PTC and studies on molecular mechanisms are vital for the successful treatment by gene therapy [5].

Long non-coding RNA (lncRNA) is a group of non-protein coding RNAs with the length of over 200 nucleotides. Growing studies have shown that dyregulated lncRNA expression is associated with cancer progression [6–9]. Several lncRNAs are verified to promote proliferation, migration, progression, invasion, apoptosis, and prognosis of papillary thyroid carcinoma, including CCND2-AS1 [10], LINC01061 [7], H19 [11], LOC100507661 [12], AFAP1-AS1 [13], MEG3 [14], and HOTTIP [15]. Nevertheless, the roles of most lncRNAs in PTC remain unclear [7]. It was proven that MCM3AP-AS1 suppresses the cell viability, migration, and tube formation of glioma-associated endothelial cells (GECs) and played a vital role in inhibiting angiogenesis of glioblastoma (GBM) in vitro [16].

Recently, several studies showed that microRNAs (miRNAs) may potentially interact with lncRNA, serving as an additional level of post-transcriptional regulation [17]. MiRNAs are small non-coding RNAs composed of ~19–25 nucleotides. miRNAs can be recognized as tumor suppressors or oncogenes [18], and they implement a wide range of biological functions under several pathophysiological conditions [19, 18]. Recent evidence suggests that miRNAs play a vital role in thyroid carcinogenesis [20]. Several studies have proven the tumor-suppressive role of miRNAs in thyroid cancer [18]. In addition, several miRNAs are used as diagnostic or prognostic markers that may significantly improve the diagnostic accuracy of PTC, including miR-146b, miR-222, miR-221, and miR-181b [20–22]. Moreover, miR-335-5p inhibits metastasis and invasion of thyroid cancer cells [23]. Also, microRNA-125b is recognized to induce autophagy in thyroid cancer [24]. In addition, miR-145 was proven to promote the expression of ZEB2 in thyroid cancer cells [25]. One miRNA of particular interest to us is miR-211, which plays a principle roles in tumorigenesis, especially in breast cancer [26], melanoma [27], cervical cancer [28], neck and head carcinomas [29], and hepatocellular carcinoma [30]. However, the role of miR-211 in PTC has not been investigated.

Extensive recent studies has proven the vital role of secreted protein acidic and cysteine rich (SPARC) genes in the regulation of cell signaling that governs malignant transformations and aggressive phenotypes, where SPARC act as a potential therapeutic target in tumor pathophysiology [31]. For example, SPARC has been identified as diagnostic markers in gastric cancer [32]. In breast cancer, SPARC was also demonstrated to inhibit breast cancer bone metastasis, making it a valuable therapeutic target [33].

Herein, the aim of the study is to investigate the role and mechanism of MCM3AP-AS1 in papillary thyroid cancer. We show that MCM3AP-AS1 in PTC was upregulated, while miR-211 was downregulated. MCM3AP-AS1 overexpression promoted papillary thyroid cancer proliferation, migration and invasion. Further, MCM3AP-AS1 was shown to be negatively correlated with miR-211-5p. Also, we found that SPARC plays an important regulating role in PTC. In addition, we confirmed the anti-tumor role of MCM3AP-AS1 silencing and the close relation among MCM3AP-AS1, miR-211-5p, and SPARC in vivo.

Methods

Clinical tissue specimens

This study was approved by the Ethics Committees of Harbin Medical University Cancer Hospital and all patients signed a written informed consent form. A total 64 pairs of papillary thyroid cancer tissues and adjacent non-tumor tissue were collected from Harbin Medical University Cancer Hospital between February 2012 and September 2014. Samples were frozen in liquid nitrogen and stored at $-80\,^\circ\mathrm{C}$.

Cell lines, culture, and oligonucleotides transfection

TPC-1, HTH83, 8505C, SW1736, and BCPAP cell lines, which are human papillary thyroid cancer cell lines, and Nthy-ori 3-1, which is normal human follicular epithelial cell line, were purchased from ATCC (Manassas, VA). All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and $37\,^\circ\mathrm{C}$. GenePharma (Shanghai, China) synthesized the oligonucleotides used in this study, including siRNA against MCM3AP-AS1 (si-MCM3AP-AS1), short-hairpin RNA plasmid directly targeting MCM3AP-AS1 (sh-MCM3AP-AS1), miR-211-5p inhibitor, miR-211-5p mimics, and their controls. Oligonucleotide transfection into cells were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The sequence of siRNA for MCM3AP-AS1 and non-coding siRNA (si-NC) were: MCM3AP-AS1: forward: 5′-GCTGCTAATGGCAACACTGA-3′, reverse: 5′-AGGTGCTGTCTGGTGGAGAT-3′; Control: forward: 5′-TTCTCCGAACGTGTCACGTTT-3′, reverse: 5′ -ACGUGACACGUUCGGAGAATT-3′; LV-sh-MCM3AP-AS1, forward: 5′-GCTGGTATTTCAATTGACTTT-3′, reverse: 5′-AGTCAATTGAA ATACCAGCTT-3′. LV-sh-NC, forward: 5′- TTCTCCGAACG TGTCACGT-3′, reverse: 5′-AAGAGGCTTGCACAGTGCA-3′.

BLAST alignment

For alignment to RNA sequences, NCBI's BLAST suite was used and the top search results with an e-value <0.01 were reported. RNA transcripts could have multiple exons

with alignment to different non-contiguous regions of a chromosome.

Quantitative reverse transcription (qRT-PCR)

The miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) was used to isolate total RNA from tissues and cells. Quality and concentration of RNA were assessed by NanoDrop 2000 (Thermo Fisher, Wilmington, DE, USA). TransScript first-strand cDNA synthesis SuperMix (TransGen, Beijing, China) was used for cDNA synthesis. Real-time PCR was conducted using SYBR green qPCR SuperMix (Applied Biosystems Life Technologies, Foster, CA, USA) in ABI prism 7500 sequence detection system (Applied Biosystems Life Technologies). Levels of expression was quantified using the $2^{-\Delta\Delta Ct}$ method and U6 and GADPH were used as internal controls. Primers used in this study included: MCM3AP-AS1: forward: 5′-TGGGATTCAGA

CGCTAACGC-3′, reverse 5′-TCCACAGCATCTTTG GCACC-3′; miR-211-5p, forward: 5′- ATGCCGCAGC AACATCCAGA-3′, reverse, 5′- AGGATGCTGCATGCA CTCGAT-3′; GAPDH, forward, 5′-TCGACAGTCA GCC GCATCTTCTTT-3′, reverse, 5′-ACCAAATCCGTTGAC TCCGACCTT-3′. Analysis was made based on six independent samples.

Luciferase reporter assay

Oligonucleotides encodingMCM3AP-AS1 cDNA fragment that contained microRNA binding sites was amplified and cloned into the pmirGLO plasmids (Promega, Madison, WI, USA). Site-directed mutagenesis PCR was used to generate mutant MCM3AP-AS1 (pmirGLO-MCM3AP-AS1-MUT) with platinum pfx DNA polymerase. Luciferase reporter plasmids and target miR-211-5p mimics or miR-NC mimics were co-transfected into cells by Lipofectamine 2000. At 48 h after transfection, relative luciferase activity was quantified using the Dual-Luciferase Reporter Assay System (Promega). Analysis was made based on six independent experiments.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8; Dojindo, JPN) assay was used to evaluate cell proliferation. Briefly, cells were seeded into 96-well plates at the density of 1×10^3 cells/well). After 24, 48, 72, or 96 h, CCK-8 agent of 10 μl was added, followed by incubation for 2 h and measurement using an enzyme immunoassay analyzer (Bio-rad, Hercules, CA, USA). Analysis was made based on four independent samples. In cell colony formation assay, cells were plated in 6-well plates (500 cells/well) and incubated in Dulbecco's Modified Eagle Medium (DMEM) with 10% of bovine calf serum at 37 °C. After 2 weeks, we fixed and stained cells

with 0.1% of crystal violet. Finally, the number of visible colonies was manually counted.

Cell migration and invasion assay

The migration and invasion of papillary thyroid cancer cells were measured using wound healing assay and transwell assay, respectively. Briefly, for wound healing assay, TPC-1 CSC and BCPAP CSC cells were seeded in 6-well plates and cultured to 90% confluence. A sterile pipette tip was then used to make a straight scratch on the cell monolayer. After 24 h, the width of the wounding scratches was measured after the scratch was photographed using a microscope equipped with a digital camera. The migration rate was expressed as relative percentage of the initial distance at 0 h following formula: migration rate = migration distance/original distance. In transwell assay, TPC-1 CSC and BCPAP CSC cells were plated in chambers (8 mm, BD Biosciences) were plated with BD BioCoat Matrigel (5×10^4 per well) in 200 ml serum free DMEM. After a 24h-incubation, the cells on the upper membrane surface were removed with a cotton tip. Then, member was fixed and stained by violet crystalline. Analysis was made based on six independent experiments.

Western blot analysis

RIPA buffer (Sigma–Aldrich, St. Louis, MO) supplemented with protease inhibitors cocktail (Roche, Diagnostics, Mannheim, Germany) was used to lyse the cells and tissues. The lysate was centrifuged at $12,000 \times g$ and BCA assay was used to quantify total protein concentration. SDS–PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was then used to resolve proteins in the lysate, followed by electro-transferring to PVDF membranes (Millipore, Bedford, MA). Non-fat milk was used to block PVDF membranes and primary antibodies, including anti-SPARC (1:1,000, Abcam, Cambridge, MA) and anti-GADPH (1:1,000, Abcam), were subsequently added. Goat anti-rabbit IgG (Abcam) was then added to the membrane and incubated at room temperature for 2 h. Finally, ECL detection kit (Beyotime Biotechnology, Shanghai, China) was used to visualized protein bands. Analysis was made based on six independent samples.

Lentivirus construction and infection

A recombinant lentiviral vector expressing MCM3AP-AS1-shRNA was constructed by Shanghai Genechem. MCM3AP-AS1-shRNA was introduced into pFU-GW-RNAi vector that carried the green fluorescent protein (GFP) reporter gene driven by the U6 promoter. TPC-1 cells were seeded at the density of 2×10^5 cells per well in 6-well plates. After 12 h, TPC-1 cells were infected with

Lv-shRNA-NC or Lv-shRNA- MCM3AP-AS1 at 10 MOI, respectively. Virus-containing culture medium was changed with fresh RPMI-1640 medium 12 h post-infection.

In vivo animal experiments

The animal study was performed according to the experimental protocols and approved by the Institutional Animal Care and Use Committee of Harbin Medical University Cancer Hospital. Briefly, 1×10^7 TPC-1 cells were injected into BALB/c nude mice (6 weeks). For the tumor growth model, cells stably transfected with lentivirus mediated sh-MCM3AP-AS1 or sh-NC were subcutaneously injected. Tumor growth was determined by caliper measurements every 3 days and tumor volume was calculated according to the following formula: volume $= 0.5 \times$ length $\times$ width $\times$ width. A total of five mice per group was used.

Immunohistochemical staining

Tumor tissue were cryosectioned at 5 μm. Goat serum (Boster, Wuhan, China) was used to block the tissue for 30 min at room temperature. Ki67 antibody (Bioss Antibodies, Inc, 1:200) was incubated with the section overnight at 4 °C. For TUNEL assay, antibodies from the colorimetric TUNEL Apoptosis Assay Kit (Beyotime, Shanghai, China) were incubated with the sections at 37 °C for 60 min. After washing by PBS, the Polink-1 HRP DAB Detection System One-step polymer detection system (ZSGB-BIO, Beijing, China) was incubated with the section for 20 min at room temperature. Hematoxylin was used to stain nucleus.

Statistical analysis

All the statistical data are presented as the means S.D. Two-tailed Student's t-test or one-way ANOVA followed by the least significant difference (LSD) post hoc test was performed for comparisons between groups. Expression correlation assays were analyzed using Pearson's coefficient correlation. Differences in patient survival were performed using the Kaplan–Meier method and analyzed by log-rank test. The relative risk for each factor was evaluated using multivariate Cox regression analysis. A value of $P < 0.05$ was considered to be statistically significant.

Results

Papillary thyroid cancer is characterized by MCM3AP-AS1 upregulation

From the clinical patients with papillary thyroid cancer, the tumor tissues and adjacent tissues of 68 patients were selected for comparison. It was found that MCM3AP-AS1 was significantly higher in patients with papillary thyroid carcinoma than that in normal tissues (Fig. 1a). Subsequently, according to the median expression of MCM3AP-AS1, patients were divided into high-expression group and low-expression group (Fig. 1b). Long-term follow-up study showed that the long-term survival rate of the high-expression group was significantly lower than that of the low-expression group (Fig. 1c).

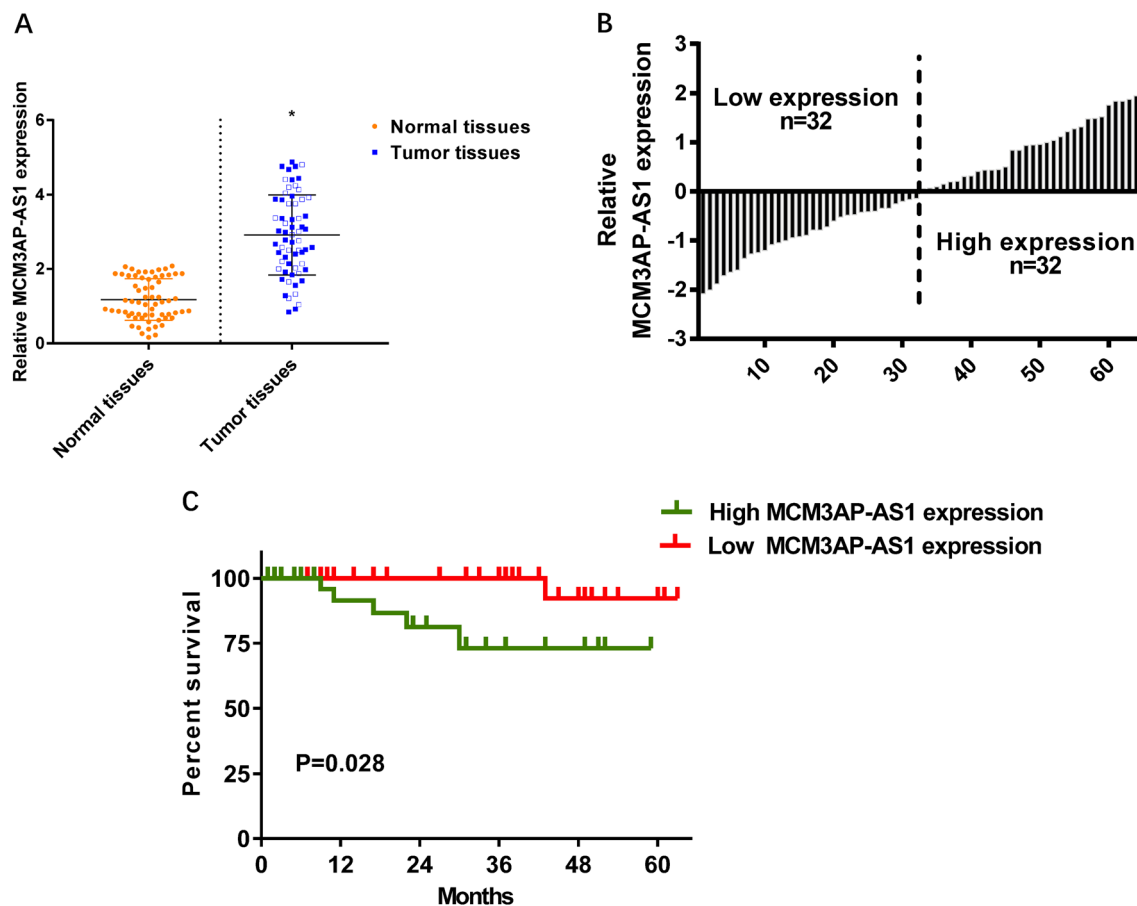### MCM3AP-AS1 promotes papillary thyroid cancer cell proliferation and metastasis

In the papillary thyroid cancer cell lines TPC-1, HTH83, 8505C, SW1736, and BCPAP, MCM3AP-AS1 expression was found to be significantly higher than that in normal human thyroid epithelial cells Nthy-ori 3-1 (Fig. 2a). The most significant increase of MCM3AP-AS1 was shown in TPC-1 and BCPAP cells, which were selected for subsequent studies. Three small interfering RNAs (siRNAs) were designed for MCM3AP-AS1, and the si-MCM3AP-AS1-1 was found to possess the highest silencing efficiency (Fig. 2b). In further studies, si-MCM3AP-AS1-1 was found to inhibit tumor cell proliferation (Fig. 2c), colony formation (Fig. 2d), migration (Fig. 2e), and invasion (Fig. 2f). Further, western blot revealed that si-MCM3AP-AS1 significantly reduced the expression of an oncogene, SPARC (Fig. 2g). Survivin expression, which was not altered by si-MCM3AP-AS1 transfection, was also analyzed as a negative control.

miR-211-5p is a regulatory target of MCM3AP-AS1

BLAST alignment analysis indicated that MCM3AP-AS1 has a binding site to miR-211-5p (Fig. 3a). Luciferase assay showed that 211-mimic transfection led to reduced MCM3AP-AS1-WT activity, but no change in MCM3AP-AS1-MUT activity (Fig. 3b). In cell experiments, si-MCM3AP-AS1 increased miR-211-5p expression (Fig. 3c), while 211-inhibitor also decreased MCM3AP-AS1 expression (Fig. 3d). In further clinical sample validation, a significant negative correlation was found between MCM3AP-AS1 and miR-211-5p expression in 68 patient samples (Fig. 3e)

The regulation of MCM3AP-AS1 on papillary thyroid cancer cells is mediated by miR-211-5p

To further validate the interaction between MCM3AP-AS1 and miR-211-5p, we revealed that the inhibitory effect of si-MCM3AP-AS1 on tumor cell proliferation/invasion can be rescued by 211-inhibitor in CCK-8 assay, cloning assay, cell scratch, and Transwell assay (Fig. 4a–c). Consistently,

Fig. 1 Papillary thyroid cancer is characterized by MCM3AP-AS1 upregulation. **a** qRT-PCR analysis of MCM3AP-AS1 levels in papillary thyroid cancer and adjacent normal tissues ($n = 68$). **b** Distribution of MCM3AP-AS1 in patients, ranked based on low to high MCM3AP-AS1 expression. Median level of MCM3AP-AS1 is indicated with dash line. The patients were divided to low- and high-expression group based on median MCM3AP-AS1 levels. **c** Survival curve of patients with high or low MCM3AP-AS1 expression

SPARC downregulation by si-MCM3AP-AS1 was abrogated by 211 inhibitor (Fig. 4d).

## MCM3AP-AS1 inhibition exerts anti-tumor effects in vivo

To validate the anti-tumor effect of MCM3AP-AS1 in vivo, mice were inoculated with cells overexpressing Lv-sh-MCM3AP-AS1 or Lv-sh-Control, which are short-hairpin RNAs to induce stable knockdown of the MCM3AP or a nonspecific gene (control), respectively. The mice were sacrificed at 8 weeks later ($n = 5$). It was found that the volume of transplanted tumor in the Lv-sh-MCM3AP-AS1 model was reduced (Fig. 5a) compared with that of the control group. Next, the expression of MCM3AP-AS1 and miR-211-5p was evaluated in mouse tumors. A decreased expression of MCM3AP-AS1 (Fig. 5b), increased expression of miR-211-5p (Fig. 5c), and decreased expression of Ki67 were observed (Fig. 5d). SPARC immunohistochemistry suggested that SPARC expression in the Lv-sh-MCM3AP-AS1 group was reduced (Fig. 5e).

Discussions and conclusions

In the present study, we showed that MCM3AP-AS1 was upregulated, while miR-211 was downregulated in PTC. MCM3AP-AS1 overexpression promoted PTC proliferation, migration, and invasion. These data potentiate the use of MCM3AP-AS1 as a potential diagnostic marker of PTC. Further, MCM3AP-AS1 was shown to be negatively correlated with miR-211-5p. This result is consistent with previously reports showing that MCM3AP-AS1 was upregulated in glioma-associated endothelial cells, whereby miR-211 was downregulated. Knockdown of MCM3AP-AS1 suppressed the cell migration, viability, and tube formation of glioma-associated endothelial cells and played a principle role in inhibiting angiogenesis of glioblastoma in vitro [16].

We also showed that validated that miR-211-5p overexpression could reverse the promoting role of MCM3AP-AS1 in PTC. This result agreed with previously study showing that the expression of miR-211 is increased by knockdown of MCM3AP-AS1 [16]. In addition, it was previously proven that miR-211-5p overexpression
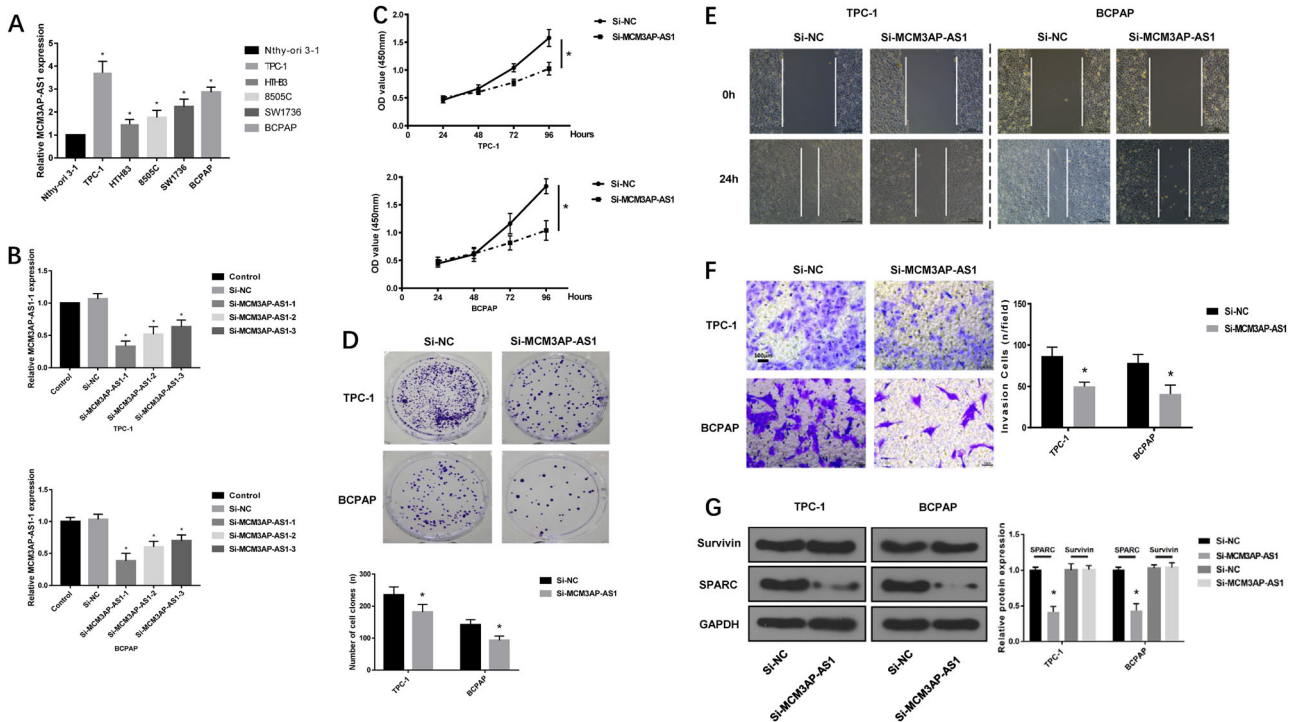
Fig. 2 MCM3AP-AS1 promotes papillary thyroid cancer cell proliferation and metastasis. **a** qRT-PCR analysis of the levels of MCM3AP-AS1 in papillary thyroid cancer cells, including TPC-1, HTH83, 8505C, SW1736, and BCPAP cells. The normal thyroid epithelial cells Nthy-ori 3-1 was used as control. $N = 6$. **b** Three siRNAs were used to silencing MCM3AP-AS1 expression. CCK-8 proliferation assay (**c**), colony formation assay (**d**), scratch wound assay (**e**), and transwell assay (**f**) showing a decreased proliferation, colony formation, migration, and invasion after MCM3AP-AS1 siRNA transfection. **g** Western blot assay of Survivin and SPARC expression in TPC-1 and BCPAP cells transfected with either si-NC or si-MCM3AP-AS1. The levels of GAPDH were used as loading controls. $N = 4$
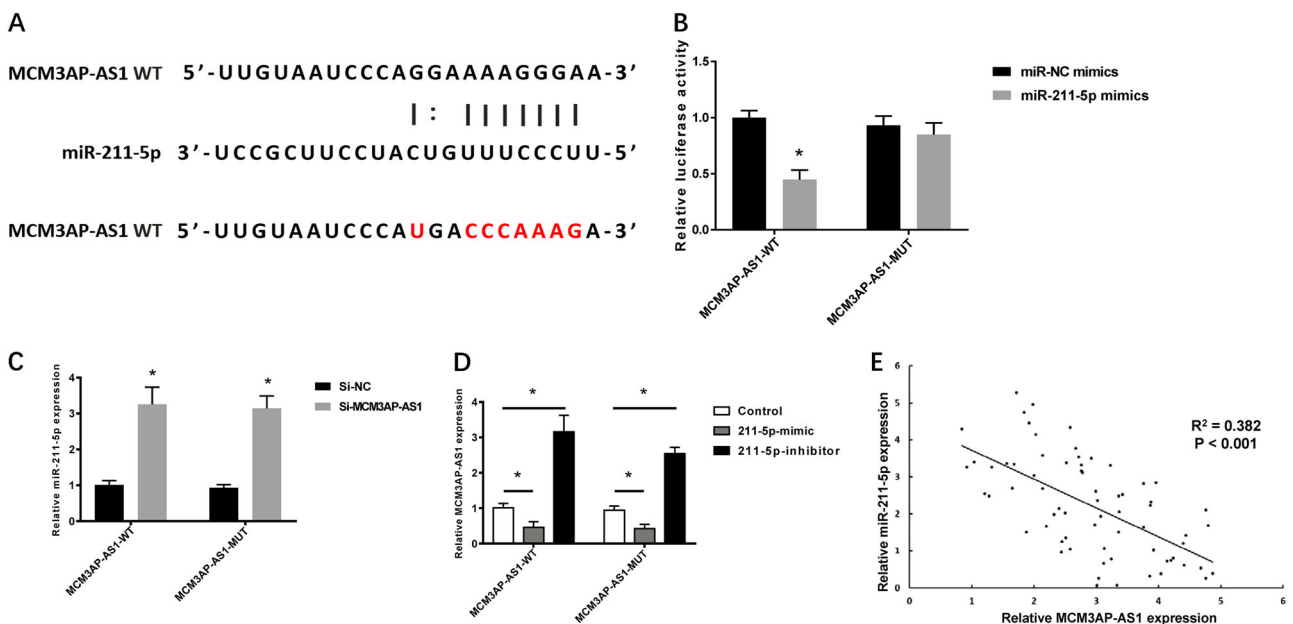

Fig. 3 miR-211-5p is a regulatory target of MCM3AP-AS1. **a** Blast alignment analysis showing a binding site between MCM3AP-AS1 WT and miR-211-5p. The sequence of the binding site was mutated in the mutant MCM3AP. **b** Luciferase assay of the interaction between miR-211 mimic and MCM3AP-AS1 WT or MCM3AP-AS1 MUT. $N = 6$. **c** miR-211 levels in cell transfected with either si-NC or si-MCM3AP-AS1. **d** MCM3AP-AS1 levels in cells transfected with either miR-211 mimic or inhibitor. $N = 6$. **e** Correlation assay between miR-211 levels and MCM3AP-AS1 levels in 68 patients with papillary thyroid cancer
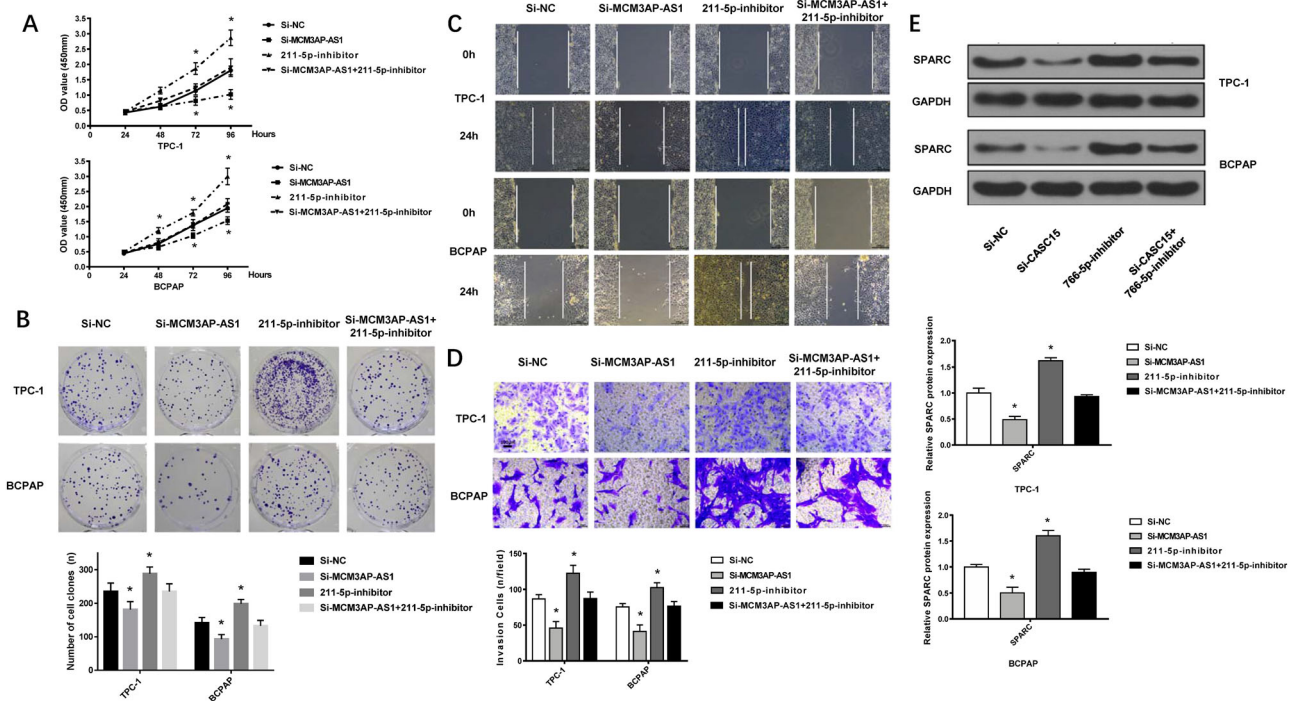
Fig. 4 The regulation of MCM3AP-AS1 on papillary thyroid cancer cells is mediated by miR-211-5p. CCK assay (**a**), colony formation assay (**b**), scratch wound assay (**c**) and transwell assay (**d**) of cells transfected with si-NC, si-MCM3AP-AS1, 211-inhibitor or the combination of si-MCM3AP-AS1 and 211 inhibitor. $N = 6$. **e** western blot analysis of SPARC. $N = 6$
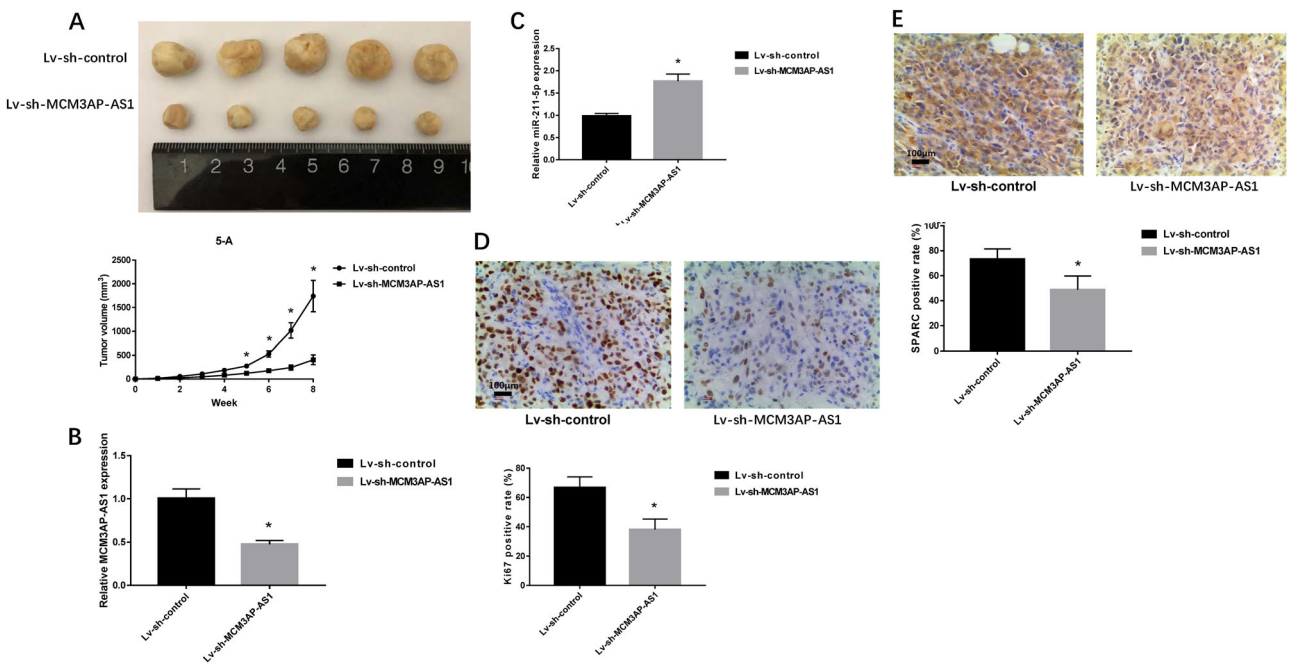
Fig. 5 MCM3AP-AS1 inhibition exerts anti-tumor effects in vivo. **a** Photograph of the harvested tumor in lv-sh-control group and lv-sh-MCM3AP-AS1 group. $N = 5$ per group. **b** Tumor growth curve. $N = 5$ per group. **c** qRT-PCR analysis of miR-211-5p levels in tumors harvested from two groups. Immunohistochemical staining of Ki67 (**d**) and SPARC (**e**)

suppresses the proliferation, migration, and invasion of thyroid tumor cells [34]. It was shown that miR-211-5p has several functional roles in tumorigenesis [35]. miR-211-5p may play a principle role in hepatocellular carcinoma metastasis by inhibiting ZEB2 expression [30]. In addition, miR-211-5p overexpression attenuated the proliferation, invasion, and migration of thyroid tumor cells by inhibiting the expression of SOX11 [34].

In vivo, we confirmed the anti-tumor role of MCM3AP-AS1 silencing and the close relation among MCM3AP-AS1, miR-211-5p, and SPARC. This is consistent the pervious study reveals that miR-211 inhibits invasion, proliferation, and migration of cervical cancer via targeting SPARC [36]. In addition, MCM3AP-AS1/miR-211 axis plays a principle role in the regulation of glioblastoma angiogenesis and also acts as new therapeutic target for the anti-angiogenic therapy of glioma [16]. Altogether, these evidences show that MCM3AP-AS1 silencing is an effective gene therapy approach for inhibiting PTC both in vitro and in vivo, and further characterization and clinical translation of the targeted silencing of MCM3AP-AS1 is warranted. It is worth noting that MCM3AP-AS1 is also involved in the regulation of other miRNAs and oncogenes, such as miR-194-5p and FOXA1 [37], which could also contribute to the anti-tumor effects of MCM3AP-AS1 silencing in PTC.

In sum, we demonstrated that MCM3AP-AS1 promotes PTC by regulating the MCM3AP-AS1/miR-211-5p/SPARC axis, which could potentially be a therapeutic target in papillary thyroid cancer.

Data availability

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The present study was approved by the Ethics Committee of Harbin Medical University Cancer Hospital.

Informed consent All patients and healthy volunteers provided written informed consent prior to their inclusion within the study.

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