LncRNA MCM3AP-AS1 serves as a competing endogenous RNA of miR-218 to upregulate GLUT1 in papillary thyroid carcinoma

Rui Nian¹
https://orcid.org/0000-0002-8316-5462

Wanjun Li¹
https://orcid.org/0000-0001-6169-8490

Xiang Li¹
https://orcid.org/0000-0003-1468-6739

Jiayu Zhang¹
https://orcid.org/0000-0002-6761-0191

Weihua Li¹
https://orcid.org/0000-0002-4992-6025

Fanfan Pan¹
https://orcid.org/0000-0003-3212-2347

Jing Cheng¹
https://orcid.org/0000-0003-2614-7847

Xin Jin¹
https://orcid.org/0000-0002-3423-0221

ABSTRACT
Objective: MCM3AP-AS1 has been characterized as an oncogenic long non-coding RNA (lncRNA) in several cancers including papillary thyroid cancer (PTC), but its role in PTC has not been fully elucidated. Considering the critical role of lncRNAs in cancer biology, further functional analysis of MCM3AP-AS1 in PTC may provide novel insights into PTC management. Subjects and methods: Paired tumor and non-tumor tissues were collected from 63 papillary thyroid carcinoma (PTC) patients. Expression levels of MCM3AP-AS1, miR-218 and GLUT1 in tissue samples were analyzed by qRT-PCR. Cell transfection was performed to explore the interactions among MCM3AP-AS1, miR-218 and GLUT1. Cell proliferation assay was performed to evaluate the effects of MCM3AP-AS1 and miR-218 on cell proliferation. Results: MCM3AP-AS1 accumulated to high levels in PTC tissues and was affected by clinical stage. MCM3AP-AS1 showed a positive correlation with GLUT1 across PTC tissues. RNA interaction prediction showed that MCM3AP-AS1 could bind to miR-218, which can directly target GLUT1. MCM3AP-AS1 and miR-218 showed no regulatory role regulating the expression of each other, but overexpression of MCM3AP-AS1 upregulated GLUT1 and enhanced cell proliferation. In contrast, overexpression of miR-218 downregulated GLUT1 and attenuated cell proliferation. In addition, miR-218 suppressed the role of MCM3AP-AS1 in regulating the expression of GLUT1 and cell proliferation. Conclusions: MCM3AP-AS1 may serve as a competing endogenous RNA of miR-218 to upregulate GLUT1 in PTC, thereby promoting cell proliferation. The MCM3AP-AS1/miR-218/GLUT1 pathway characterized in the present study might serve as a potential target to treat PTC.

Keywords
MCM3AP-AS1; papillary thyroid cancer (PTC); GLUT1; miR-218

INTRODUCTION
Papillary thyroid carcinoma (PTC) is a type of well-differentiated thyroid carcinoma and accounts for more than 80% of all thyroid carcinoma cases (1). Due to the well-differentiated characteristic, PTC usually grows slowly in local region (2). Therefore, most PTC patients can be treated with optimized surgery in combination with levothyroxine suppression therapy and/or radiiodine treatment, and the treatment outcomes are generally satisfactory (3). However, metastasis may occur in extreme cases, and the survival of patients with metastatic PTC is still poor (4).
In addition, the incidence rate of PTC has been rapidly increasing over the past several decades (5,6). Therefore, novel preventive and therapeutic approaches are still needed.

Accelerated glucose metabolism provides energy for the growth of tumors, and inhibition of glucose metabolism is considered as a potential therapeutic approach for cancer therapy (7). Glucose transporter 1 (GLUT1) plays critical roles in the early steps of glucose metabolism by mediating the transportation of glucose across cell membrane (8). GLUT1 is usually upregulated in cancers, and high expression levels of GLUT1 predict poor survival of PTC patients (9). Certain tumor suppressive miRNAs, such as miR-218, can suppress cancer development by targeting GLUT1 (10). Extensive studies have been performed to characterize the long non-coding RNAs (lncRNAs)/mRNAs/miRNAs related competing endogenous RNAs (ceRNAs) network in various carcinoma cells (11). Generally, lncRNAs can serve as the ceRNAs of mature miRNAs in cytoplasm to suppress their role in inhibiting gene expression, thereby by indirectly regulating the expression of downstream tumor suppressors and oncogenes (11). Our bioinformatics analysis showed that miR-218 could form a strong base pairing with lncRNA (>200 nt) MCM3AP-AS1 (2539 bp, Accession: KJ903636.1). MCM3AP-AS1 was reported to play an oncogenic role in many cancers (12-14). In most cases, MCM3AP-AS1 upregulates the expression of oncogenes by serving as the ceRNA of miRNAs. In papillary thyroid cancer (PTC), MCM3AP-AS1 was reported to promote proliferation and invasion of cancer cells through regulating the miR-211-5p/SPARC axis (15). However, its role in PTC has not been fully elucidated. We speculated that MCM3AP-AS1 may serve as the ceRNA of miR-218 to regulate GLUT1, thereby participating in PTC. This study was therefore carried out to investigate the interactions among MCM3AP-AS1, miR-218 and GLUT1 in PTC. MCM3AP-AS1 may serve as a ceRNA of miR-218 to upregulate GLUT1 in PTC, thereby promoting cell proliferation.

SUBJECTS AND METHODS

PTC patients and specimen collection

This study enrolled a total of 63 PTC patients (21 males and 44 females, age range from 21 to 55 years old, mean age 35.5 ± 6.7 years old) selected from 144 PTC patients who were admitted at the 3201 Medical College Affiliated Hospital between April 2016 and May 2019. Inclusion criteria: 1) newly diagnosed cases of PTC; 2) complete medical record of patients; 3) therapies were not initiated. Exclusion criteria: 1) recurrent PTC; 2) patients with other clinical disorders; 3) initiated therapies. This study was approved by the Ethics Committee of aforementioned hospital (Ethics approval no. IUR-TYC2432). All patients were informed of the details of the experimental design and signed the written informed consent. All patients were subjected to biopsy that was performed under the guidance of ultrasound using a fine needle. PTC tumor tissues and non-tumor tissues were collected from each patient by dissecting biopsy. All tissue specimens were confirmed by histopathological exams.

Vectors and miRNA mimic

PcDNA3.1 vector (GenePharma, Shanghai, China) was used to construct vectors expressing MCM3AP-AS1 and GLUT1 by inserting the full-length cDNA or MCM3AP-AS1 (2539 bp), and GLUT1 (1479 bp) into vector. Negative control (NC) miRNA and miR-218 mimic were also purchased from GenePharma.

Cell line and transient cell transfection

PTC cell lines IHH-4 (Stage IV) and MDA-T120 (stage IV) were purchased from ATCC (Manassas, VA, USA) and used as the cell model of PTC. Cells were cultivated in a mixture containing 10% FBS and 90% DMEM at 37 °C with 5% CO₂ and 95% humidity.

To perform transfection, cells were harvested at 80% confluence. Cells were counted and 40 nM miRNAs (NC miRNA was used as NC group) or 10 nM vectors (empty vector was used as NC group) were transfected into 3 × 10⁶ IHH-4 and MDA-T120 cells through transient transfections using Lipofectamine 2000 (GenePharma). Cells were incubated with the transfection mixture for 6 h. After that, cells were washed with fresh cell culture medium. Cells were harvested at 24 h post-transfection to perform the subsequent experiments. Untransfected cells were used as the Control (C) cells.

Dual-luciferase reporter assay

Dual-luciferase reporter assay was performed to investigate the interaction between MCM3AP-AS1 and miR-218 in IHH-4 cells using the Promega Dual-
Luciferase™ Reporter (DLR™) Assay System (Promega) following the manufacturer’s instructions. The binding site of miR-218 on MCM3AP-AS1 was cloned into pGL3-Promoter Vector at 5’ upstream of the luciferase gene. Two different combinations of transfection were performed: 1) MCM3AP-AS1 + miR-218-WT; 2) MCM3AP-AS1 + miR-218-MUT (mutant site was marked in Figure 2D). Firefly luminescence was used to normalize the renilla luminescence.

qRT-PCR

Ribozol (Sigma-Aldrich) was used to extract total RNAs from ground tissue samples (0.03 g) and IHH-4 cells (3 × 10⁵). RNA samples were precipitated and washed using 85% ethanol to harvest miRNAs. RNA samples were digested with DNase I at 37 °C for 60 min to remove genomic DNA. Prior to the following applications, RNA samples were stored at -80 °C. Reverse transcriptions were performed using the TruScript Reverse Transcriptase Kit (Norgenbiotek). To measure the expression levels of MCM3AP-AS1 and GLUT1, KAPA SYBR FAST qPCR Kit (Roche) was used to prepare all qPCR reaction mixtures. GAPDH was used as the endogenous control. β-actin was also used as an endogenous control and similar results were obtained. To measure the expression levels of miR-218, All-in-One™ miRNA qRT-PCR Detection Kit (Genecopoeia) was used to perform the addition of poly (A), miRNA reverse transcriptions and qPCR assays. In qPCR assays, U6 was used as the endogenous control. Primer amplification efficiency in all cases was between 98.8 and 99.7%. qPCR reactions were performed in 3 replicates. The relative expression levels were calculated using the 2^(-ΔΔCT) method. For the comparison of expression levels among multiple genes, the gene in the sample with the biggest ΔCT value was set to value “1”, and all other samples were normalized to this sample to calculate the relative expression levels. Primer sequences were: 5’-CTGCTAATGGCAACACTGA-3’ (forward) and 5’-AGGTGCTGTCTGGTGGAGA-3’ (reverse) for MCM3AP-AS1; 5’-CAGGAGGCAATTGGCAATGAGCTGA-3’ (forward) and 5’-GAAGGCTGGGGCTCATTT-3’ (reverse) for GAPDH; 5’-CACCATTGGCAATGGAGCGGTTC-3’ (forward) and 5’-TTGGCGAGCACATATACTAAAAT-3’ (reverse) for GLUT1; 5’-CGCTTTACGAATTTGCGTGC-3’ (forward) and 5’-TTTGCGTTCGCTAACCAG-3’ (forward) and reverse poly(T) for miR-218.

Western blot analysis

The expression levels of GLUT1 protein in IHH-4 cells were measured at 24 h post-transfection. Total proteins were extracted from 3 × 10⁴ IHH-4 cells using RIPA solution (GenePharma). Protein samples were quantified using BCA assay (GenePharma). Protein samples were incubated in boiling water for 12 min for denaturation, followed by electrophoresis using 10% SDS-PAGE gel (80 min at 100 V). Proteins were then transferred to PVDF membranes (20 min at 45 V), and PBS containing 5% non-fat milk was used to block the membranes at 22 °C for 2 h. Next, rabbit anti-GAPDH (1:1,800, ab37168, Abcam) or anti-GLUT1 (1:2,000, ab15309, Abcam) primary antibodies were used to incubate the membranes at 4 °C for 16 h, followed by incubation with goat HRP (IgG) secondary antibody (1:1,800; ab6721; Abcam) at 22 °C for 2 h. Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific) was then used to incubate the membranes at 22 °C for 1 min. Images were taken using myECL Imager (Thermo Fisher Scientific) after exposure for 10 min. Image J v1.46 software was used to process all data.

Cell proliferation analysis

The effects of cell transfection on the proliferation of IHH-4 and MDA-T120 cells were assessed by CCK-8 assay. Briefly, 1 ml aforementioned cell culture medium was used to re-suspend cell pellets (containing 3 × 10⁴ cells) to prepare single cell suspensions. Cells were cultivated in cell culture plate (96-well) under the aforementioned conditions. Each transfection group included 3 replicate wells. Each well was added with 10 μL CCK-8 solution (Sigma-Aldrich) at 4 h before the termination of cell culture. Following that, 10 μL DMSO was added, and OD values at 450 nm were measured.

FISH

Fluorescence in situ hybridization (FISH) was performed as previously described (16). Briefly, IHH-4 cells grown on the slides were washed with PBS and then fixed in 4% paraformaldehyde. After treatment with protease reagent, the slides were incubated with prehybridization buffer at 40°C for 4 h, and
then hybridized with digoxin-labeled probe at 40 °C overnight. FISH probe sequence of MCM3AP-AS1 was 5’ (digoxigenin)-TAATGTCTGTTACATGGTA TCTGTGGGTCAGGAATCCAGGTG-3’.

Statistical analysis
All experiments were performed in 3 biological replicates. Mean values were calculated and used for data analyses. Differences between two types of tissue from PTC patients were compared by paired t-test. Differences among multiple patient or cell groups were compared by ANOVA (one-way) combined with Tukey test. Pearson’s correlation coefficient was used to analyze correlations. P < 0.05 was considered statistically significant.

RESULTS
MCM3AP-AS1 was upregulated in PTC and affected by clinical stages
The expression levels of MCM3AP-AS1 in both PTC and non-tumor tissues were measured by qRT-PCR. The sample with the lowest expression level was set to value “1”. Other samples were normalized to this sample. Compared with non-tumor tissues, the expression levels of MCM3AP-AS1 were significantly increased in PTC tissues (Figure 1A, p < 0.05). Based on clinical findings, the 63 patients were staged according to AJCC staging system. There were 18, 20, 17 and 8 cases at clinical stage I-IV, respectively. As shown in Figure 1B, the expression levels of MCM3AP-AS1 were significantly increased with the increasing of clinical stage (p < 0.05).

MCM3AP-AS1 may bind to miR-218, but no interaction between MCM3AP-AS1 and miR-218 was observed in IHH-4 cells
FISH was performed to analyze the subcellular localization of MCM3AP-AS1, and the results showed that MCM3AP-AS1 was localized in cytoplasm (supplemental data-1). RNA interaction was predicted using the online tool http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp. It showed that MCM3AP-AS1 and miR-218 may form multiple base pairs (Figure 2A). Although miR-218 may bind to multiple sites of MCM3AP-AS1, only the one with the highest affinity was present. The binding energy was -13.26070 kcal/mol, indicating a strong base pairing between them. To further evaluate the interaction between MCM3AP-AS1 and miR-218, IHH-4 cells were transfected with MCM3AP-AS1 expression vector or miR-218 mimic. Compared with NC (NC miRNA or empty pcDNA3.1 vector), the expression levels of MCM3AP-AS1 and miR-218 were significantly increased (Figure 2B, p < 0.05). However, overexpression of MCM3AP-AS1 and miR-218 did not affect the expression of each other (Figure 2C, p < 0.05). On the other hand, luciferase assay results indicated the sponge relationship between MCM3AP-AS1 and miR-218. To perform dual luciferase activity assay, the binding site of miR-218 on MCM3AP-AS1 was cloned into pGL3-Promoter Vector at 5’ upstream of the luciferase gene. Overexpression of miR-218-WT could attenuate the luciferase activity of MCM3AP-AS1, but miR-218-MUT could not (Figure 2D, p < 0.05). The principle is that the binding of miR-218-WT could downregulate the expression of downstream luciferase gene, leading to reduced luciferase activity.

The expression of MCM3AP-AS1 was correlated with the expression of GLUT1 in PTC tissues
The expression levels of GLUT1 (a downstream target of miR-218) in both PTC and non-tumor tissues were measured by qRT-PCR. Compared with non-tumor tissues, the expression levels of GLUT1 were significantly higher in PTC tissues (Figure 3A, p < 0.05). The correlation between the expression of GLUT1 and MCM3AP-AS1 was analyzed by performing Pearson’s Correlation Coefficient. It was observed that the expression of GLUT1 was significantly and positively correlated with the expression of MCM3AP-AS1 (Figure 3B).

MCM3AP-AS1 regulated the expression of GLUT1 in IHH-4 cells through miR-218
Western blot analysis showed that overexpression of MCM3AP-AS1 significantly upregulated GLUT1 at both mRNA (Figure 4A) and protein (Figure 4B) levels compared with NC (NC miRNA or empty pcDNA3.1 vector) (p < 0.05) (original blots imagines were shown in supplementary Figure 1). However, overexpression of miR-218 significantly downregulated GLUT1 at both mRNA and protein levels. In addition, co-transfection experiment showed that overexpression of miR-218 attenuated the effects of overexpression of MCM3AP-AS1 on the expression of GLUT1.
Figure 1. MCM3AP-AS1 was upregulated in PTC and affected by clinical stages. The expression of MCM3AP-AS1 in both PTC and non-tumor tissues were measured by qRT-PCR. The expression levels of MCM3AP-AS1 were compared between two types of tissue by performing paired t-test (A). ANOVA (one-way) combined with Tukey test was used to compare the expression levels of MCM3AP-AS1 among patients at different clinical stages (B). Mean values of 3 biological replicates were presented, *, p < 0.05.

Figure 2. MCM3AP-AS1 might bind to miR-218, but no interaction between MCM3AP-AS1 and miR-218 was observed in IHH-4 cells. We performed RNA interaction predictions using http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp. MCM3AP-AS1 and miR-218 might form strong base pairing (A). IHH-4 cells were transfected with MCM3AP-AS1 expression vector or miR-218 mimic. Overexpression of MCM3AP-AS1 and miR-218 was confirmed by qRT-PCR at 24 h post-transfection (B). The interaction between MCM3AP-AS1 and miR-218 was analyzed by qRT-PCR (C). Mean values of 3 biological replicates were presented, *, p < 0.05. (D) The luciferase result between MCM3AP-AS1 + miR-218-MUT or MCM3AP-AS1 + miR-218-WT. Mean values of 3 biological replicates were presented, *, p < 0.05.
**MCM3AP-AS1 in papillary thyroid carcinoma**

**Figure 3.** The expression of MCM3AP-AS1 was correlated with the expression of GLUT1 in PTC tissues. The expression of GLUT1 (a downstream target of miR-218) in both PTC and non-tumor tissues were measured by qRT-PCR. The expression levels of GLUT1 were compared between two types of tissue by performing paired t-test (A). The correlation between GLUT1 mRNA and MCM3AP-AS1 was analyzed by performing Pearson’s Correlation Coefficient (B). Mean values of 3 biological replicates were presented, *, p < 0.05.

**Figure 4.** MCM3AP-AS1 regulated the expression of GLUT1 in IHH-4 cells through miR-218. qRT-PCR and Western blot were performed to analyze the effects of overexpression of MCM3AP-AS1 on GLUT1 at both mRNA (A) and protein (B) levels. Mean values of 3 biological replicates were presented, *, p < 0.05. Check the additional file for the original, uncropped gels or blots. The original blots were presented in Figure 1.

**DISCUSSION**

In the present study, we found that MCM3AP-AS1 was upregulated in PTC and might upregulate GLUT1 by sponging miR-218 to promote the proliferation of PTC cells.

The functions of MCM3AP-AS1 have only been investigated in a few cancers (12,13,15). In liver cancer, MCM3AP-AS1 is upregulated and can regulate the miR-194-5p/FOXA1 axis to promote tumor growth (12). In glioblastoma, MCM3AP-AS1 is also upregulated in papillary thyroid carcinoma.
Figure 5. MCM3AP-AS1 promoted the proliferation of IHH-4 and MDA-T120 cells through miR-218 and GLUT1. CCK-8 assay was performed to analyze the effects of MCM3AP-AS1, miR-218, GLUT1, MCM3AP-AS1 + miR-218 and GLUT1 + miR-218 overexpression on the proliferation of IHH-4 and MDA-T120 cells at 24 h post-transfection. Mean values of 3 biological replicates were presented, *, p < 0.05.

and negatively regulates the expression of miR-211, which in turn activates the PI3K/AKT and ERK1/2 signaling pathways through KLF5 (13). These studies revealed the oncogenic function of MCM3AP-AS1 in cancer biology. Besides, MCM3AP-AS1 was reported to be upregulated in PTC and it promotes proliferation and invasion of cancer cells through regulating the miR-211-5p/SPARC axis in PTC (15). In this study, we confirmed that MCM3AP-AS1 was upregulated in PTC, and overexpression of MCM3AP-AS1 resulted in the increased proliferation of PTC cells. Therefore, our data suggest that MCM3AP-AS1 plays an oncogenic role in PTC by promoting cancer cell proliferation.

MiR-218 is a well-characterized tumor suppressive miRNA that suppresses cancer development and progression by affecting cancer cell behaviors, such as proliferation (17,18). Downregulation of miR-218 is closely correlated with the progression of PTC (19). Consistently, our study also observed the inhibitory effects of miR-218 on PTC cell proliferation. We found that miR-218 could form strong base paring with MCM3AP-AS1. However, overexpression of miR-218 in PTC cells did not affect the expression of MCM3AP-AS1. It is known that lncRNAs may sponge miRNAs to reduce their effects on downstream targets (20). It has been reported that miR-218 can target GLUT1 to inhibit bladder cancer (10). In PTC, increased expression levels of GLUT1 could promote tumor growth, leading to poor prognosis (9). This study also reported the downregulation of GLUT1 in PTC cells after overexpression of miR-218. Therefore, miR-218 may also target GLUT1 in PTC. In addition, overexpression of MCM3AP-AS1 resulted in upregulation of GLUT1. Our data supported the speculation that MCM3AP-AS1 might sponge miR-218 to upregulate GLUT1.

It is worth noting that, based on the predicted secondary structure of MCM3AP-AS1 by RNA fold (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi), the binding site of miR-218 on MCM3AP-AS1 was mapped to a loop. However, it is known that bases in loop regions may also form noncanonical base pairs with biological functions (21). Future studies are still needed to further elucidate the interaction between miR-218 and MCM3AP-AS1.

In conclusion, MCM3AP-AS1 plays an oncogenic role in PTC possibly by sponging miR-218 to upregulate GLUT1 and promote cancer cell proliferation. The MCM3AP-AS1/miR-218/GLUT1 axis may serve a potential target to treat PTC.

Ethics approval and consent to participate: this study was approved by Ethics Committee of 3201 Medical College Affiliated Hospital. Written informed consent was obtained from all individual participants included in the study. Procedures operated in this research were completed in keeping with the standards set out in the Announcement of Helsinki and laboratory guidelines of research in China.

Consent for publication: not applicable.

Availability of data and material: the datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Funding: not applicable.

Authors’ contributions: Rui Nian, Wanjun Li: study concepts, literature research, clinical studies, data analysis, experimental studies, manuscript writing and reviewing; Xiang Li, Jiayu Zhang: study design, literature research, experimental studies, manuscript editing; Weihua Li, Fanfan Pan: definition of intellectual content, clinical studies, data acquisition and statistical analysis; Jing Cheng: data acquisition, manuscript preparation and data analysis; Xin Jin: data acquisition and statistical analysis. All authors have read and approve the submission of the manuscript.

Acknowledgements: not applicable.

Disclosure: no potential conflict of interest relevant to this article was reported.

REFERENCES


**SUPPLEMENT DATA 1**

Supplement data 1. Subcellular location of MCM3AP-AS1 in IHH-4 cells. RNA FISH was employed to detect the MCM3AP-AS1 expression in the IHH-4 cells. DAPI fluorescence indicate the nuclei (left). MCM3AP-AS1 was detected in cytoplasm (middle). The merged showed in left. scale bar = 100 μm.