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Evaluation of hsa_circ_0000018/let-7f-5p/FAM96A axis in lung adenocarcinoma progression

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Abstract.
BACKGROUND: Circular RNAs (circRNAs) are critical regulators of lung adenocarcinoma (LA) progression. Although a molecular marker targeting hsa_circ_0000018 has been developed and used for diagnosing colon cancer, the role of this circRNA in LA progression has not been explored till now.
OBJECTIVES: This study aimed to elucidate the role and regulatory mechanisms of hsa_circ_0000018 in LA progression.
METHODS: LA tissues and corresponding adjacent non-tumor tissues were collected from 36 patients to confirm the levels of circRNAs, microRNAs (miRNAs), and messenger RNAs (mRNAs) using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). We also cultured two LA cell lines (A549, PC-9), and the human normal lung epithelial cell line BEAS-2B. Cell function experiments were conducted to assess malignancy in LA cells, including proliferation, migration, and invasion, following forced hsa_circ_0000018 expression. The correlation between hsa_circ_0000018, let-7f-5p, and family with sequence similarity 96 member A (FAM96A) was confirmed by using starBase (miRNA-circRNA interaction database), luciferase assay, and western blotting.
RESULTS: Expression of hsa_circ_0000018 and FAM96A was reduced, whereas that of let-7f-5p was upregulated in LA. Cell function assays revealed that upregulation of hsa_circ_0000018 had a suppressive effect on the proliferation, migration, and invasion of LA cells. Additionally, hsa_circ_0000018 sponge binds let-7f-5p, resulting in upregulation of FAM96A expression.
CONCLUSION: Our data reveal hsa_circ_0000018 as a tumor suppressor in LA that targets the let-7f-5p/FAM96A axis. Our findings enrich the known regulatory network of circRNAs in LA.

Keywords: hsa_circ_0000018, lung adenocarcinoma, let-7f-5p, FAM96A, circRNAs

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1. Introduction

Lung cancer is a highly prevalent form of cancer worldwide and is further classified into small-cell and non-small cell lung cancer (NSCLC) categories [1]. Lung adenocarcinoma (LA) is the most common subtype of lung cancer, accounting for 45% of all lung cancer cases globally [2]. With improvements in the diagnosis and treatment of LA, patients with LA have longer survival times. However, the prognosis of LA patients remains poor because most cases are diagnosed with LA at late stage when cancer treatments are challenging to deliver [3]. Therefore, it is necessary to identify the critical regulators of LA progression.

Circular RNAs (circRNAs) are a type of non-coding RNA with a covalently bonded closed loop structure that lacks a 5'-cap and a 3'-polyadenylated tail [4]. The circRNA-microRNA (miRNA)- messenger RNA (mRNA) regulatory network is known to play a role in tumor progression, and circRNAs are implicated in this process [5–7]. For example, hsa_circ_104348 is an oncogenic circRNA in hepatocellular carcinoma that regulates the miR-184-3p/RTKN2 axis [7]. The hsa_circ_101237-miR-490-3p-mitogen-activated protein kinase-1 (MAPK1) network participates in NSCLC tumorigenesis by regulating cell malignancy [8]. CircRNAs circ_0029426 and circ_0007618 show potential as biomarkers for the diagnosis and prognosis of LA [9]. In addition, the knockdown of hsa_circ_0020850 represses LA development by regulating miR-195-5p to enhance insulin receptor substrate 2 (IRS2) expression [10]. Hsa_circ_0020850 suppresses LA progression by targeting the miR-6783-3p/DKK1 axis [11]. Hsa_circ_0000018 (alternative name: hsa_circRNA_00054), located on chr1: 15860731-15863309, is downregulated in colon cancer, and a molecular marker targeting hsa_circ_0000018 has been developed for the diagnosis of colon cancer [12]. After detecting the hsa_circ_0000018 expression in lung adenocarcinoma, we found that it was also downregulated in LA. However, a literature search revealed that its function has not been explored in any cancer. Therefore, we hypothesized that the abnormal expression of hsa_circ_0000018 may play a key role in LA and decided to explore its effect and mechanism of action in this cancer.

MiRNAs are short non-coding RNAs that range from 19 to 23 nucleotides in length and can bind to the mRNAs of their target genes, thereby modulating their expression [13]. The miRNA let-7f-5p has been found to act as an oncogenic agent in prostate cancer [14] and a tumor suppressor in osteosarcoma [15]. In lung cancer, let-7f-5p is upregulated in typical and atypical carcinoid tumors; however, its function and mechanism in LA have not been investigated [16]. Using the online tool starBase (miRNA-circRNA interaction database), we predicted that let-7f-5p binds to hsa_circ_0000018. Therefore, we explored the expression of let-7f-5p and its relationship with hsa_circ_0000018 expression in LA.

Family with sequence similarity 96 member A (FAM96A) is a protein belonging to the cytosolic Fe/S protein family and plays a crucial role in biological processes. Previous studies have confirmed that FAM96A suppresses tumor growth in both hepatocellular carcinoma [17] and gastrointestinal stromal tumors [18] via a proapoptotic process. However, the function of FAM96A in LA has not been elucidated.

Based on these previous studies, we speculated that hsa_circ_0000018, let-7f-5p, and FAM96A may be critical regulators of LA. To confirm this hypothesis, we performed a series of cell function experiments to explore the regulatory networks involving hsa_circ_0000018, let-7f-5p, and FAM96A. Our research has the potential to enhance an understanding of the complex regulatory mechanisms underlying LA and could have implications for the development of more effective diagnostic and therapeutic strategies.

2. Material and methods

2.1. Clinical tissues

Our study involved the collection of LA tissues and corresponding adjacent non-tumor tissues from 36 patients who had been diagnosed with LA at Puren Hospital in China between January 2021 and January 2022. The Ethics Committee of Puren Hospital approved the study protocol (Approval Number: prll2021019), and all participants provided written informed consent. Patients who were diagnosed with LA and did not undergo any treatment were included, whereas those with a history of LA or other diseases were excluded. Additional information regarding the clinical characteristics of the patients is provided in Supplementary Table 1.

2.2. Cell lines

The normal human lung epithelial cell line BEAS-2B (Catalog number: CL-0496) LA cell lines A549 (Catalog number: CL-0016), and PC-9 (Catalog number: CL-

0668) were purchased from Procell (China). BEAS-2B cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, ProCell), whereas A549 and PC-9 cells were maintained in Ham's F-12K medium (ProCell) and RPMI-1640 medium (ProCell), respectively. All cells were maintained in 10% fetal bovine serum (FBS; Procell) in a cell culture incubator in an atmosphere of 5% CO_2 and at 37°C.

2.3. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

The expression of hsa_circ_0000018, FAM96A, and let-7f-5p in LA was confirmed using qRT-PCR. RNA was extracted using RNAiso Plus (TAKARA, Japan) and cDNA was synthesized using the SuperScript Reverse Transcriptase Kit (Vazyme, Nanjing, China). qRT-PCR was conducted with the help of SYBR Premix Ex Taq kit purchased from TAKARA (Japan) under the following conditions: 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 30 s. The expression of hsa_circ_0000018, FAM96A, and let-7f-5p was calculated by using the $2^{-\Delta\Delta Ct}$ method as described previously (20) with GAPDH as an internal reference for hsa_circ_0000018 and FAM96A, and U6 for let-7f-5p. Primer sequences are listed in Supplementary Table 2. The experiment was independently repeated thrice.

2.4. RNA enzyme digestion assay

RNA enzyme digestion assays are used to confirm the closed-loop structure of RNA molecules because RNA enzymes can digest linear RNA, not circRNAs. Briefly, the RNA from A549 and PC-9 cells was incubated with RNase R (Epicenter Technologies, USA) at 37°C for 30 min to assess the stability of hsa_circ_0000018 and linear RNA DNAJC16 [19]. After incubation, qRT-PCR was performed to detect the levels of hsa_circ_0000018 and DNAJC16 in LA cells. The experiment was independently repeated thrice.

2.5. Cell transfection

The hsa_circ_0000018 overexpression vector (pcDNA 3.1-circ) was constructed by RiboBio (China) using the pcDNA 3.1 circRNA Mini vector. The pcDNA 3.1 circRNA mini vector without the hsa_circ_0000018 overexpression sequences was used as a negative control. Let-7f-5p mimic, mimic-NC, siRNA targeting FAM96A (si-FAM96A), and si-NC (negative control siRNA) were constructed by RiboBio. A549 and PC-9 cells at 60% confluence were transfected with pcDNA 3.1-circ, pcDNA 3.1, let-7f-5p mimic, mimic-NC, si-FAM96A, or si-NC using Lipo6000 (Beyotime, China) and incubated for 48 h. The transfection efficiency was determined by qRT-PCR. The experiment was independently repeated thrice.

2.6. Cell proliferation assay

Cell proliferation was assessed using the Cell Counting Kit-8 (CCK8) assay. In a 96-well plate, 2×10^3 cells were seeded and after transfection for 0, 24, 48, and 72 hours, CCK-8 solution (10 μL/well, Beyotime, China) was added and cells were incubated for another 2 h. The optical density of the cells at 450 nm was measured using a microplate reader, and cell proliferation was determined by plotting a growth curve [20]. The experiment was independently repeated thrice.

2.7. Transwell assay to assess cell migration and invasion

A transwell assay was performed to evaluate the migratory and invasive capabilities of LA cells as described previously [10]. Transfected LA cells (1×10^5 cells/mL) in a serum-free medium were added to the upper chamber. In contrast, the medium supplemented with 10% FBS was added to the bottom chamber. After 24 h of incubation, the migrated cells present on the lower surface of the membrane were fixed, stained, and observed under a light microscope. For the invasion assay, Matrigel (BD Biosciences, USA) was added to the transwell plate before adding the transfected cells. All the other procedures were identical to those used for the migration assay. The experiment was independently repeated thrice.

2.8. Luciferase assay

The binding sites for hsa_circ_0000018, let-7f-5p, and FAM96A were predicted using StarBase database (https://starbase.sysu.edu.cn/index.php). Based on these predictions, wild-type (WT) vectors of hsa_circ_0000018 and FAM96A carrying binding sites for let-7f-5p were constructed using psiCHECK-2 vectors from RiboBio (China). The mutant (MUT) vectors for hsa_circ_0000018 and FAM96A that lacked the binding sites for let-7f-5p were constructed with psiCHECK-2 vectors by RiboBio. The WT or MUT vectors were transfected into LA cells along with the let-7f-5p mimic or mimic-NC using Lipo6000 (Beyotime, China). Lu-

ciferase activity was verified using the Dual-Luciferase Reporter Assay System (Beyotime) after a 48-hour transfection period as described previously [10]. The experiment was independently repeated thrice.

2.9. Western blotting

The RIPA buffer (Beyotime, China) was used to extract proteins from LA cells that were quantified using a bicinchoninic acid protein assay kit (Beyotime). Next, 20 μg of protein per well was separated on a 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was transferred to polyvinylidene difluoride membranes. Following the blocking of membranes with 5% fat-free milk for 3 h, primary antibodies, including anti-FAM96A (Catalog number: PA5-66628, Thermo Fisher, USA) or anti-GAPDH (Catalog number: sc-47724, Santa Cruz, USA) antibodies, were added and the membranes were incubated for 12 h at 4°C. The membranes were then incubated with corresponding fluorescent anti-rabbit or anti-mouse secondary antibodies (LI-COR Biosciences, Germany) for 2 h at room temperature. Finally, Odyssey 3.2 (LI-COR Biosciences) fluorescence imager was used to acquire images of the western blots as described previously [21]. The experiment was independently repeated thrice.

2.10. Statistical methods

Statistical analysis was conducted by using GraphPad Prism 7.0 software and data are shown as mean $\pm$ SD of all experiments (in triplicate). A paired t-test was performed to calculate the differences between the two groups, and an analysis of variance followed by Dunnett's or Tukey's multiple comparison test was conducted to calculate the differences between the two groups. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Downregulation of hsa_circ_0000018 in LA tissues and cells

The initial approach used to investigate the role of hsa_circ_0000018 in LA was to assess its expression using qRT-PCR. The results showed that hsa_circ_0000018 expression was reduced by 50% in LA tissues compared to that in adjacent normal tissues (Fig. 1A) and in LA cells (A549 and PC-9) compared to that in BEAS-2B cells (Fig. 1B). A review of the structure of hsa_circ_0000018 (Fig. 1C) showed that its length was 407 nt, and that DNAJC16 was a linear gene of hsa_circ_0000018. In addition, RNase R digestion reduced DNAJC16 levels to a greater extent than hsa_circ_0000018 digestion, proving that hsa_circ_0000018 had a stable closed-loop structure (Fig. 1D). These data proved that hsa_circ_0000018, with a stable closed-loop structure, was downregulated in LA.

3.2. hsa_circ_0000018 overexpression inhibits LA progression in vitro

After constructing the hsa_circ_0000018 overexpression vectors using pcDNA 3.1, we found that pcDNA 3.1-circ upregulated hsa_circ_0000018 expression in both A549 and PC-9 cells (Fig. 2A). A series of cell experiments were conducted to demonstrate alterations in LA cell function after overexpression of hsa_circ_0000018. The CCK8 assay showed that hsa_circ_0000018 overexpression attenuated the proliferation of A549 and PC-9 cells (Fig. 2B). In addition, LA cells transfected with pcDNA 3.1-circ showed impaired migration and invasion abilities, as demonstrated by the transwell assay results (Fig. 2C and D). The results of *in vitro* experiments suggested that hsa_circ_0000018 was an oncogenic circRNA in LA cells.

3.3. hsa_circ_0000018 targets let-7f-5p/FAM96A axis in LA

Prediction analysis using starBase revealed the presence of binding sites between let-7f-5p and hsa_circ_0000018 (Fig. 3A). We constructed hsa_circ_0000018-WT and hsa_circ_0000018-MUT vectors with and without binding sites, respectively, to perform a luciferase assay. In the hsa_circ_0000018-WT group, the let-7f-5p mimic reduced luciferase activity, whereas no such effect was observed in the hsa_circ_0000018-MUT group (Fig. 3B). Similarly, starBase predicted the presence of binding sites between FAM96A and let-7f-5p (Fig. 3C), and luciferase activity was reduced only in the FAM96A-WT+let-7f-5p mimic group (Fig. 3D). qRT-PCR confirmed the upregulation of let-7f-5p and the downregulation of FAM96A in LA tissues (Fig. 3E). Moreover, let-7f-5p expression was found to have a negative correlation with both hsa_circ_0000018 and FAM96A expression in LA tissues, while a positive correlation was observed between hsa_circ_0000018 and FAM96A expression (Fig. 3F). These data confirmed that hsa_circ_0000018 targets let-7f-5p to positively regulate FAM96A expression in LA cells.

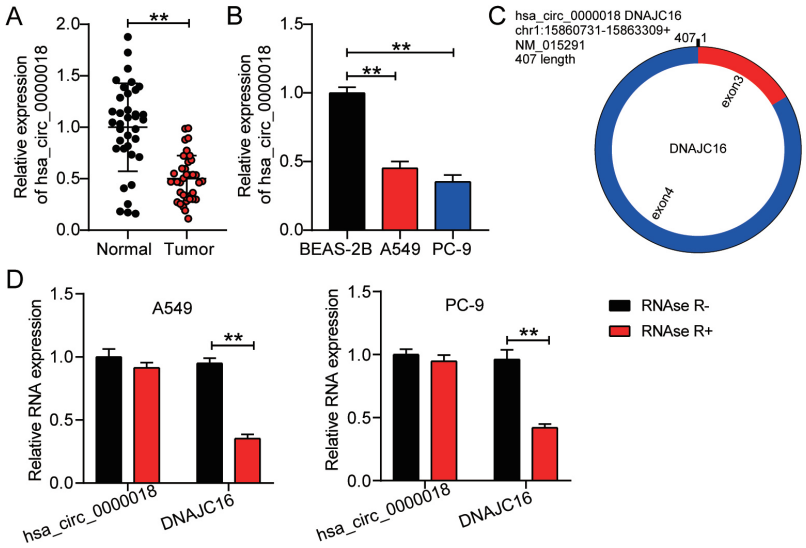

Fig. 1. The downregulation of hsa_circ_0000018 in LA. (A) qRT-PCR was used to analyze hsa_circ_0000018 expression in LA tissues and adjacent normal tissues. (B) qRT-PCR was used to analyze hsa_circ_0000018 expression in LA cell lines (A549 and PC-9) and human normal lung epithelial cell line BEAS-2B. (C) The construction of hsa_circ_0000018. (D) The stability of hsa_circ_0000018 was determined by RNA enzyme digestion assay. $^{**}P < 0.01$.

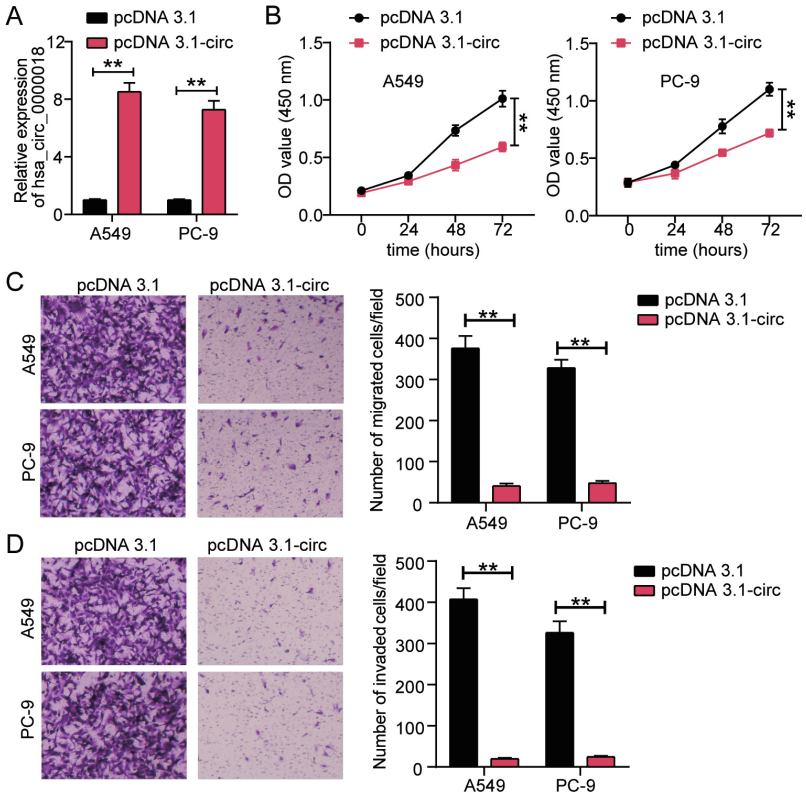

Fig. 2. Hsa_circ_0000018 overexpression inhibits LA progression *in vitro*. (A) The transfection efficiency of pcDNA 3.1-circ was verified in LA cell lines (A549 and PC-9) using qRT-PCR. (B) The effect of pcDNA 3.1-circ transfection on cell proliferation was assessed using the CCK8 assay in LA cells. (C-D) The migration and invasion abilities of LA cells were evaluated using Transwell assays after transfection with pcDNA 3.1-circ to determine the effect of hsa_circ_0000018 on cell migration (C) and invasion (D). $^{**}P < 0.01$.

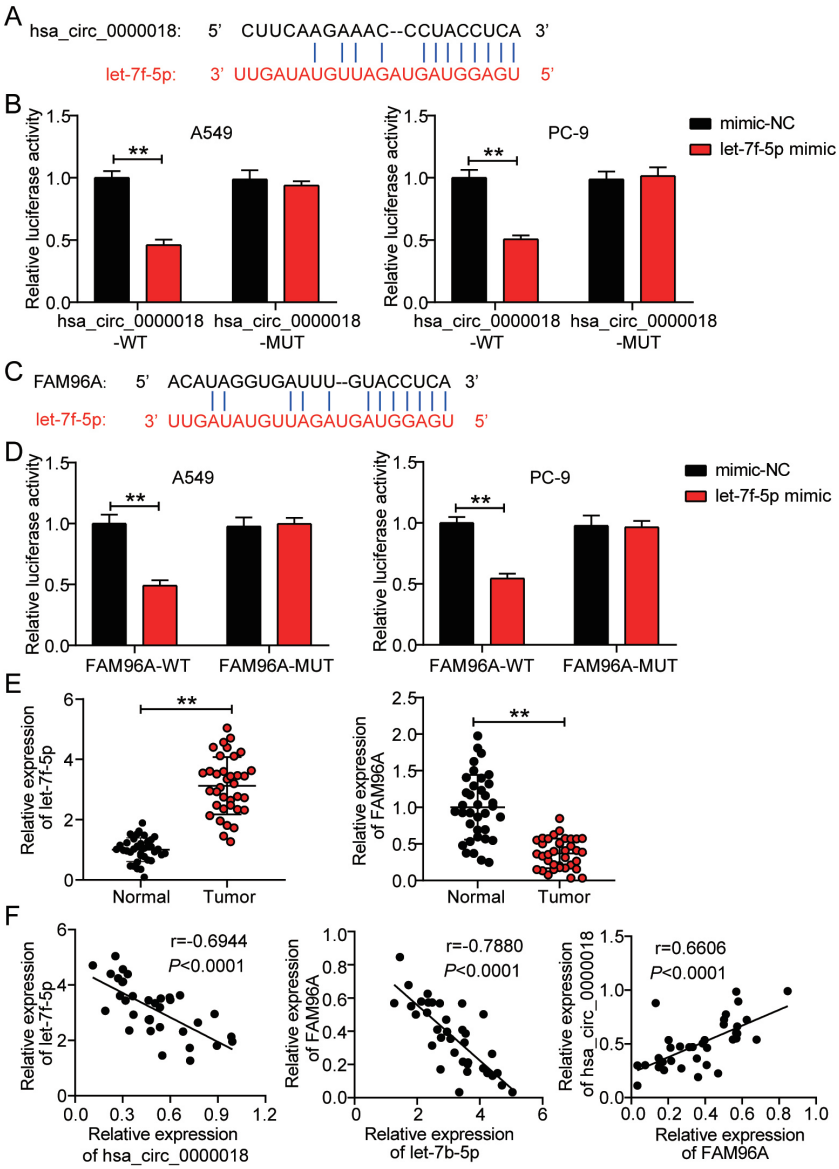

Fig. 3. Hsa_circ_0000018 targets let-7f-5p/FAM96A axis in LA. (A) The prediction of potential binding sites between hsa_circ_0000018 and let-7f-5p using starBase. (B) Luciferase assay further validated the existence of binding sites between hsa_circ_0000018 and let-7f-5p. (C) The prediction of binding sites between FAM96A and let-7f-5p using the starBase database. (D) Luciferase assay further verified the existence of binding sites between FAM96A and let-7f-5p. (E) The expression of let-7f-5p and FAM96A in LA tissues and adjacent normal tissues was detected by using qRT-PCR. (F) Pearson correlation analysis was used to quantify the correlation between hsa_circ_0000018, let-7f-5p, and FAM96A. $^{**}P < 0.01$.

3.4. FAM96A knockdown reverses the effect of hsa_circ_0000018 overexpression on LA cells

Following transfection of pcDNA 3.1-circ and si-FAM96A into LA cells, western blotting confirmed that pcDNA 3.1-circ enhanced the level of FAM96A protein by $>$ 2.5-fold, whereas si-FAM96A relieved the effect of pcDNA 3.1-circ in LA cells (Fig. 4A). The results of the CCK8 assay indicated that the suppression of cell proliferation caused by pcDNA 3.1-circ was partially restored by FAM96A knockdown (Fig. 4B). The decrease in cell migration and invasion caused by pcDNA 3.1-circ was partially reversed by si-FAM96A (Fig. 4C and D). These results indicate that FAM96A knockdown could relieve the effect of hsa_circ_0000018 on LA cells because of their regulatory relationship.

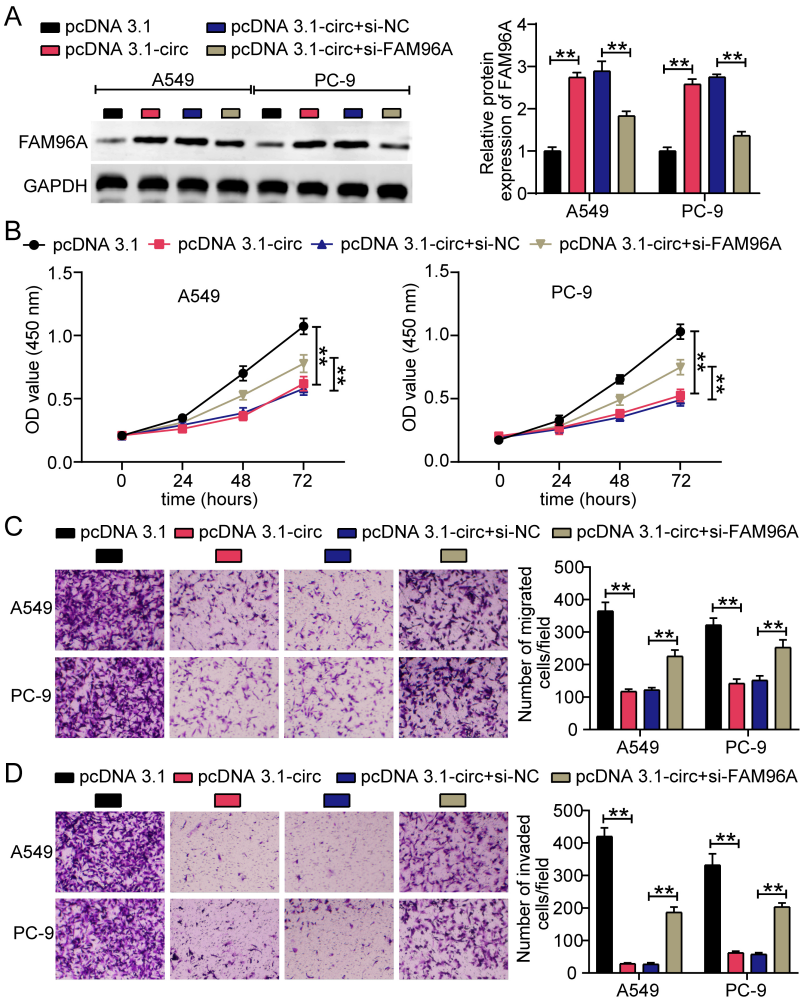

Fig. 4. FAM96A knockdown rescued the inhibitory effect of hsa_circ_0000018 overexpression on LA cells. (A) Western blotting was conducted to verify the expression of FAM96A protein in transfected A549 and PC-9 cells. (B) The cell proliferation ability of transfected A549 and PC-9 cells was evaluated using CCK8 assay. (C-D) The cell migration (C) and invasion (D) abilities of transfected A549 and PC-9 cells were identified using transwell assays. pcDNA 3.1-circ, hsa_circ_0000018 overexpression vector. si-NC, negative control siRNA. si-FAM96A, siRNA targeting FAM96A. $^{**}P < 0.01$.

4. Discussion

According to a previous report, LA accounted for approximately 40% of all lung cancer cases diagnosed in 2019 worldwide [22]. Several studies have shown that circRNAs are critical regulators of tumorigenesis and LA progression. Zhou et al. observed that circRNA-ENO1 upregulates enolase 1 (a glycolytic enzyme) and accelerates LA progression by regulating glycolysis, cell proliferation, and epithelial-mesenchymal transition. In contrast to circRNA-ENO1, circDCUN1D4 suppresses tumor metastasis in LA by forming an RNA-protein ternary complex with HuR and TXNIP [23]. Hsa_circ_0000018, also known as hsa_circRNA_000541, is downregulated in colon cancer, and a molecular marker targeting hsa_circ_0000018 has been developed for the diagnosis of colon cancer [12]. Whether hsa_circ_0000018 promotes or inhibits tumorigenicity in LA has not yet been investigated. Here, we show for the first time that hsa_circ_0000018 is downregulated in LA and that its overexpression can effectively reduce the proliferation, migration, and invasion of LA cells. The findings of this study on the function of hsa_circ_0000018 in LA are consistent with those of a previous study on its function in colon cancer, suggesting that a molecular marker targeting hsa_circ_0000018 may also be developed for the diagnosis of LA. Additionally, many

studies have shown that circRNAs can sponge the miRNA-mRNA axis, thereby participating in LA progression [10,24,25]. For example, hsa_circMMD_007 promotes LA progression by acting as an endogenous sponge for miR-197-3p [26]. In this study, we observed that hsa_circ_0000018 hindered cell malignancy in LA by acting as a sponge for let-7f-5p.

The miRNA let-7f-5p has been implicated in distinct roles in a variety of cancers. For example, let-7f-5p is an oncogenic miRNA in prostate cancer, yet functions as a tumor suppressor in osteosarcoma [15]. Di Fazio et al. analyzed let-7f-5p expression in both typical and atypical carcinoid tumors in lung cancer, confirming that let-7f-5p was upregulated in most tumor samples compared to normal lung tissues. However, the specific functions and mechanisms of action of let-7f-5p in LA have not been explored. The results of this study are consistent with the findings reported by Di Fazio et al., indicating an upregulation of let-7f-5p in LA samples. This study also showed that FAM96A is a target gene of let-7f-5p in LA cells, and that let-7f-5p expression was negatively correlated with both hsa_circ_0000018 and FAM96A in LA samples. Therefore, we hypothesized that let-7f-5p acts as a bridge connecting hsa_circ_0000018 and FAM96A in LA, thereby regulating FAM96A expression. Western blotting confirmed this hypothesis by showing that hsa_circ_0000018 overexpression enhanced FAM96A protein expression.

FAM96A, a cytosolic Fe/S protein, is a pro-apoptotic protein [27]. Evidence supports FAM96A as a tumor suppressor in gastrointestinal stromal tumors, showing that FAM96A re-expressed in gastrointestinal stromal tumors induces cell apoptosis and diminishes tumorigenicity [18]. FAM96A inhibits tumor growth in nude mice, thereby playing an anti-cancer role in hepatocellular carcinoma [17]. Although there have been no studies on the function of FAM96A in LA, we speculate that FAM96A may play an anticancer role in LA, based on a previous study. In our study, we demonstrated that FAM96A is downstream of hsa_circ_0000018 and that its expression can be enhanced by upregulating hsa_circ_0000018. Cell function experiments showed that FAM96A knockdown partially relieved the negative effects of hsa_circ_0000018 overexpression in LA cells.

Although our study elucidated the effect and underlying mechanism of hsa_circ_0000018 on LA cells, further investigation is required to determine whether hsa_circ_0000018 modulates LA progression *in vivo*. The clinical role of hsa_circ_0000018 in LA, such as in prognosis and drug treatment, warrants further investigation.

5. Conclusion

Our study revealed the role of hsa_circ_0000018 in LA and showed that hsa_circ_0000018 hindered LA progression *in vitro* by targeting the let-7f-5p/FAM96A axis. Our findings enrich the known regulatory network of circRNAs in LA and may aid in its diagnosis and treatment.

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Availability of data and materials

Not applicable.

Authors contributions

Interpretation or analysis of data: MZ, DDH, and JJQ.
Preparation of the manuscript: QL.
Revision for important intellectual content: WH.
Supervision: all authors.
All authors have read and approved this manuscript.

Ethics approval and consent to participate

All procedures conducted in this study were in compliance with the principles outlined in the Declaration of Helsinki and were approved by the Ethics Committee of Puren Hospital (Approval number: prll2021019). Additionally, this study obtained written informed consent from all patients.

Consent for publication

Consent for publication was obtained from the participants.

Conflict of interest

The authors have no conflicts of interest related to this research.

Acknowledgments

Not applicable.

Supplementary data

The supplementary files are available to download from http://dx.doi.org/10.3233/CBM-230111.

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