Original Article

Circular RNA hsa-circ-000881 suppresses the progression of lung adenocarcinoma *in vitro* via a miR-665/PRICKLE2 axis

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Background: Circular RNA (circRNA) has become a new focus in the field of tumor biology research in recent years. Many circRNAs have been showed to play an important role in the progression of lung adenocarcinoma (LUAD). In this work, we studied the oncological role of hsa-circ-000881 in LUAD and attempted to explore the related mechanism.

Methods: The relative expressions of hsa-circ-000881, miR-665, and PRICKLE2 were detected by RT-qPCR or western blot. Functional assays were conducted to analyze the role of hsa-circ-000881 in the proliferation, migration, and invasion of LUAD cells. A luciferase reporter assay was performed to verify whether hsa-circ-000881, miR-665, and PRICKLE2 interact with each other.

Results: Circ-000881 was remarkably downregulated in LUAD. Overexpression of circ-000881 attenuated cell growth, migration, and invasion, whereas its knockdown enhanced the malignancy of LUAD cells. The results of luciferase reporter assay and bioinformatics analysis confirmed that circ-000881 served as a sponge for miR-665, and PRICKLE2 was a direct target of miR-665.Overexpression of miR-665 or silencing of PRICKLE2 abolished circ-000881-mediated inhibition of malignant tumor behavior in LUAD cells.

Conclusions: Circ-000881 has inhibitory effects on LUAD via a miR-665/PRICKLE2 axis, suggesting that circ-000881 may be an underlying therapeutic target for LUAD.

Keywords: Circ-000881; miR-665; lung adenocarcinoma (LUAD); PRICKLE2

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Introduction

Lung cancer is most prevalent cancer type in the world both in incidence and mortality (1). Non-small cell lung cancer (NSCLC) accounts for more than half of all lung cancer cases, with its most common subtype, lung adenocarcinoma (LUAD), comprising 40% of all lung cancers (2,3). Patients with adenocarcinoma often develop distant metastases early on in their disease (4). Early diagnosis of LUAD is an issue that urgently needs to be addressed. Many patients with LUAD missed the best time for surgical treatment when they were diagnosed (5). In the last decade, progress has been made in the treatment of LUAD, but mortality is still high (6). Biological research on molecular markers of tumor genesis and development can provide new ideas on more efficient means of diagnosis and therapeutic strategies for patients with LUAD.

Circular RNAs (circRNAs) belong to the functional noncoding RNA (ncRNA) family, the members of which have been widely studied in recent decades. With their high

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level of stability and highly conserved structure, circRNAs have a low likelihood of degradation (7). Since the discovery of circRNAs, many researchers have attempted to investigate the whether or not they are involved in the occurrence and development of human diseases, especially cancers, and some studies have produced exciting results (8). Wang *et al.* reported that hsa-circ-0001038 was upregulated in cervical cancer, which was correlated with an unfavorable outcome for patients (9). In their study, Sang *et al.* observed that high expression of circRNA ciRS-7 facilitated the malignant biological behavior of esophageal squamous carcinoma cells by obviously promoting cell growth, migration, and invasion (10). In another study, down-regulation of hsa-circ-0091570 was often observed in patients with hepatocellular carcinoma who had a higher tumor grade, and accelerated tumor growth *in vivo* (11). Dysregulation of circRNAs, such as hsa-circ-0001588, hsa-circ-0000211, and circASPH, has also been confirmed in LUAD (2,4,12).

Recently, some circRNAs have been proven to possess protein translation ability (13). In cancers, circRNAs usually interact with microRNAs (miRNAs) to produce a crucial promotion or suppression effect (14). For instance, it was reported that circ-RanGAP1 sponges miR-877-3p to promote malignant biological behaviors in gastric cancer cells (15). The interaction between circ-MYLK and miR-29a was demonstrated by Zhong *et al.* to facilitate the progression of bladder carcinoma by inducing epithelial–mesenchymal transition (16). In another study, circ-0000977 was also found to regulate the progression of pancreatic ductal adenocarcinoma through interaction with miR-874-3p (17). However, so far, the role of circRNA in the progression of LUAD has remained unclear.

In the present research, we analyzed the biological function of circRNA circ-000881 in LUAD, along with the underlying mechanism, using The Cancer Genome Atlas (TCGA) database.

We present the following article in accordance with the MDAR reporting checklist (available at http://dx.doi.org/10.21037/atm-21-844).

Methods

Patients and tissue samples

A total of 40 pairs of LUAD and para-carcinoma normal tissues were collected from patients who had undergone surgery at our hospital. None of the patients had a history of other tumors or had received radiotherapy or chemotherapy preoperatively. All tissue samples were immediately frozen in liquid nitrogen and stored in a freezer at –80 ℃. Ethics approval for this study was acquired from the Medical Ethics Committee of Qilu hospital, and informed consent was obtained from all participants. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

Bioinformatic analysis

We used the Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/) database to analyze circ-000881 and miR-665 expression in tumor and normal tissues. We used TargetScan (http://www.targetscan.org/) to predict possible binding sites between circ-000881 and miR-665. Possibly target genes of miR-665 were predicted by using Starbase (http://starbase.sysu.edu.cn/), The Cancer Genome Atlas (TCGA), TargetScan and miRDB (http://mirdb.org/).

Cell culture

Human bronchial epithelial cells (HBE) and lung cancer cells (A549, HCC827, H23, H1975, and H1299) were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Science (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; KeyGEN, Jiangsu, China) containing 10% fetal bovine serum (FBS, GIBCO, Shanghai, China) in a incubator at 37 ℃ with 5% CO_2.

Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from tissues or cultured cells with TRIzol reagent (Invitrogen, CA, USA). Subsequently, reverse transcription was performed to obtain cDNA by using the PrimeScript 1st strand cDNA Synthesis Kit (TaKaRa, Dalian, China). Then, Quantitative real-time PCR (qRT-PCR) was accomplished using the SYBR Premix Ex Taq II kit (Takara). U6 (for miR-665) and GAPDH (for circ-00081 and PRICKLE2) were used as an internal control. The $2^{-\Delta\Delta Ct}$ method was chosen to calculate the relative messenger RNA (mRNA) expression. The sequences of primers are listed in *Table 1*.

Western blot

Cellular protein was extracted with radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). A BCA

protein assay kit (Tiangen, Beijing, China) was employed to determine protein concentration. Equal amounts of protein were denatured, separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blocking with 5% nonfat skim milk at room temperature for 1 hour, the membranes were incubated with primary antibody overnight at 4 ℃. Next, the membrane was incubated with the secondary antibody at room temperature for 1 hour. The protein bands were visualized by enhanced chemiluminescence (ECL, Thermo Fisher Scientific). The quantities of the protein bands were analyzed by ImageJ software. The used antibodies are listed in *Table 2*.

Cell transfection

Circ-000881 siRNA, PRICKLE2 siRNA, miR-665 mimics, and their negative controls were purchased from GenePharma (Shanghai, China). To overexpress circ-000881, the sequence of circ-000881 was introduced into pcDNA3.1 vector (Geneseed, Guangdong, China). Transfection was performed using Lipofectamine 3000 (Invitrogen). The transfection efficiency was evaluated by RT-qPCR, and the cells were collected for subsequent experiments.

Table 1 The sequences of primers were used in this study

Name	Sequences
miR-665	Forward: 5'-TTAGAGGCAGAGACCCCCCGT-3'
	Reverse: 5'–TGGGTAGAGGCATAGACCCGT-3'
U6	Forward: 5'-CCCTTCGGGGACATCCGATA-3'
	Reverse: 5'-TTTGTGCGTGTCATCCTTGC-3'
circ-00081	Forward: 5'-AGGGACCGAACGGACTGTAG-3'
	Reverse: 5'-ACAAAACCGTAACAGCGAGC-3'
PRICKLE2	Forward: 5'-CTGCACAAAGGCCCTCTCAG-3'
	Reverse: 5'-CTGCGGGCTAACAGAGAAGT-3'
GAPDH	Forward: 5'-GCAACTAGGATGGTGTGGCT-3'
	Reverse: 5'-TCCCATTCCCCAGCTCTCATA-3'

Cell proliferation assays

After transfection, a cell counting Kit-8 (CCK-8) assay was performed. First, A549 and HCC827 cells were seeded into 96-well plates and incubated at 37 ℃ with 5% CO_2 for the indicated times (0, 24, 48, and 72 hours). Then, the cells were treated with CCK-8 reagent (Dojindo, Mashikimachi, Japan) for 2 hours. The optical density value was detected using a microplate reader at 450 nM. A colony formation assay was also performed to assess cell proliferation. Cells (500 cells/well) were seeded into 6-well plates and cultured in an incubator (37 ℃, 5% CO_2). After 14 days of incubation, 4% paraformaldehyde was used to fix the cells, which were then stained with 1% crystal violet. Finally, a microscope was used to count the number of cell clones.

Wound-healing assay

A wound-healing experiment was used to detect the migration ability of cells. Briefly, after transfection, HCC827 and A549 cells were seeded in 6-well plates and then incubated with serum-free DMEM. When cell confluency reached 90%, the monolayer was scratched with a sterile pipette tip. The wounds were observed and photographed at 0 and 48 hours, and Image J software was used to analyze the migration rate.

Transwell assay

After transfection, A549 and HCC827 cells (1×10^5 cells) were seeded into the upper chambers, which had been

Table 2 The antibodies were used in this study

Antibody	Supplier name
Primary antibody	
PRICKLE2	ab211419; Abcam, Cambridge, UK
GAPDH	ab8245, Abcam
Secondary antibody	
HRP-labeled Goat Anti-Rabbit IgG(H+L)	A0208; Beyotime, China
HRP-labeled Goat Anti-Mouse IgG(H+L)	A0216; Beyotime, China

coated with Matrigel (BD Biosciences, New York, NY, USA) and filled with serum-free medium. Medium supplemented with 10% FBS was added to the lower compartment. After incubation for 2 days, the cells on the lower surface were fixed and stained with 0.1% crystal violet for 15 min. Non-migrated cells were wiped away, and migrated cells were counted under a microscope in high power fields.

Luciferase reporter assay

A549 and HCC827 cells (1×10^4 cells per well) were seeded into 96-well plates. Circ-000881 or PRICKLE2 wild-type and mutant luciferase reporter plasmids (QIAGEN, Shanghai, China) were constructed. Each kind of plasmids was subsequently co-transfected with miR-665 mimic or mimic NC into A549 and HCC827 cells. After 48 hours of transfection, a dual-luciferase reporter assay (Promega) was carried out to assess luciferase activity.

Statistical analysis

We conducted statistical analyses using the GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA) software, and data were expressed as means ± standard deviations. Statistical differences were analyzed using Student's *t*-test, one-way analysis of variance (ANOVA), or Pearson's correlation. P<0.05 was regarded as a statistically significant difference.

Results

Circ-000881 was downregulated in LUAD tissues and cells

First, TCGA database analysis showed circ-000881 expression to be downregulated in LUAD tissues (P=0.0012, *Figure 1A*). Therefore, we collected 40 pairs of LUAD tissues and adjacent normal tissues to measure circ-000881 expression with RT-qPCR. The results indicated that circ-000881 was expressed at a low level in tumor tissues compared with para-carcinoma tissues (P<0.01, *Figure 1B*). The expression of circ-000881 was also assessed in HBE and A549, HCC827, H23, H1975, and H1299 cells. Compared with that in HBE cells, circ-000881 expression was downregulated in A549, HCC827, H23, H1975, and H1299 cells (P<0.01, *Figure 1C*).

Circ-000881 acted as a suppressor in A549 and HCC827 cells

Among the lung cancer cells, HCC827 cells and A549 cells exhibited low and high levels of circ-000881 expression, respectively. Therefore, we selected A549 cells for circ-000881 silencing and HCC827 for circ-000881 overexpression. RT-qPCR confirmed that the transfection was successful (*Figure 2A*). Next, we examined the effects of circ-000881 on the biological functions of HCC827 and A549 cells. The CCK-8 assay results demonstrated that after the transfection of overexpression vector, the proliferative capabilities of HCC827 cells were remarkably decreased, while after transfection with si-circ-000881, A549 cells became obviously less proliferative (P<0.01, *Figure 2B*). The outcomes of colony formation assays arrived at the same conclusion (P<0.01, *Figure 2C*). Furthermore, overexpression of circ-000881 diminished the migratory abilities and invasive capabilities of HCC827 cells, while circ-000881-silenced A549 cells became more invasive and could migrate more easily (P<0.01, *Figure 2D*,*E*).

Circ-000881 interacted with miR-665 in LUAD

To further explore how circ-000881 achieves its inhibitory effect in LUAD, we investigated whether circ-000881 contains the binding sequence of miR-665 using a bioinformatics tool (TargetScan 7.2). The result confirmed that circ-000881 contains the binding sites of miR-665 (*Figure 3A*). The luciferase reporter assay results demonstrated that miR-665 mimic reduced the luciferase activity of A549 and HCC817 cells transfected with circ-000881 wild-type luciferase reporter plasmids (P<0.01, *Figure 3B*), but the same effect was not observed in the mutant group. Moreover, analysis of the TGCA database showed miR-665 expression to be increased in many cancers, including LUAD, which suggested that it might play a role in tumor promotion (*Figure 3C*). Subsequently, we detected miR-665 expression at the RNA level both in clinical LUAD samples and in para-carcinoma tissues. MiR-665 was highly expressed in LUAD tissues but expressed at low levels in para-carcinoma tissues (P<0.01, *Figure 3D*). A high expression of miR-665 was accompanied by a low expression of circ-000881 in LUAD tissues, suggesting there to be a negative regulatory mechanism between them (P=0.0004, *Figure 3E*). Together, these results suggested that circ-000881 served as a sponge for miR-665 in LUAD.

PRICKLE2 is a direct target of miR-665

By using bioinformatics tools (Starbase, TCGA, TargetScan, and miRDB), we found that MOCS1,

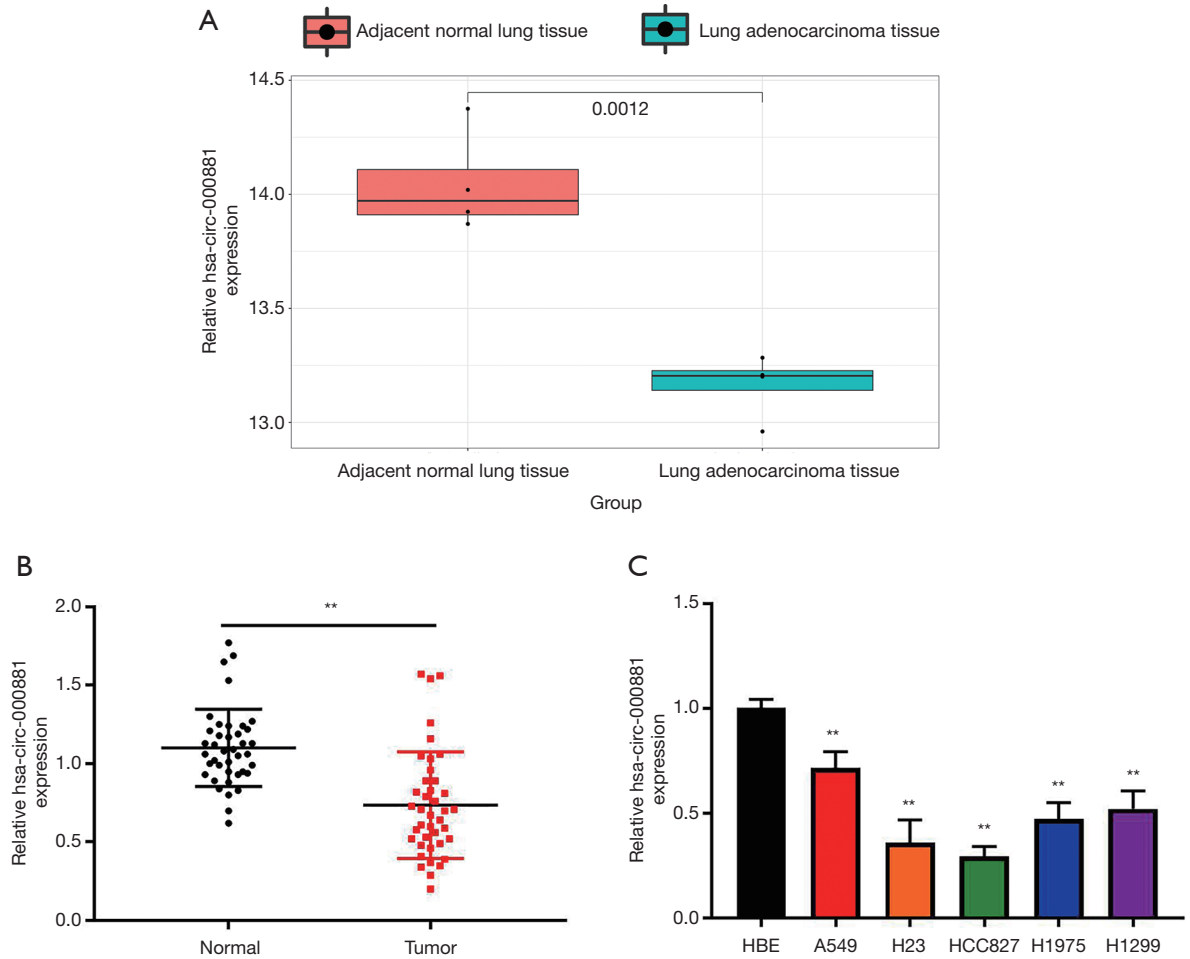
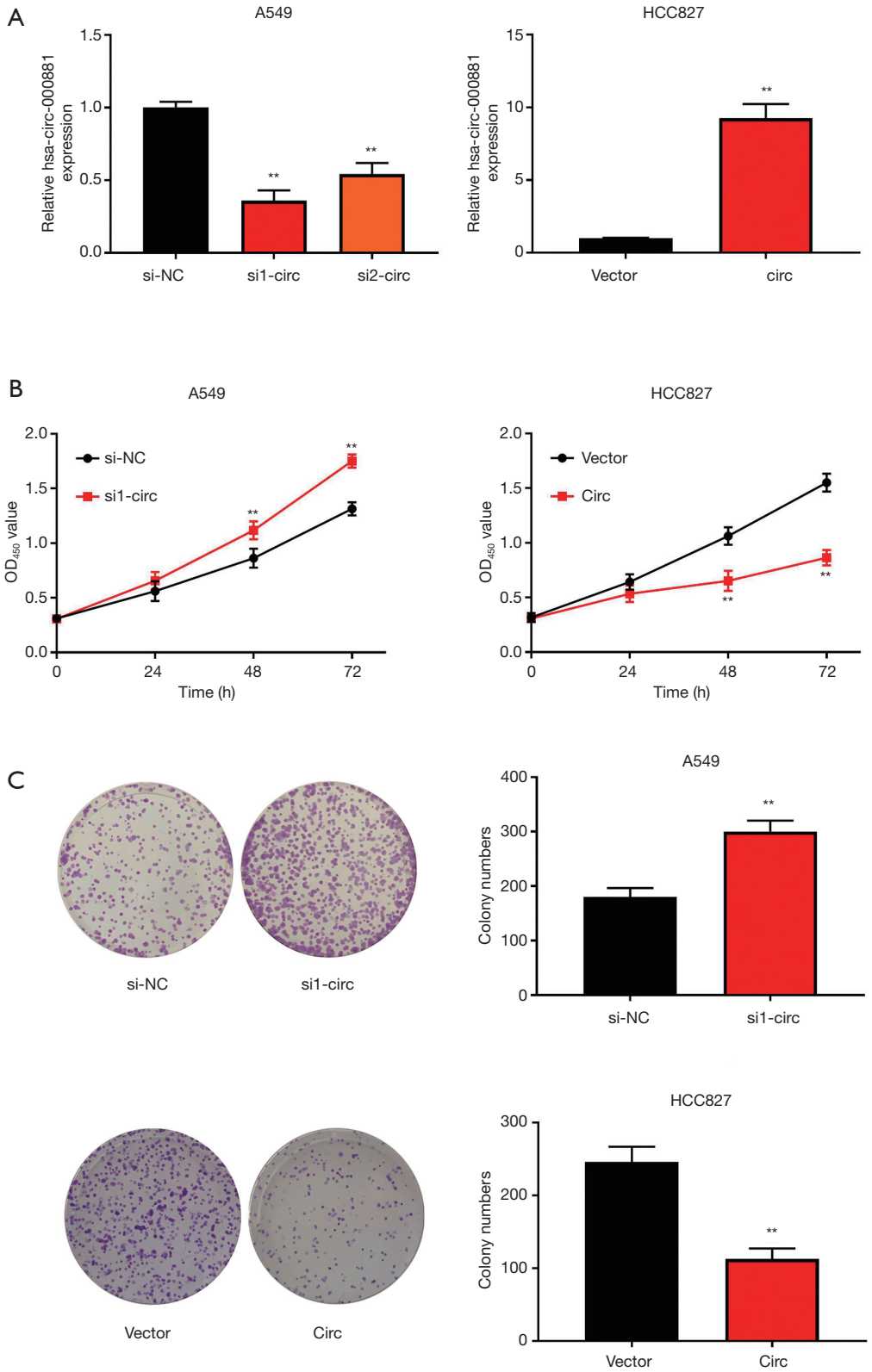


Figure 1 Circ-000881 is downregulated in lung adenocarcinoma (LUAD) tissues and cells. (A) The Cancer Genome Atlas (TCGA) analysis showed tumor tissues to have lower circ-000881 expression than normal tissues. (B) RT-qPCR was used to measure circ-000881 expression in 40 pairs of LUAD tissues and para-carcinoma tissues. (C) RT-qPCR was used to measure circ-000881 expression in human bronchial epithelial cells (HBE) and non-small cell lung cancer (NSCLC) cells (HCC827, H23, A549, H1975, and H1299). **P<0.01 *vs.* Normal or HBE group.

CDC25A, PRELP, PRICKLE2, KCNN3, and TGFBR2 might be potential targets of miR-665 (*Figure 4A*). Among these genes, PRICKLE2 has rarely been the focus of studies on cancer, which intrigued us enormously. Therefore, we immediately investigated the potential interaction between miR-665 and PRICKLE2. *Figure 4B* shows the potential binding sites of miR-665 and PRICKLE2. The luciferase reporter assay showed that miR-665 mimic reduced the luciferase activity of the PRICKLE2 wild-type luciferase reporter plasmids in A549 and HCC817 cells (P<0.01, *Figure 4C*), but had no significant effect on the mutated luciferase reporter plasmids, indicating that miR-665 could bind to PRICKLE2. This observation indicated that miR-665 could bind to PRICKLE2. Furthermore, analysis of the expression of PRICKLE2 in LUAD was carried out using data from the TCGA database. PRICKLE2 had a low expression in LUAD tissues, and patients with higher expression of PRICKLE2 displayed longer overall survival (*Figures 4D,E*), which suggested that PRICKLE2 might play a tumor-inhibitor role. PRICKLE2 expression was significantly decreased in LUAD tissues (P<0.01, *Figure 4F*). Furthermore, the expression of PRICKLE2 was negatively correlated with miR-665 in LUAD tissues (P<0.0001, *Figure 4G*). From these observations, it is reasonable to draw the conclusion that miR-665 can bind to PRICKLE2 directly in LUAD.



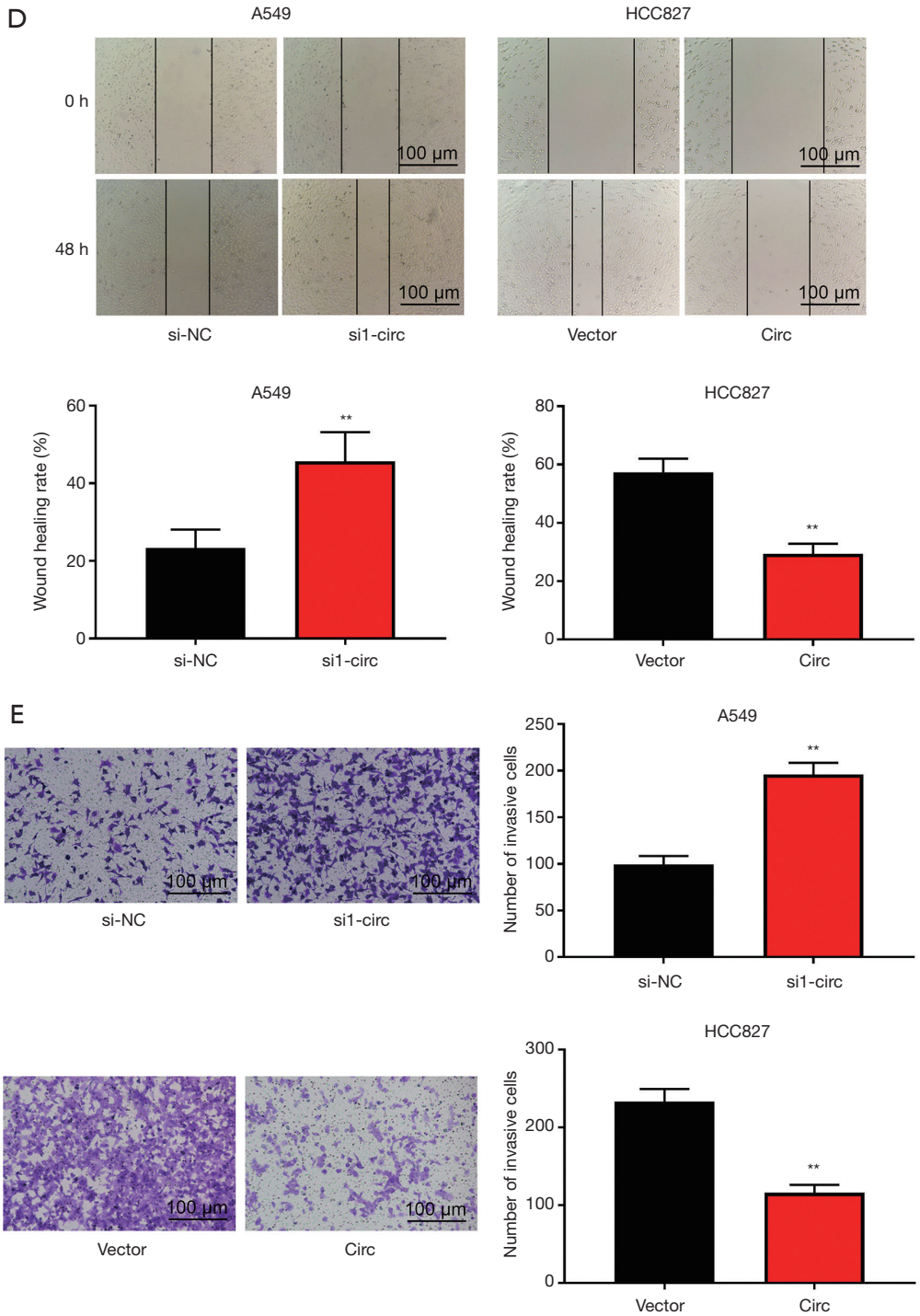


Figure 2 Circ-000881 plays an inhibitory role in A549 and HCC827 cells. (A) Reverse transcription and quantitative polymerase chain reaction (RT-qPCR) was used to measure circ-000881 expression (B) Cell counting Kit-8 assays were performed to assess cell viability. (C) Cell proliferation properties were detected in A549 and HCC827 cells after transfection with si-cric-000881 or si-NC or overexpression plasmid or vector. (D) Wound-healing assays were carried out to investigate the migration capabilities of circ-000881 knockdown A549 cells and circ-000881 overexpression HCC827 cells. (E) Cell invasion ability was examined by conducting Transwell assays. Cells were fixed and stained with 0.1% crystal violet. **P<0.01 *vs.* si-NC or vector group.

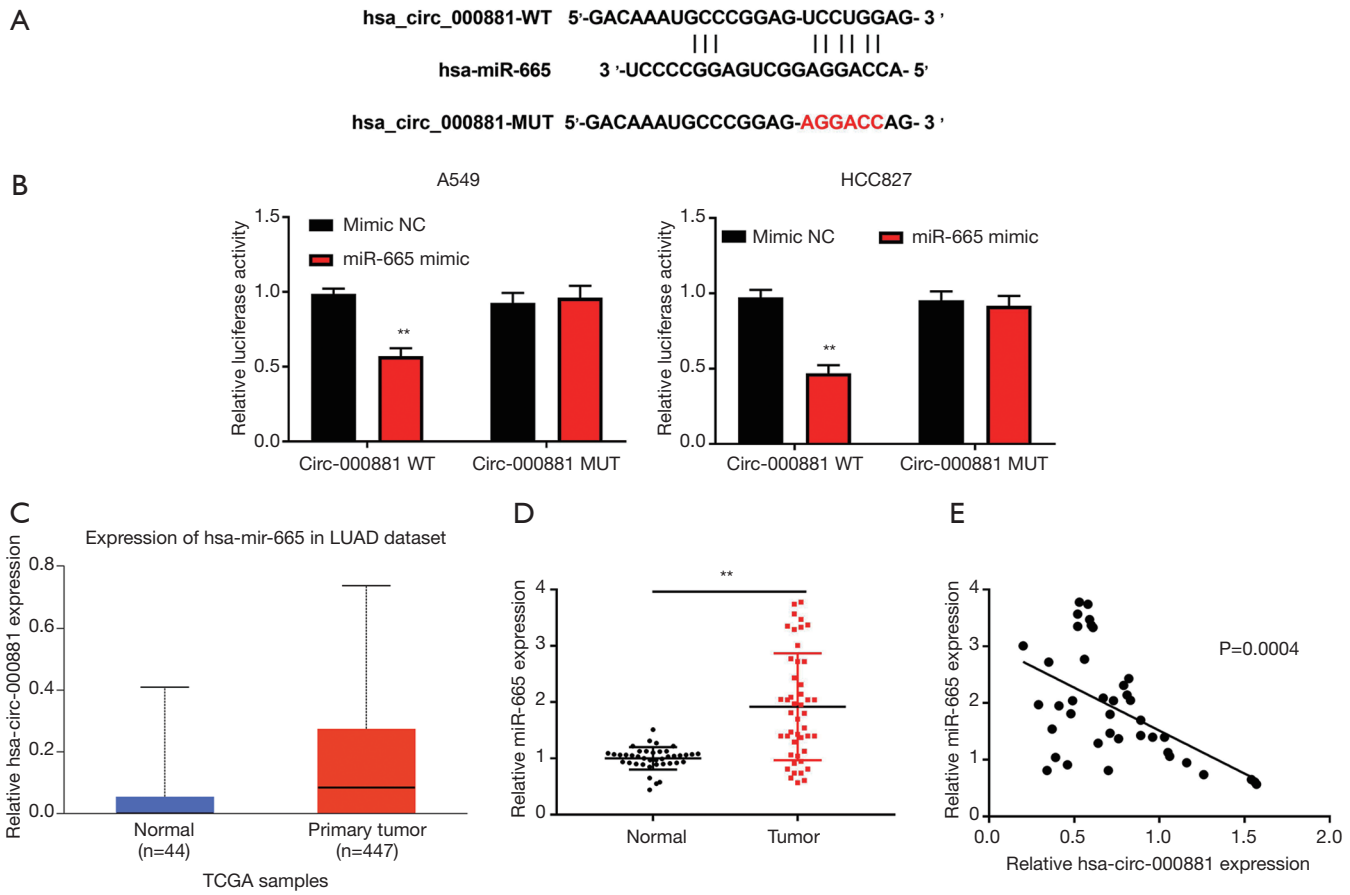


Figure 3 Circ-000881 serves as a sponge for miR-665 in lung adenocarcinoma (LUAD). (A) Prediction of possible binding sites between circ-000881 and miR-665 by TargetScan software. (B) The interaction between miR-665 and circ-000881 in A549 and HCC817 cells was analyzed by luciferase reporter assay. (C) Analysis of miR-665 expression in LUAD patients using the The Cancer Genome Atlas (TCGA) database. (D) Expression of miR-665 in 40 pairs of clinical specimens was measured by (RT-qPCR). (E) MiR-665 shows a negative correlation with circ-000881 in LUAD tissues. **P<0.01 *vs.* Normal or circ-000881-MUT group.

Circ-000881 exerted inhibitory effects on lung cancer cells by regulating a miR-665/PRICKLE2 axis

To investigate whether a circ-000881/miR-665/PRICKLE2 regulatory pathway is present in LUAD, we performed a series of assays. The expression of PRICKLE2 in HCC827 cells increased with circ-000881 overexpression, while silencing of circ-000881 decreased PRICKLE2 expression in A549 cells (P<0.01, *Figure 5A*). We also investigated the effects of circ-000881, miR-665, and PRICKLE2 on the cell proliferation, migration, and invasion abilities of HCC827 cells. We found that overexpression of miR-665 could abolish circ-000881-mediated inhibition of cell malignant biological behavior (*Figure 5B-D*). Similarly, silencing of PRICKLE2 also abolished circ-000881-mediated inhibition of cell proliferation, migration, and invasion (*Figure 5B-D*). Taken together, these results revealed that circ-000881 suppressed LUAD progression by regulating a miR-665/PRICKLE2 axis.

Discussion

With the development of personalized medicine, great advances have been made in the understanding of the carcinogenesis and treatment of NSCLC. However, the clinical outcome for patients is still unsatisfactory due to the lack of specific and efficient early diagnostic approaches (18). LUAD is one of the most common types of NSCLC, especially in women (19). Therefore, mechanistic research of the occurrence and development of LUAD is of great importance for developing new therapeutic strategies for the disease.

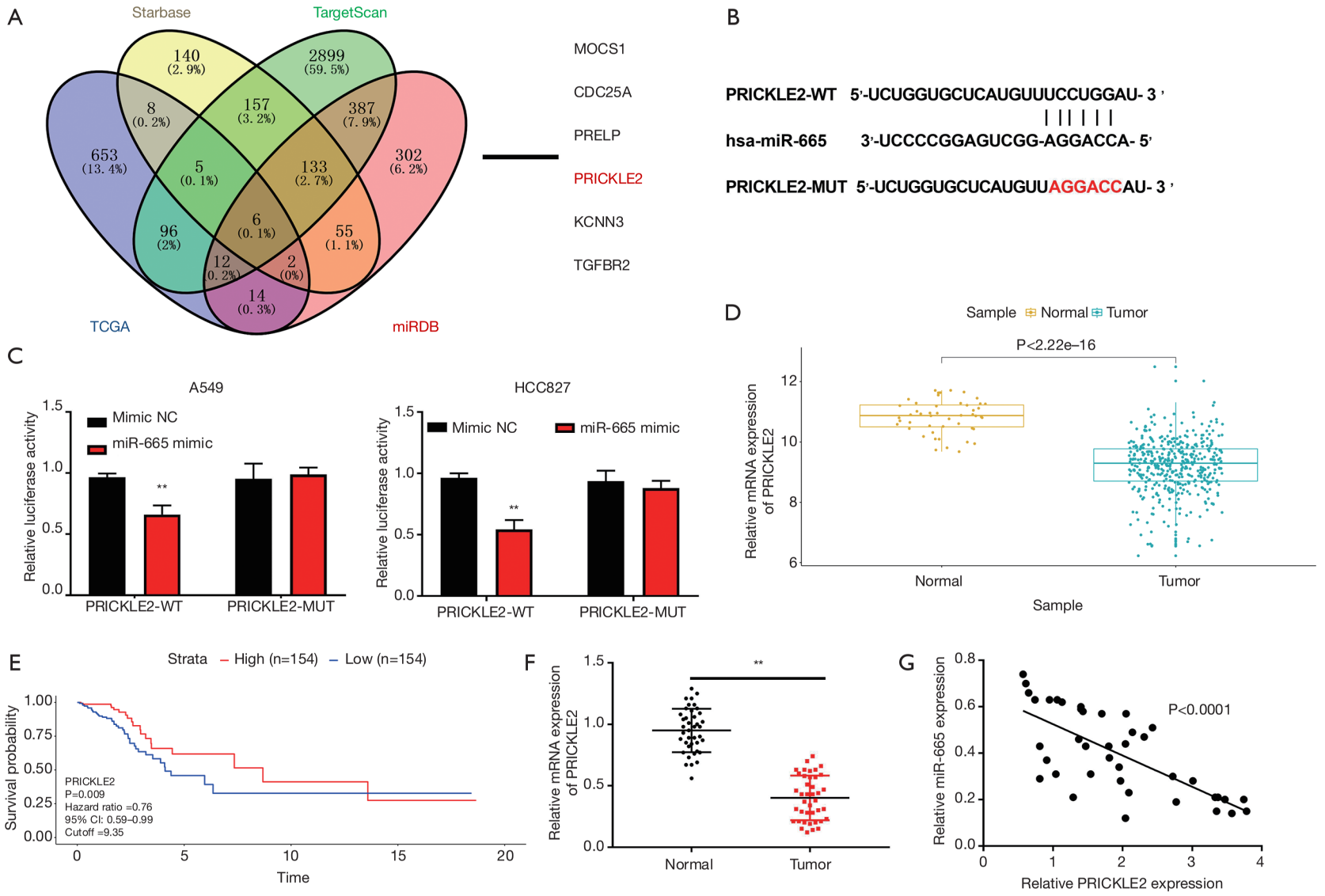


Figure 4 PRICKLE2 directly interacts with miR-665 in lung adenocarcinoma (LUAD). (A) Possibly target genes of miR-665 were predicted by using bioinformatics tools [Starbase, The Cancer Genome Atlas (TCGA), TargetScan and miRDB]. (B) Prediction of possible binding sites between PRICKLE2 and miR-665. (C) MiR-665 directly interacts with PRICKLE2 in A549 cells and HCC817 cells. (D) Analysis of PRICKLE2 expression in LUAD patients by TCGA database. (E) Results of TCGA analysis showing patients with low PRICKLE2 expression to have better survival than patients with high PRICKLE2 expression. (F) The levels of PRICKLE2 mRNA expression in 40 couples of LUAD tissues and para-carcinoma tissues were measured. (G) A negative correlation between the expressions of miR-665 and PRICKLE2 in LUAD tissues is shown. **P<0.01 *vs.* Normal or PRICKLE2-MUT group.

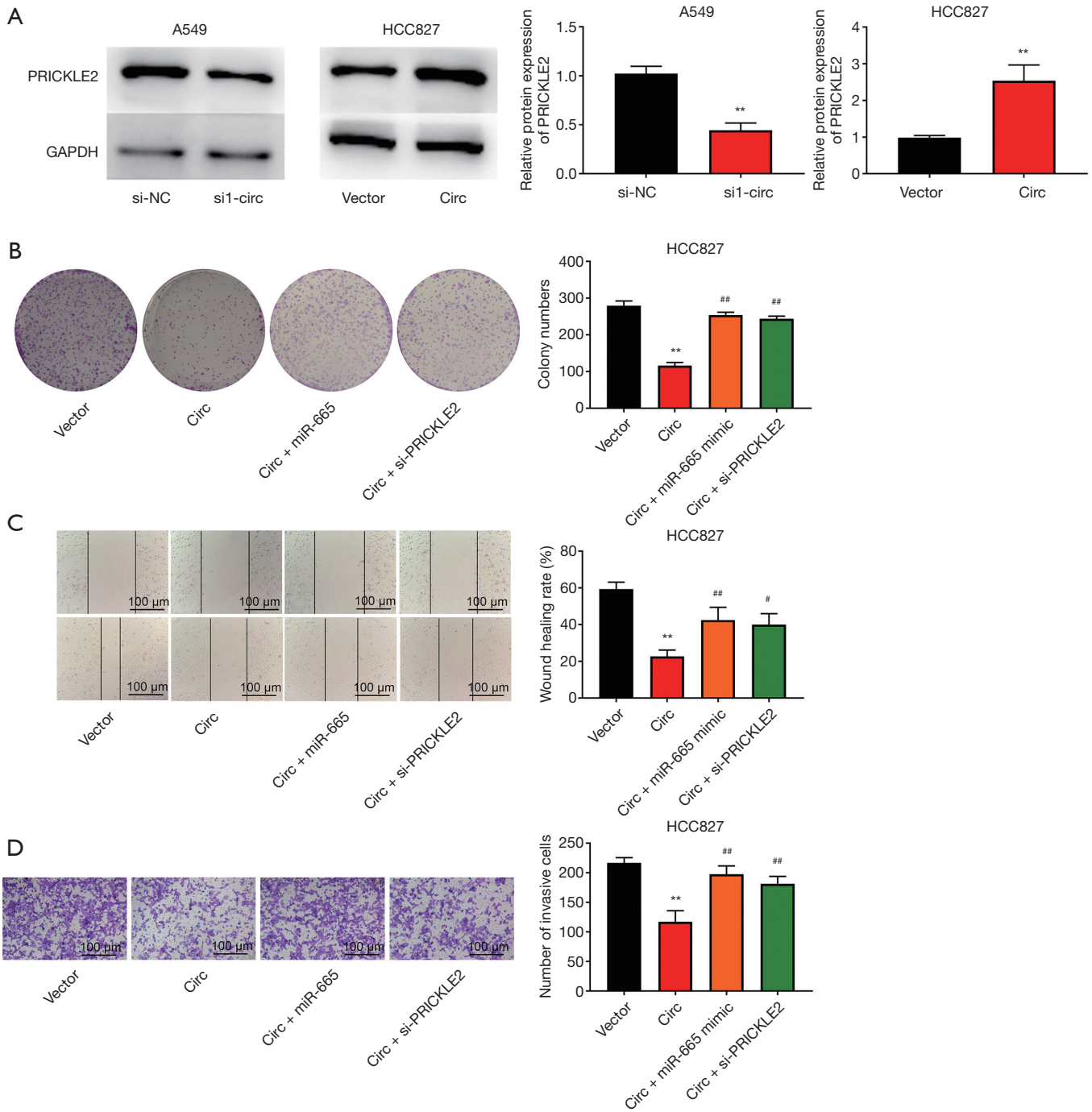


Figure 5 Circ-000881 inhibits the proliferation, migration, and invasion of lung cancer cells via regulating the miR-665/PRICKLE2 axis. (A) Expression of PRICKLE2 at the protein level was detected in HCC827 and A549 cells. (B) Colony formation assay was conducted to examine the proliferation of HCC827 cells. (C) Wound-healing assay was used to detect the migration ability of HCC827 cells. (D) Transwell assay was used to detect the invasion ability of HCC827 cells. Cells were fixed and stained with 0.1% crystal violet. **P<0.01 *vs.* si-NC or vector group, #P<0.05 and ##P<0.01 *vs.* circ group.

In recent years, circRNAs have been widely researched. The dysregulation of circRNAs has been revealed to lead to the promotion of oncogenesis and progression in several cancers (20-22). We already know that some circRNAs are dysregulated in LUAD, but the understanding of roles of circRNAs in cancer progression is far from sufficient. For this reason, we investigated the influence of circ-000881 on the development of LUAD and attempted to gain an understanding of the pathway and molecular mechanism of its action. Firstly, significant downregulation of circ-000881 expression in LUAD tissues and cells was observed. Subsequent functional cellular experiments revealed that overexpression of circ-000881 diminished the migratory, proliferative, and invasive abilities of HCC827 cells, whereas silencing of circ-000881 accelerated the aggressive malignant biological behavior of A549 cells. These observations confirmed the certain participation of circ-0000881 in the progression of LUAD.

Many studies have proved that circRNAs regulate gene expression by sponging miRNAs and play an important part in cancer development (23). For instance, circ-0000515 was recognized to be a tumor promoter in cervical cancer through its sponging of miR-326 (24). Furthermore, Yao *et al.* identified that circ-LDLRAD3 enhanced the malignant characteristics of pancreatic cancer cells, including their proliferative and migratory abilities, through sponging miR-137-3p (25). During our study of tumor inhibition by circ-000881, miR-665 greatly aroused our interest because of its potential binding site with circ-000881, which was revealed by bioinformatics analysis. Many studies have demonstrated miR-665 to be abnormally expressed in tumors such as ovarian cancer (26), gastric adenocarcinoma (27) and hepatocellular carcinoma (28). Liu *et al.* also found that miR-665 expression was upregulated in small cell lung cancer tissues (29). Xia *et al.* demonstrated miR-665 up-regulation in NSCLC tissues and cells, with the overexpression of miR-665 promoting cell proliferation, migration, and invasion (30). Likewise, we also found that miR-665 expression was significantly upregulated in LUAD. Furthermore, overexpression of miR-665 abolished the circ-000881-mediated inhibition of lung cancer cell proliferation, migration, and invasion. Taking the above results into consideration, we can reasonably conclude that circ-000881 suppresses the progression of LUAD by sponging and inhibiting miR-665.

By combining with the 3'-untranslated regions of their downstream target genes, miRNAs can regulate gene expression (2). Among the potential target genes of miR-665, PRICKLE2 has not been thoroughly studied. The PRICKLE2 gene is located on human chromosome 3p14 and is expressed in the testes, eyes, and brain. It exerts a variety of functions such as neurite formation, tumor formation, and metastasis (31). Senchenko *et al.* reported that PRICKLE2 shows methylation/deletion in 22% of cervical adenocarcinomas and 49% of squamous cell carcinomas, and has tumor suppression functions, which is promising for the discrimination of these diseases (32). Rudenko *et al.* found that PRICKLE2 downregulation was associated with the malignant biological behavior of clear-cell renal cell carcinoma (33). Consistent with these reports, we discovered that PRICKLE2 expression was downregulated in LUAD tissues. Also, we found that silencing of PRICKLE2 abolished circ-000881-mediated inhibition of cell proliferation, migration, and invasion. Taken together, the findings of this study reveal the involvement of a circ-000881/miR-665/PRICKLE2 network in LUAD.

Conclusions

Circ-000881 expression is significantly downregulated in LUAD tissues and cells. Circ-000881 plays an inhibitory role in the proliferation, invasion, and migration of LUAD cells via the miR-665/PRICKLE2 pathway. Our findings may be useful in identifying a potential therapeutic target for LUAD.

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Footnote

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Data Sharing Statement: Available at http://dx.doi.org/10.21037/atm-21-844

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at http://dx.doi.org/10.21037/atm-21-844). The authors have no conflicts of interest to declare.

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. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). Ethics approval for this study was acquired from the Medical Ethics Committee of Qilu hospital, and informed consent was obtained from all participants.

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