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Detecting the Regulatory Mechanism of circ-CCND1 in Lung Adenocarcinoma

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Abstract

Numerous circular RNAs (circRNAs) have been investigated in human malignancies, including lung adenocarcinoma. Previous investigations reported that circ-CCND1 (CCND1, deubiquitinylation of the G1 cyclin D1) functions in cancer progression, but its role as well as its regulatory mechanism remain unclear. The present study therefore aimed to identify the role of circ-CCND1 in lung adenocarcinoma progression. The circ-CCND1 expression and characteristics were separately analyzed using qRT-PCR. We used the CCK-8 assay, colony formation assay, western blotting, Transwell assay, and the luciferase reporter assay to characterize tumor growth and metastasis in vivo. The results demonstrated that circ-CCND1 expression increased in lung adenocarcinoma cell lines, and downregulated circ-CCND1-suppressed cell proliferation and migration. Bioinformatics analyses suggested that miR-503-5p and Sirtuin 5 (SIRT5) were circ-CCND1 downstream targets. The miR-503-5p downregulation or SIRT5 overexpression reversed migration and proliferation after knockdown of circ-CCND1. In vivo tests confirmed that downregulation of circ-CCND1 suppressed tumor formation and metastasis in nude mouse xenografts. In addition, western blotting showed that knockdown of circ-CCND1 suppressed the SIRT5-mediated, autophagy related proteins, Beclin-1 and light chain 3 (LC3) expressions. Taken together, this study showed that circ-CCND1 downregulation suppressed proliferation and migration through miR-503-5p/SIRT5-mediated autophagy regulation by sponging.

Keywords: Lung Adenocarcinoma; circ-CCND1; miR-503-5p; SIRT5; Autophagy

Introduction

Lung Cancer (LC) is a major cause of worldwide death. Approximately 85% LCs involve Non-Small Cell Lung Cancer (NSCLC), which include lung adenocarcinoma and lung squamous. Though there have been advances in early detection and standard treatments, NSCLC is usually diagnosed in the advanced stage, which has a poor prognosis [1]. Metastases accounts for 90% of human cancer related deaths [2]. Chemotherapy failure is the main reason for recurrences and poor prognoses in many cancers such as colorectal cancer [3], gastric cancer [4], liver cancer [5], and NSCLC [6], and thus, the identification of regulatory mechanisms and a search for therapeutic targets are important.

Autophagy is a lysosomal degradation pathway, which maintains intracellular homeostasis, and which is evolutionarily conserved in baseline conditions and adaptive responses to stress, by removing damaged organelles as well as protein aggregates [7]. Autophagy functions both negatively and positively in cancer therapy [8, 9], and thus, it is imperative to identify the autophagy roles in NSCLC therapies. A previous study found that target autophagy-related genes (LC3 and Beclin-1) may have a protective role in suppressing the progression of NSCLC [10].

It has been reported that circular RNA (circRNA) functions in the regulation of many diseases [11]. CircRNA belongs to a newly identified class of endogenous non-coding RNAs (ncRNAs), which are characterized by having head-to-tail bonds between exons formed by back splicing [12, 13]. CircRNAs have been detected in various cancer tissues using high-throughput sequencing technology and bioinformatics methods [14]. Previous studies have found that circ-CCND1 enhances laryngeal squamous cell carcinoma proliferation by interacting with miR-646 and HuR [15]. But if circ-CCND1 played a role in the progress of lung cancer still unknown. Thus, the aimed of this study was to identify the role of circ-CCND1 and its potential regulatory mechanism during lung cancer.

Materials and Methods

Ethics statement

Four-week-old BALB/c nude mice weighing 15-20 g (SLARC, Shanghai, China) were used. All animal experiments were approved by the Ethics Committee of our Hospital. All surgery and euthanasia were performed under sodium pentobarbital anesthesia (50 mg/kg) by intraperitoneal injection, and all efforts were made to minimize suffering. All mice were sacrificed by using the CO_2 asphyxia method after experiment. (The air displacement rate of CO_2 was 20% of container volume per minute).

Cell line and culture

We obtained the normal human lung epithelial cell line, BEAS-2B, and the NSCLC cell lines, A549, PC9, and H1650, from Cell Bank of the Chinese Academy of Sciences, and cultured them in Dulbecco's Modified Eagle's Medium (Life Technologies, Carlsbad, CA, USA) supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin,

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and 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified atmosphere with 5% CO_2.

Bioinformatics analyses

The circRNA/miRNA target genes were predicted using the web-based tool, Circular RNA Interactome. We predicted the interactive correlation between miR-503-5p and SIRT5 using http://www.targetscan.org/ .

RNA interference and overexpression

We purchased miR-503-5p inhibitors (5'-CUGCAGAACUGUUCCCGCUGCUA-3'), miR-503-5p inhibitor negative control (5'-UUGUACUACACAAAAGUACUG-3'), miR-503-5p mimics (5'-GGGGUAUUGUUUCCGCUGCCAGG-3'), miR-503-5p mimics NC (5'-TGACTGTACTGACTCGACTG-3'), siRNA against circ-CCND1 (5'-CCAGAAGGGAAAGCUUCAUUU-3'), si-circ-CCND1 negative control (NC) (5'-UUCUCCGAACGUGUCACGUTT-3') and SIRT5 overexpression vector (cDNA sequence were insert into cDNA3.1 vector, empty cDNA3.1 vector was used for NC) were from GenePharma (Shanghai, China). Transfections were performed following the supplier's protocols. In brief, we transferred cells in 6-well culture plates and transfected them using Lipofectamine 2000 (Invitrogen). For xenograft experiments, a lentiviral-mediated circ-CCND1-silencing (sh-circ-CCND1) A549 cell line was constructed. Brief description, oligonucleotides specific for short hairpin RNAs (shRNAs) against circ-CCND1 (sh-circ-CCND1) (5'-CCAGAAGGGAAAGCUUCAUUU-3') or negative control (sh-NC) (5'-UUCUCCGAACGUGUCACGUTT-3') were inserted into GV298 (Genechem Co., Ltd). Lentiviral vectors were co-transfected with packaging plasmids psPAX2 and pMD2G into HEK293T cells. Infectious lentiviruses were harvested at 36 and 60 h after transfection, followed with concentration by ultracentrifugation (2 h at 120,000 g). Stable cell lines were obtained by selection with puromycin.

Quantitative real-time polymerase chain reaction (qRT-PCR)

We isolated total RNA using the TRIzol reagent (Invitrogen), and performed relative RNA expression quantitation using SYBR Premix EX Taq (Takara, Shiga, Japan) and $2^{-\Delta\Delta Ct}$ method. We utilized actin and U6 as internal references for circRNA/mRNA and miRNA, respectively. The primers utilized to assay hsa_circ_0023305 (circ-CCND1) expression included forward, 5'-TTCCCAGCACCAACATGTAA-3' and reverse, 5'-AGGAAGCGGTCCAGGTAGTT-3'. The SIRT5 primers were forward, 5'-AGAGAGCTCGCCCACTGTGATTTA-3' and reverse, 5'-AGGGTCCCTGGAAATGAAACCTGA-3'. The miR-503-5p primers were forward, 5'-CCTATTTCCCATGATTCCTTCATA-3' and reverse, 5'-GTAATACGGTTATCCACGCG-3'. The U6 primers were forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'. The actin primers were forward, 5'-GACCTGACTGACTACCTCATGAAGAT-3' and reverse, 5'-GTCACACTTCATGATGGAGTTGAAGG-3'.

Dual-luciferase reporter assay

The circ-CCND1 binding site and the SIRT5 3'-UTR, termed circ-CCND1-WT, circ-CCND1-Mut, SIRT5-3'UTR WT, and SIRT5-3'UTR-Mut, were inserted into the pGL3 promoter vector KpnI and HindIII sites (Realgene, Nanjing, China) using the dual-luciferase reporter assay. We transferred cells into 24-well plates and transfected 80 ng plasmid, 5 ng Renilla luciferase vector pRL-SV40, 50 nM miR-503-5p mimics, and negative control into cells using Lipofectamine 2000 (Invitrogen). We collected the cells and analyzed them 2 d after transfection using the Dual-Luciferase Assay (Promega, Madison, WI, USA), following the manufacturer's instructions. We independently repeated the experiments three times.

CCK-8 assay

We performed NSCLC cell proliferation assays using the CCK-8 assay kit (Dojindo, Japan), following standard procedures. We seeded A549 cells in 96-well plates at a density of 1 × 103 cells/well. We added cells to 10 μL CCK-8 solution at 37°C for 1 h and incubated them at 37°C, then measured the absorbance at 450 nm. We repeated all experiments three times.

Cell migration assay

We assayed cell migration in 24-well Transwell chambers with 8 μm pore size membranes (BD Biosciences, Franklin Lakes, NJ, USA). We added 1 × 105 cells in 200 μL serum-free medium to the upper chamber. We then filled the lower chamber with 500 μL complete medium as a chemo attractant. We fixed cells that invaded the lower chamber after 1 d with 4% paraformaldehyde for 0.5 h and stained them with Crystal Violet for 10 min.

Western blot assay

We extracted protein from cells or tissues with RIPA lysis buffer and performed western blot assays following a previous protocol [16]. We obtained anti-Beclin-1 (#3738, 1:1,000), anti-LC3 (#12741T, 1:1,000), and anti-actin (#8H10D10, 1:1,000) primary antibodies from Cell Signaling Technology (Beverly, MA, USA) and stained protein blots following the manufacturer's instructions. We visualized immunoreactivity using a chemiluminescence detection kit (ECL; Western Blotting Substrate, Donghuan Biotech, Dongguan, China).

Tumor xenograft formation and metastasis assays

We injected 1×10^7 viable cells from wild-type or si-circ-CCND1 A549 cells into the right flanks of nude mice. We detected tumor sizes every 5 d using Vernier caliper, and calculated the volume using the formula: $0.5 \times \text{length} \times \text{width}^2$.

For metastasis analysis, we transfected wild-type and si-circ-CCND1 A549 cells (2 × 105) with luciferase expression vectors, and intravenously injected the cells into mice tails. After 30 d, A549 cell metastasis was analyzed by bioluminescence imaging following an intravenous injection of luciferin (150 mg luciferin/kg body weight) into the tails.

Statistical analyses

We analyzed the data using Prism software (GraphPad, La Jolla, CA, USA). The Student's t-tests was unpairedwhich used to determine statistical significance in two groups and one-way ANOVA with the post hoc Bonferroni test was used for three or more groups. We expressed the results as the mean ± Standard Deviation (SD).$P < 0.05$ indicated statistical significance.

Results

The circ-CCND1 expression was increased in NSCLC cells lines, and downregulated circ-CCND1 suppressed cell proliferation and migration

The present study of qRT-PCR detection show that circ-CCND1 expression also increased in NSCLC cells lines when compared with normal lung epithelial cells (BEAS-2B). In addition, using qRT-PCR, A549 cells had a higher level of circ-CCND1 expression (Figure 1A). A549 cells were therefore used for the following study. Bioinformatics

analysis showed that circ-CCND1 (hsa_circ_0023305) was derived from the CCND1 gene, whose mature spliced sequence length was 3,882 bp. The gene was located on chromosome 11: 69457798-69469242. The qRT-PCR detection verified that circ-CCND1 expression decreased after transfection with siRNA against circ-CCND1, when compared with the Negative Control (NC) (Figure 1B). The CCK8 (Figure 1C) and cloning formation assays (Figure 1D and 1E) showed that circ-CCND1 silencing suppressed cell proliferation in A549 cells. The Transwell assay for migration analysis showed that circ-CCND1 downregulation suppressed A549 cell migration (Figure 1F and 1G), suggesting that circ-CCND1 functioned in lung adenocarcinoma progression, and that down regulation of circ-CCND1 decreased cell migration and proliferation.

The miR-503-5p and SIRT5 were circ-CCND1 downstream targets

Bioinformatics analyses suggested that miR-503-5p was the circ-CCND1 downstream target (Figure 2A) and miR-503-5p inhibited luciferase activity in WT cell lines (Figure 2B). Bioinformatics analysis also showed that SIRT5 was a miR-503-5p target, indicating that miR-503-5p interacted directly with the SIRT5 3'-UTR to suppress its mRNA expression (Figure 2C). In the luciferase reporter assay, miR-503-5p inhibited luciferase activity in WT, but not mutant (MUT) cell lines (Figure. 2D). The qRT-PCR detection showed that miR-503-5p expression was inhibit after treatment with inhibitor (Figure. 2E). The result also show that SIRT5 expression was increased after transfected with SIRT5 overexpression vector (Figure 2F). The qRT-PCR showed that circ-CCND1 silenced miR-503-5p expression promotion and decreased SIRT5, LC3, and Beclin-1 expressions. The miR-503-5p downregulation suppressed miR-503-5p expression, but recovered SIRT5, LC3, and Beclin-1 expression levels after circ-CCND1 silencing. However, the miR-503-5p silencing or SIRT5 overexpression did not reverse circ-CCND1 levels (Figure 2G-K). The combined results indicated that miR-503-5p and SIRT5 were circ-CCND1 downstream targets, and that circ-CCND1 silencing inhibited lung adenocarcinoma cell migration and proliferation through targeting the miR-503-5p/SIRT5 axis.

The miR-503-5p downregulation or SIRT5 overexpression reversed the migration and proliferation abilities after circ-CCND1 knockdown

In this study, we treated A549 cells with miR-503-5p, SIRT5, or circ-CCND1, to determine the effects on cell migration and proliferation. A549 cells transfected with si-circ-CCND1 and miR-503-5p were inhibited, and single or combined SIRT5 overexpression was also detected using the Transwell assay. The cloning formation assay showed that circ-CCND1 silencing suppressed cell proliferation, but miR-503-5p downregulation or SIRT5 overexpression reversed A549 cell proliferation (Figure 3A and 3B). The Transwell assay also showed that miR-503-5p silencing or SIRT5 overexpression reversed cell migration after knockdown of circ-CCND1 (Figure 3C and 3D). Western blotting showed that circ-CCND1 silencing suppressed autophagy of LC3-II/LC3-I and Beclin-1 expression. The miR-503-5p silencing or SIRT5 overexpression reversed the relative autophagy protein expressions after circ-CCND1 silencing (Figure 3E-G). In addition, downregulation by miR-503-5p or SIRT5 overexpression

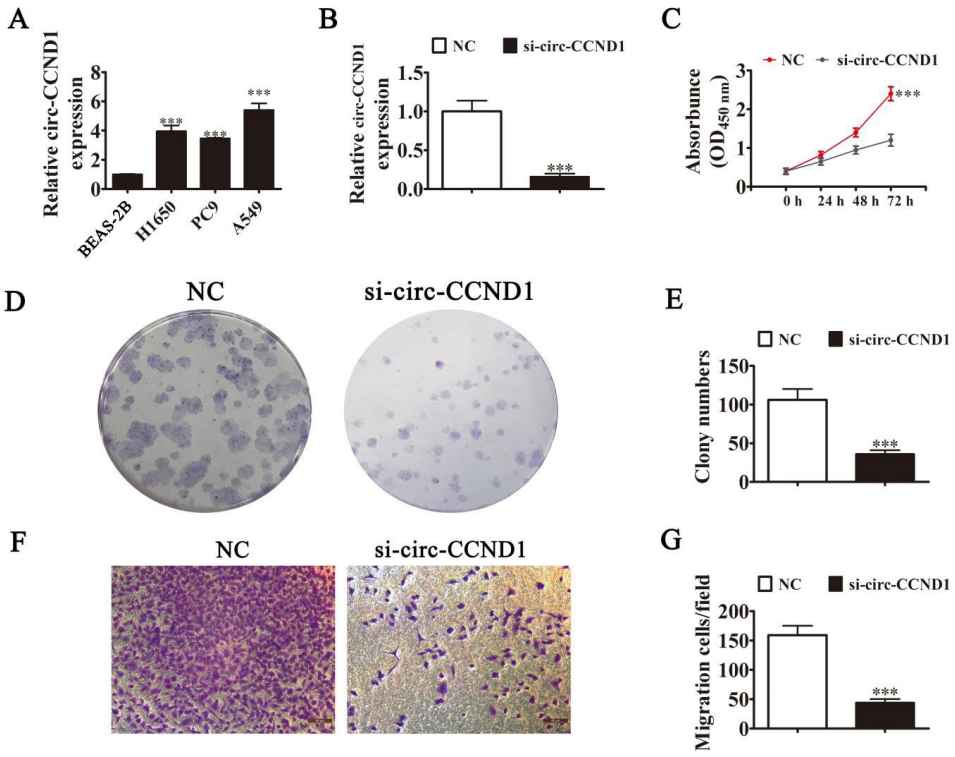

Figure 1: The circ-CCND1 expression was increased in non-small cell lung cancer cell lines, and downregulation of circ-CCND1 suppressed cell proliferation and migration. (A) The qRT-PCR showed the expression of circ-CCND1 in A549, PC9, H1650, and normal lung epithelial cells (BEAS-2B). Data are expressed as the mean ± SD. ***P < 0.001 vs. BEAS-2B. (B) The qRT-PCR showed the expression of circ-CCND1 in A549 cells after transfection with siRNA against circ-CCND1 (si-circ-CCND1) or thesi-circ-CCND1 negative control (NC). Data are expressed as the mean ± SD. ***P < 0.001 vs. the NC. (C) CCK8 detection showed the proliferation ability of A549 cells between si-circ-CCND1 and si-circ-CCND1-NC. Data are expressed as the mean ± SD. ***P < 0.001 vs. the NC. (D and E) The colony formation assay showed proliferation in A549 cells between si-circ-CCND1 and si-circ-CCND1-NC. Data are expressed as the mean ± SD; ***P<0.001 vs. NC. (F and G) Cell migration and invasion were assessed in A549 cells between si-circ-CCND1 and si-circ-CCND1-NC using Transwell assays. Data are expressed as the mean ± SD. ***P < 0.001 vs. the NC.

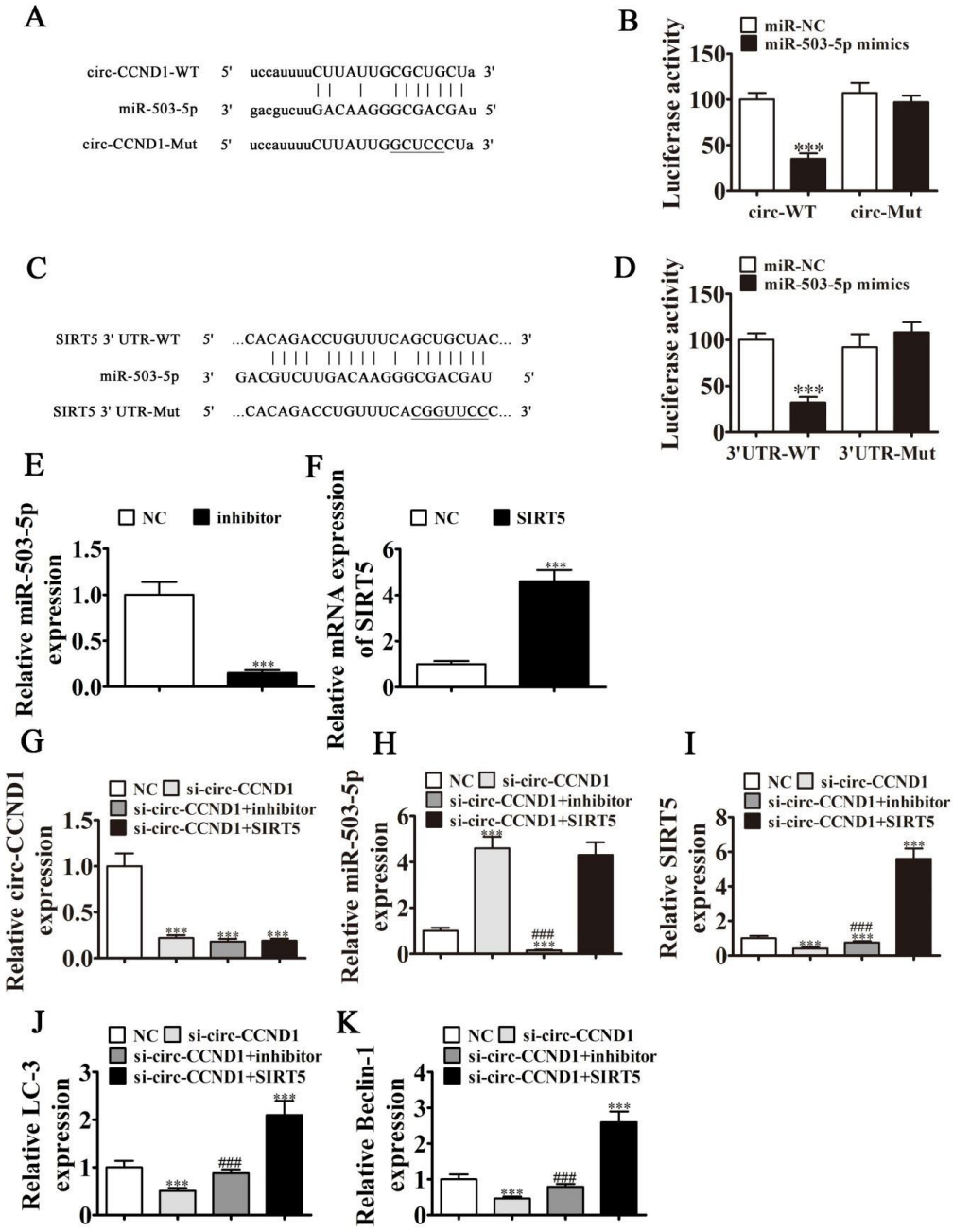

Figure 2: The miR-503-5p and SIRT5 were downstream targets of circ-CCND1. (A) The predicted binding sites of miR-503-5p in circ-CCND1. The Mutated (Mut) version of circ-CCND1 is also shown. (B) The relative luciferase activity was determined 48 h after transfection with the miR-503-5p mimic/mimic negative control (NC) or with the circ-CCND1 wild-type/Mut in HEK293T cells. Data are expressed as the mean ± SD; ***P < 0.001. (C) The predicted binding sites of miR-503-5p within the 3'-UTR of SIRT5. The mutated version of the 3'-UTR of SIRT5 is also shown. (D) The relative luciferase activity was determined 48 h after transfection with the miR-503-5p mimic/mimic NC or with the 3'-UTR-SIRT5 wild-type/Mut in HEK293T cells. Data are expressed as the mean ± SD; ***P < 0.001. (E and F) qRT-PCR detection show the expression of miR-503-5p (A) and SIRT5 (B). Data are expressed as the mean ± SD; ***P < 0.001 vs. the NC. (G-K) The qRT-PCR detection showed the expression of circ-CCND1 (G), miR-503-5p (H), SIRT5 (I), LC3 (J), and Beclin-1 (K) in A549 cells after transfection with si-circ-CCND1, and the miR-503-5p vector alone or combined with inhibited SIRT5 overexpression. si-circ-CCND1 control was used for NC. Data are expressed as the mean ± SD. ***P < 0.001 vs. the NC. ###P < 0.001 vs. si-circ-CCND1.

reversed migration and proliferation after knockdown of circ-CCND1, by regulation of autophagy.

Downregulation of circ-CCND1 suppressed tumor formation and metastasis in nude mouse xenografts

The circ-CCND1 interference lentiviral A549 cell line (lentiviral A549stable cell lines were obtained by selection with puromycin. MOI=4.6 pfu/cell) was used for tumor formation analyses in nude mice xenografts. After 5 days, we measured the tumor volume using Vernier calipers. The results showed that circ-CCND1 knockdown suppressed tumor growth, including the tumor weight and volume (Figure 4A-C). The qRT-PCR assay confirmed that miR-503-5p expression in tumor tissues significantly increased in circ-CCND1-silenced group (Figure 4D). However, circ-CCND1 silencing decreased SIRT5 expression (Figure 4E). Western blot analysis also showed that knockdown of the circ-CCND1 suppressed the autophagy-related

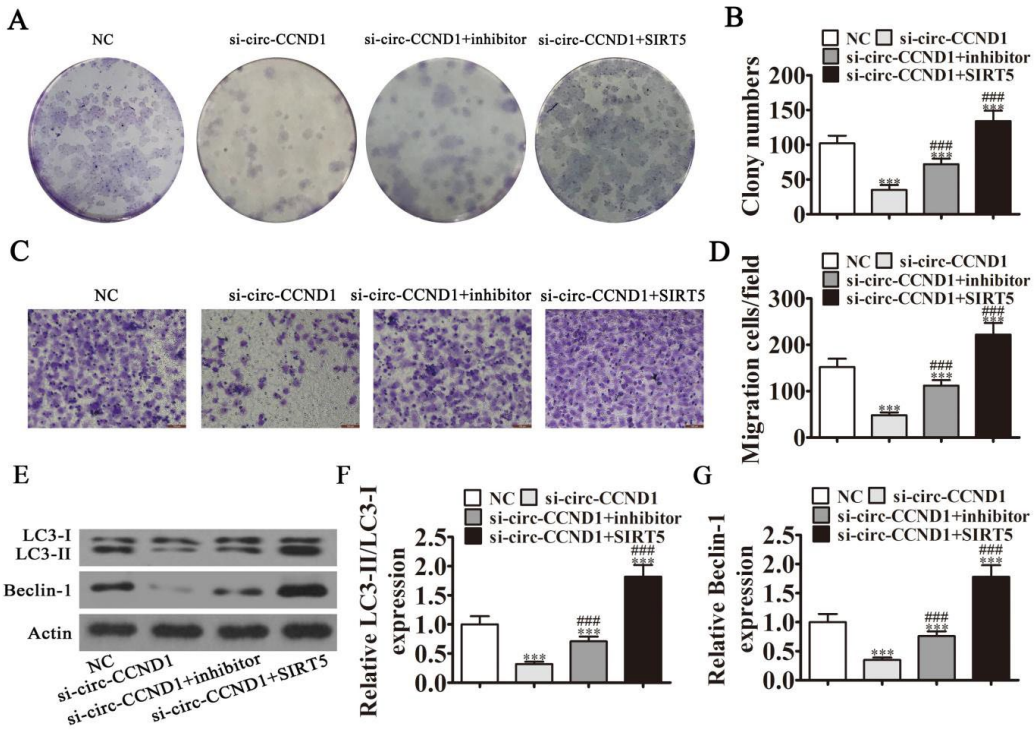

Figure 3: Downregulation of miR-503-5p or SIRT5 overexpression reversed the proliferation and migration abilities after knockdown of circ-CCND1. (A and B) The colony formation assay showing proliferation in A549 cells. si-circ-CCND1 control was used for NC. Data are expressed as the mean ± SD; ***P < 0.001 vs. the normal control (NC). ###P < 0.001 vs. si-circ-CCND1. (C and D) Cell migration and invasion were assessed in A549 cells using Transwell assays. si-circ-CCND1 control was used for NC. Data are expressed as the mean ± SD. ***P < 0.001 vs. the NC. ###P < 0.001 vs. si-circ-CCND1. (E-G) Western blot analysis showing the expression of the autophagy related proteins, Beclin-1, and LC3. si-circ-CCND1 control was used for NC. Data are expressed as the mean ± SD. ***P < 0.001 vs. the NC. ###P < 0.001 vs. si-circ-CCND1.

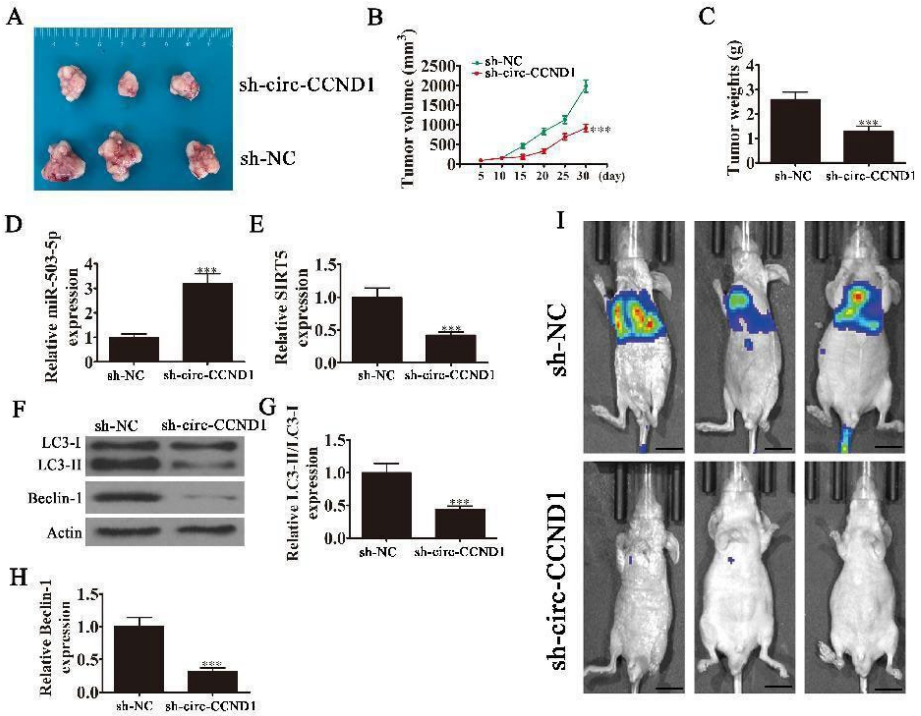

Figure 4: Downregulation of circ-CCND1 suppressed tumor formation and metastasis in nude mouse xenografts. (A) Representative images of nude mouse xenografts of A549 cells. (B) Tumor volumes in mice were measured every 5 days between sh-circ-CCND1 and sh-NC. Data are expressed as the mean ± SD. ***P< 0.001 vs. the sh-NC. (C) The tumor weight was measured 30 day after grafting between sh-circ-CCND1 and sh-NC. Data are expressed as the mean ± SD. ***P< 0.001 vs. the sh-NC. (D and E) The qRT-PCR assay of miR-503-5p (D) and SIRT5 (E) expressions between sh-circ-CCND1 and sh-NC. Data are expressed as the mean ± SD. ***P < 0.001 vs. the sh-NC. (F-H) Western blot analysis showed the expressions of the autophagy related proteins, Beclin-1 and LC3 between sh-circ-CCND1 and sh-NC. Data are expressed as the mean ± SD. ***P < 0.001 vs. the sh-NC. ###P < 0.001 vs. si-circ-CCND1. (I) Live imaging shows the effect of circ-CCND1 on A549 cell metastasis 30 days after intravenous tail injections.

protein expressions, LC3-II/LC3-I and Beclin-1 at the protein level (Figure 4F-H). Live imaging analyses showed the effect of circ-CCND1 on A549 cell metastasis at 30 days after intravenous tail injections. The results showed that circ-CCND1 silencing inhibited A549 cell metastasis (Figure 4I), suggesting that downregulation of circ-CCND1 suppressed tumor formation and metastasis in nude mouse xenografts by regulation of autophagy.

Discussion

Previous studies reported that circ-CCND1 had an essential proliferation-promoting role for cancer [15]. The present study showed that circ-CCND1 was increased in NSCLC cell lines, especially in A549 cells. The circ-CCND1 was a circRNA, which was derived from the CCND1 gene. The cell cycle regulator, CCND1, is reported to be a tumor suppressor gene [17, 18]. The present study found that circ-CCND1 was also associated with NSCLC progression. The circ-CCND1 downregulation suppressed proliferation and migration, as shown using in vivo and in vitro experiments. An increasing number of studies have suggested that circRNA functions as a ceRNA to regulate cancer progression via sponging miRNA [19, 20]. The present study also analyzed the circ-CCND1 target using bioinformatics analyses. The results confirmed that miR-503-5p was a circ-CCND1 downstream target, and the luciferase reporter assay showed the interactive association between miR-503-5p and circ-CCND1. The miR-503-5p has been reported to be associated with tumor progression [21, 22], and its overexpression suppressed ovarian cancer proliferation [23]. The present study showed that circ-CCND1 downregulation promoted miR-503-5p expression, and miR-503-5p inhibition recovered proliferation and migration abilities after circ-CCND1 silencing, suggesting that circ-CCND1 regulated the lung adenocarcinoma progression by sponging miR-503-5p.

Further studies found that miR-503-5p regulated the NSCLC progression by targeting SIRT5, and the luciferase reporter assay was used to characterize interaction between SIRT5 and miR-503-5p. A previous study found that SIRT5 silencing significantly reduced the migration ability of gastric cancer cells [24]. SIRT5 promotes cisplatin resistance in ovarian cancer [25], and promotes hepatocellular carcinoma progression via regulation of mitochondrial apoptosis [26]. We verified that downregulation of circ-CCND1 decreased SIRT5 expression, and that overexpression of SIRT5 recovered the proliferation and migration abilities after circ-CCND1 silencing. Further studies have found that SIRT5 can regulate autophagy. Autophagy is controlled by specific genes known as autophagy-related genes. In addition, many other genes can also affect autophagy. Accumulating evidence has proven that SIRTs have a regulatory function in autophagy. This study found that knockdown of circ-CCND1 decreased cell proliferation and migration by suppression of SIRT5-mediated autophagy. Autophagy has opposing, context-dependent roles in cancer, and interventions both stimulating and inhibiting autophagy are proposed as cancer therapies [27, 28]. Previous studies also found that SIRT5 promotes cell proliferation and invasion in hepatocellular carcinomas via targeting E2F1 [29], and that SIRT5 can regulate autophagy [30], which can directly influence lung adenocarcinoma cell growth and drug resistance [31]. Previous studies found that SIRT5 induces autophagy in tumor cells through the AMPK-mTOR pathway [32]. This study found that circ-CCND1 downregulation suppressed proliferation and migration via SIRT5-mediated autophagy regulation in lung adenocarcinoma, by sponging miR-503-5p.

In summary, the present study found that downregulation of circ-CCND1 functioned in inhibiting lung adenocarcinoma cell progression. Further studies found that downregulation of circ-CCND1 inhibited activation of autophagy by sponging miR-503-5p and enhancing SIRT5 expression. This study provides insights into the complexity of autophagy in tumor progression. Thus, circ-CCND1 may serve as a potential biomarker for lung adenocarcinoma diagnosis and prediction of prognosis, and targeting miR-503-5p/SIRT5 may be a promising therapeutic strategy for lung adenocarcinoma patients.

Ethical Statement

All animal experiments were approved by the Ethics Committee of Xinhua Hospital of Shanghai Jiaotong University, Shanghai, China.

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Acknowledgement

None

Conflict of Interest

None

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