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Circ-PITX1 promotes non-small-cell lung cancer progression through regulating ETS1 expression via miR-615-5p

Yang Guo | **Jianfang Pan** | **Xiaofei Gao** | **Yan Zheng**

Department of Pulmonary and Critical Care Medicine, Zhejiang Jinhua Guangfu Tumor Hospital, Jinhua, China

Correspondence
Yan Zheng, Department of Pulmonary and Critical Care Medicine, Zhejiang Jinhua Guangfu Tumor Hospital, No. 1296, Huancheng Road, Wucheng District, Jinhua 321000, China.
Email: 15957955617@163.com

Abstract

Background: Circular RNAs (circRNAs), produced by reverse splicing, act as important players in human cancers. We aimed to assess the biological functions of circRNA pituitary homeobox 1 (circ-PITX1) in non-small-cell lung cancer (NSCLC).

Methods: qRT-PCR was employed to determine RNA expression. Biological behaviors of NSCLC cells were assessed by CCK-8, colony formation, EdU assay, flow cytometry, wound healing, and transwell assays. Glutamine catabolism was examined via the measurement of glutamine consumption, α-ketoglutarate levels, as well as ATP levels. Protein levels were detected by western blot assays. Dual-luciferase reporter assay and RNA immunoprecipitation (RIP) assay were performed to reveal the mechanism responsible for circ-PITX1 regulating NSCLC cell malignancy. The murine xenograft model was established to investigate circ-PITX1's effect on tumor formation.

Results: Circ-PITX1 was overexpressed in NSCLC tissue samples and cells. Its low expression repressed NSCLC cell proliferation and motility. Moreover, our data revealed its downregulation inhibited glutamine catabolism and tumor formation and promoted cell apoptosis. In addition, circ-PITX1 bound to miR-615-5p, and its inhibitory effect on tumor cellular behaviors could be reversed after decreasing miR-615-5p expression. The miRNA targeted E26 transformation specific-1 (ETS1), whose upregulation abolished miR-615-5p overexpression-induced effects in NSCLC cells. Furthermore, circ-PITX1 positively modulated ETS1 production through interaction with miR-615-5p.

Conclusion: Circ-PITX1 facilitated NSCLC progression via modulating miR-615-5p/ETS1 pathway.

KEYWORDS
circ-PITX1, ETS1, miR-615-5p, NSCLC

INTRODUCTION

Lung cancer is an aggressive malignancy that seriously threatens people's health, and the World Health Organization divides lung cancer into non-small-cell lung cancer (NSCLC) and small-cell lung cancer according to its biological, therapeutic, and prognostic characteristics.[1] Despite some advanced techniques in clinical treatment, including target therapy, immune therapy, and other therapies, prognosis of NSCLC remains poor because of the absence of early symptoms in most patients, as well as the metastasis.[2,3] Thus, it is imperative to find a novel method for improving prognosis.

CircRNAs are noncoding RNAs that are circular RNAs without 5′–3′ polarity, enabling circRNAs resistant to exonuclease and thus very stable.[4] CircRNAs modulate cell proliferation, metastasis, and apoptosis in many cancers, inducing NSCLC.[5] For instance, circ_0004015 promoted NSCLC cell proliferation as well as gefitinib resistance.[6] Moreover, circPVT1 knockdown repressed NSCLC cell growth and mobility.[7] As for circRNA pituitary homeobox 1 (circ-PITX1; also known as

hsa_circ_0074027, chr5: 134363423-134369964), it derives from PITX1 gene and circ-PITX1 could promote the progression of NSCLC.[8] As indicated, glutamine is responsible for the survival and proliferation of cancer cells.[9] Hence, we explored circ-PITX1's role in NSCLC and the inner mechanism.

CircRNAs affect target gene levels and miRNA functions by acting as decoys for miRNAs.[10] MiRNAs, small ncRNAs, play critical roles in cellular functions.[11] MiR-615-5p is a tumor-suppressing miRNA in many tumors, including lung cancer.[12–14] Furthermore, E26 transformation specific-1 (ETS1) is highly expressed in diverse malignancies, including NSCLC.[15,16] As predicted through bioinformatics tool, both ETS1 and circ-PITX1 carried complementary binding sequences of miR-615-5p. Thus, the authors supposed that there was a circ-PITX1/miR-615-5p/ETS1 pathway in NSCLC cells and identified this hypothesis through a series of assays.

MATERIALS AND METHODS

Clinical samples

NSCLC tissues ($n = 60$) and adjacent normal tissues ($n = 60$) were obtained from NSCLC patients who had undergone surgery at Zhejiang Jinhua Guangfu Tumor Hospital during 2018–2020 and promptly frozen in liquid nitrogen. Characteristic information of NSCLC patients is shown in Supporting Information Table S1. Each patient had signed informed consent. This research acquired approval from the Research Ethics Committee of Zhejiang Jinhua Guangfu Tumor Hospital.

Cell culture and transfection

Human bronchial epithelial cell line (HBE1, BeNa Culture Collection, Beijing, China) and NSCLC cell lines (A549, H522, H1581, and HCC827, BeNa Culture Collection) were cultured in complete DMEM (BeNa Culture Collection) with standard culture conditions (5% CO_2, 37°C).

The shRNAs targeting circ-PITX1 (sh-circ-PITX1) or ETS1 (sh-ETS1), circ-PITX1 or ETS1-overexpressing vector (circ-PITX1 or ETS1), and miR-615-5p mimic or inhibitor were commercially obtained from Genecreat. According to the recommendations, cells were introduced with these vectors or oligonucleotides with the addition of Lipofectamine 3000 (Invitrogen).

RNA isolation and qRT-PCR

After collection of RNA samples and incubation with TRIzol reagent (Invitrogen) as per the guidebook, the study isolated RNA at 4°C. cDNA was generated using high-capacity RT reagents (TaKaRa). After measurement of cDNA concentration, qRT-PCR was carried out on an IQ5 thermocycler with the addition of SYBR Green reagents (TaKaRa). Circ-PITX1 (5′-GCGTCCCTGTGTATGTTGGA-3′ and 5′-GTCTGTC TTAAAGCGACAGCG-3′), PITX1 (5′-GTACGCACTTCA-CAAGCCAGCA-3′ and 5′-GCTCGGTGAGGTTGGTCCA CA-3′), miR-615-5p (5′-ATAATAGGGGGTCCCCGGT GCT-3′ and 5′-CAGTGCGTGTCGTGGAGT-3′), ETS1 (5′-GAGTCAACCCAGCCTATCCAGA-3′ and 5′-GAGCGTC TGATAGGACTCTGTG-3′), β-actin (5′-GCCGGGACCT-GACTGACTAC-3′ and 5′-TCTCCTTAATGTCACGCAC-GAT-3′), U6 (5′-CTCGCTTCGGCAGCACATATACT-3′ and 5′-ACGCTTCACGAATTTGCGTGTC-3′). Gene expression was evaluated by the $2^{-\Delta\Delta Ct}$ method.

Stability analysis of circRNA

The assay regarding the use of RNase R (Seebio) was conducted to evaluate circ-PITX1 ability against exonuclease. RNA was incubated with or without RNase R at 37°C for half an hour. Afterwards, RNA subjected to qRT-PCR.

In addition, cells were exposed to Actinomycin D (Seebio) to represses the transcription. After that, qRT-PCR was used for circ-PITX1 and PITX1 level analysis.

Cell proliferation assays

Transfected cells (A549 and H1581) were plated into 96-well plates, followed by continuous incubation for 48 h. Next, CCK-8 (Beyotime) was added and subjected to 3 hours of incubation. These culture plates were put in the microplate reader for cell viability analysis.

Transfected cells (A549 and H1581) were plated into six-well plates, followed by incubation for 15 days. Thereafter, paraformaldehyde (Beyotime) was used to fix cell colonies, which were then stained using crystal violet (Beyotime). After removing these staining solutions, the colonies were counted and imaged.

A Cell Proliferation Kit (Beyotime) was utilized for detecting DNA synthesis. Briefly, NSCLC cells were treated with EdU and fixed using 4% paraformaldehyde. Thereafter, Click Additive solution was put into per well for 0.5 h. After incubation with DAPI (Wuhan Khayal Biotechnology), EdU-positive cells were counted and captured for cell proliferation analysis.

Flow cytometry analysis

A549 and H1581 cells were digested using trypsase and washed using PBS, followed by resuspending in binding buffer (Vazyme). Every sample was incubated with Annexin V-FITC (Vazyme). After these samples were exposed to PI (Vazyme), a flow cytometer was used to examine apoptotic cells.

Wound healing assay

NSCLC cells were evenly spread in six-well plates. After confirming that the cells had grown fully, white needles were used to make a cross on the bottom of the wells. PBS was used to clean the well thoroughly and photos were taken under optical microscopy. At 24 h, fresh complete medium was replaced and photos were taken again. Image J software was used for data analysis.

Transwell assay

Matrigel (BD Biosciences) was diluted at a ratio of 1:8 using serum-free medium. The diluted Matrigel solution was plated onto the bottom membrane of the Transwell chamber (Costar). After adding complete culture medium to stop digestion, the supernatant was discarded. Resuspended cells using serum-free DMEM were then seeded into the Transwell chamber. After incubating the cells in the cell culture incubator, non-invasive cells were removed, being careful to handle gently throughout the process. The cells were fixed with formaldehyde and the chamber was air-dried. The cells in the chamber were observed under a microscope.

Measurement of glutamate, α-KG, and ATP levels

Glutamine consumption, α-ketoglutarate (α-KG) production, and ATP production were measured by Glutamate Assay Kit (Abcam), α-KG Assay Kit (Sigma-Aldrich), and ATP Detection Assay Kit (Abcam), respectively, as per respective guidebooks.

Western blot

Extraction of total protein from tissue samples and cells was conducted using RIPA lysis buffer (Vazyme). After quantification of protein concentration, protein (40 μg/lane) was loaded onto SDS-PAGE and blotted onto nitrocellulose membranes, followed by blocking with 5% skim milk (Yili). Next, the membranes were interacted with appropriate primary antibodies against glutaminase-1 (GLS1) (#56750S, 1:1000, CST), ETS1 (#14069, 1:1000, CST; Abcam) or β-actin (ab227387, 1:2000; Abcam). The ECL luminous liquid was prepared in a 1:1 ratio and dropped onto the strips. They were then placed in the imaging system and photographed.

Bioinformatics prediction and dual-luciferase reporter assay

Circular RNA interactome or TargetScan was utilized to predict the relationship between miR-615-5p and circ-PITX1 or ETS1. Dual luciferase reporter gene plasmids including circ-PITX1 wt, ETS1 3′UTR wt, circ-PITX1 mut, and ETS1 3′UTR mut were provided by GeneCopoeia. The experimental groups were circ-PITX1 wt/mut + miR-615-5p overexpression group/control group; ETS1 3′UTR wt/mut + miR-615-5p overexpression group/control group. Cell transfection was performed as described above. After 48 h, the supernatant was discarded. PLB (LMAI Bio) was added to each well. Supernatant was collected and added to 96-well enzyme plates for detection. Stop & Glo Reagent (LMAI Bio) was added to each well, and data was measured after 2 s. All the above operations were performed in the dark.

RIP assay

A549 and H1581 cells were lysed as per the guidebook of complete RNA immunoprecipitation (RIP) lysis buffer. After lysis, 0.1 mL of cell lysates was incubated with the magnetic beads which linked with antibody specific to Ago2 or IgG antibody. The immunoprecipitated RNAs were eluted with proteinase K to remove the non-specific binding, and qRT-PCR was performed for circ-PITX1, miR-615-5p, and ETS1 expression analysis.

Tumor formation assay in vivo

BALB/c nude mice (male, 20–25 g, $n = 12$) were commercially provided by Vital River. Genomeditech commercially provided lentivirus expressing sh-circ-PITX1 or sh-NC. The prepared two strains of A549 cells were cultured in 100-mm culture dishes. When they were in the logarithmic growth phase, the cells were digested, centrifuged, and resuspended in PBS, then 5×10^6 cells were taken from each group, centrifuged, and placed on ice. In each group, six nude mice were subcutaneously implanted with 100 μL of cell suspension and tagged with an ear tag. When the subcutaneous tumors reached approximately 100 mm^3 in size, the tumor size was measured every 7 days. After 35 days, the mice were euthanized by cervical dislocation, photographed, and the tumors were removed. The subcutaneous tumor tissues were soaked in polyformaldehyde fixative for 24 h, embedded in paraffin, and sliced. Approval was obtained from the Animal Care and Use Committee of Zhejiang Jinhua Guangfu Tumor Hospital.

Immunohistochemistry analysis

Tumor samples were incubated with anti-Ki67 (ab15580, 1:200; Abcam), followed by addition of secondary antibody (ab171870, 1:1000; Abcam). These slides were then stained using diaminoaniline (DAB; Beyotime) and counterstained

with hematoxylin (Beyotime). Finally, the sections were observed under a microscopy.

Statistical analysis

All data were expressed as the mean ± standard deviation and analyzed by GraphPad Prism. Differences were analyzed via Student's *t*-test or a one-way ANOVA. The value of $p < 0.05$ indicated statistical significance.

RESULTS

Circ-PITX1 was upregulated in NSCLC tissues

Circ-PITX1 level was upregulated in NSCLC tissues (Figure 1a). Circ-PITX1 was upregulated in NSCLC cells (A549, H522, H1581, and HCC827), especially in A549 and H1581 cells, when compared with HBE1 cells (Figure 1b). Besides, the NSCLC tissues were divided into high circ-PITX1 expression and low circ-PITX1 expression groups based on the median value of its expression. The analysis of correlation between circ-PITX1 expression and clinicopathological characteristics of NSCLC patients showed that circ-PITX1 expression was associated with tumor size, TNM stage and lymphatic metastasis (Table 1). As expected, PITX1 mRNA levels rather than circ-PITX1 expression were reduced by RNase R (Figure 1c,d). Half-life of linear PITX1 transcript was only about 12 h, while that of circ-PITX1 transcript exceeded 24 h (Figure 1e,f).

Circ-PITX1 knockdown inhibited malignant growth of NSCLC cells

Circ-PITX1 expression was significantly reduced after transfection with sh-circ-PITX1 (Figure 2a).

TABLE 1 Correlation between circ-PITX1 expression and clinicopathological characteristics of NSCLC patients.

Clinicopathologic parameters	Case	Circ-PITX1 expression		*p* value[a]
		Low ($n = 31$)	High ($n = 29$)	
Gender				0.3473
Male	28	15	13	
Female	32	16	16	
Age (years)				0.3205
<55	38	21	17	
≥55	22	10	12	
Tumor size				0.0185*
<3 cm	39	19	20	
≥3 cm	21	12	9	
TNM stage				0.0151*
I–II	41	28	13	
III	19	3	16	
Lymphatic metastasis				0.0069*
Negative	20	13	7	
Positive	40	18	22	

*$p < 0.05$.
[a]Chi-square test.

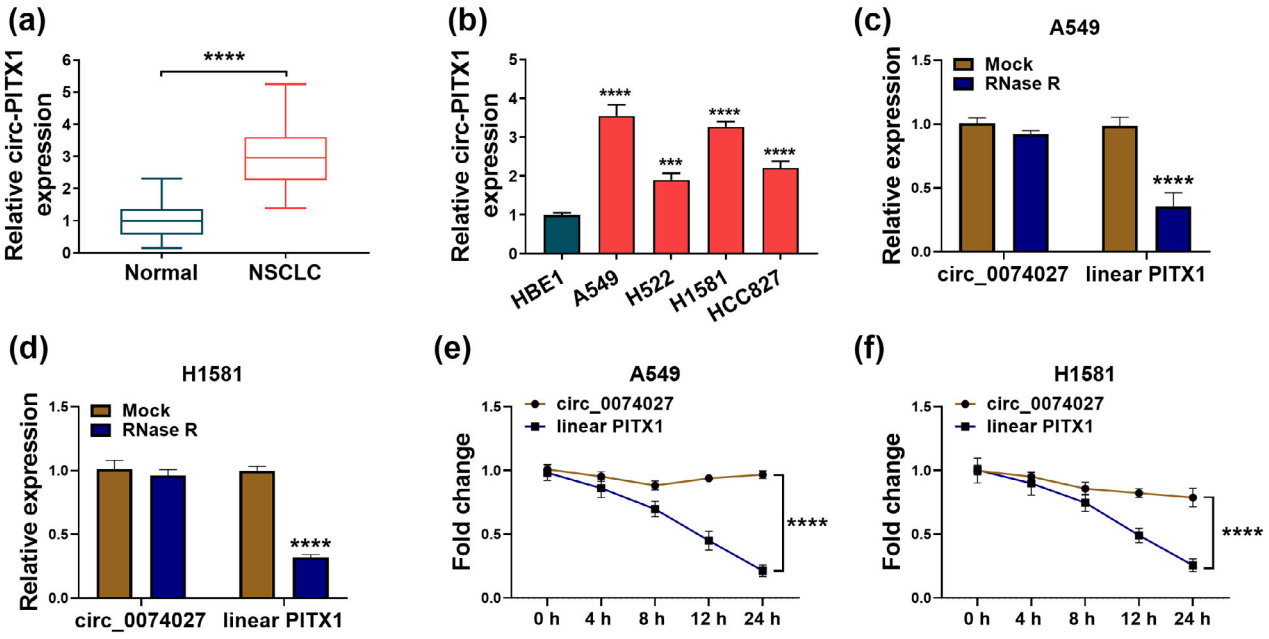

FIGURE 1 Circ-PITX1 was overexpressed in NSCLC tissues and cells. (a) The expression of circ-PITX1 was detected by qRT-PCR in NSCLC tissues ($n = 60$) and adjacent normal tissues ($n = 60$). (b) The level of circ-PITX1 was measured by qRT-PCR in NSCLC cell lines (A549, H522, H1581, and HCC827) and human bronchial epithelial cell line (HBE1). (c–f) After treatment of RNase R and Actinomycin D, circ-PITX1 and PITX1 levels were detected by qRT-PCR in A549 and H1581 cells. ***$p < 0.001$, ****$p < 0.0001$.

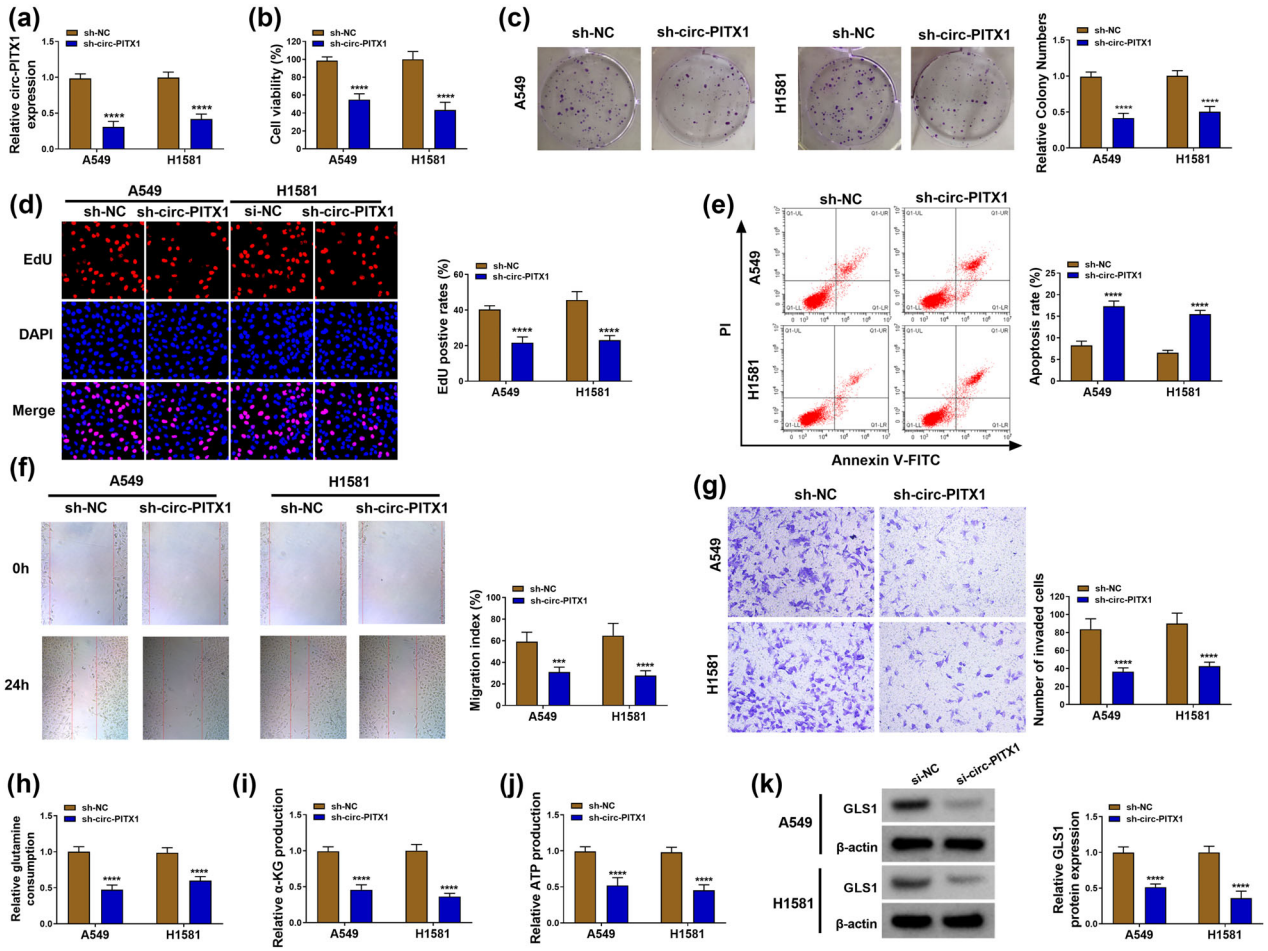

FIGURE 2 Circ-PITX1 downregulation suppressed proliferation, migration, invasion, and glutamine metabolism, and induced apoptosis in NSCLC cells. A549 and H1581 cells were transfected with sh-circ-PITX1 or sh-NC. (a) The expression of circ-PITX1 was detected by qRT-PCR. (b) Cell viability was assessed by CCK-8 assay. (c) The colony numbers were determined by colony formation assay. (d) EdU assay was used to assess DNA synthesis. (e) Flow cytometry analysis was used to determine apoptosis rate. (f) Wound healing assay was employed to assess cell migration ability (×40). (g) Cell invasion ability was measured by transwell assay (×100). (h–j) Glutamine consumption, α-KG production, and ATP production were examined by Glutamate Assay Kit, α-KG Assay Kit, and ATP Detection Assay Kit, respectively. (k) The protein expression of GLS1 was analyzed by western blot assay. ***$p < 0.001$, ****$p < 0.0001$.

Additionally, its low expression reduced cell viability and colony formation (Figure 2b,c). The study also revealed its inhibitory effect on DNA synthesis (Figure 2d). Moreover, circ-PITX1 silencing increased cell apoptosis and hindered cell motility (Figure 2e–g). We found that circ-PITX1 knockdown suppressed glutamine consumption and α-KG production (Figure 2h,i). Meanwhile, the assay revealed that ATP production was suppressed by circ-PITX1 knockdown (Figure 2j). Furthermore, circ-PITX1 silence inhibited the protein expression of GLS1 (Figure 2k).

Circ-PITX1 bound to miR-615-5p

The miRNAs with circ-PITX1-binding sites were predicted through the Circular RNA interactome online database. The miRNAs with scores above 90 were selected for the study, including miR-524-3p, miR-525-3p, miR-584, miR-604, and miR-615-5p. As shown in Figure S2a, circ-PITX1 depletion also upregulated miR-615-5p expression in A549 cells. Thus, this miRNA was chosen for the study. The study discovered circ-PITX1 potentially bound to miR-615-5p (Figure 3a). Our data also indicated that miR-615-5p mimics decreased the luciferase activity of circ-PITX1 wt but not that of circ-PITX1 mut (Figure 3b). Circ-PITX1 and miR-615-5p expression were markedly increased in Ago2 group compared to IgG group (Figure 3c). Next, we discovered miR-615-5p was reduced in NSCLC tissues and cells (Figure 3d,e). miR-615-5p expression was negatively correlated with circ-PITX1 level in NSCLC tissues (Figure 3f). The data indicated in Figure 3g revealed that circ-PITX1 was successfully overexpressed in A549 and H1581 cells. Moreover, miR-615-5p expression was negatively regulated by circ-PITX1 (Figure 3h). In summary, circ-PITX1 could bind with miR-615-5p.

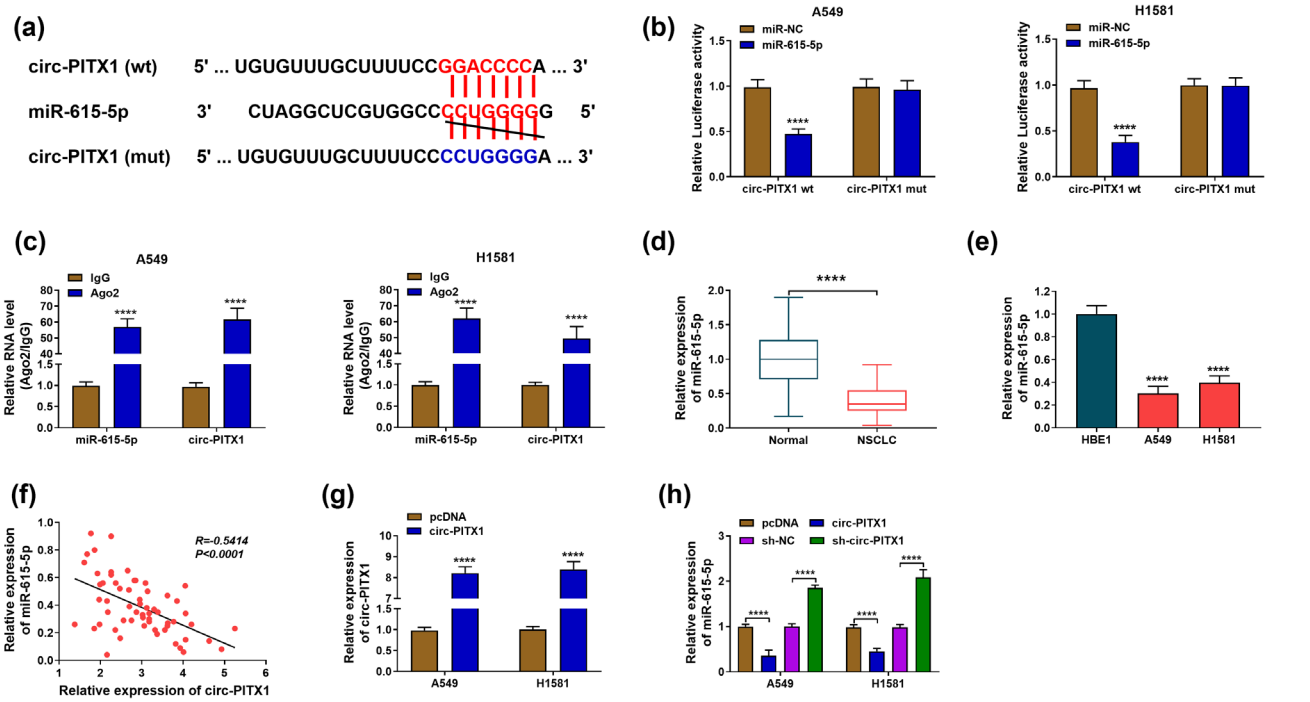

FIGURE 3 Circ-PITX1 directly targeted miR-615-5p in NSCLC cells. (a) The complementary binding sequence of miR-615-5p and circ-PITX1 was predicted by Circular RNA interactome. (b) A549 and H1581 cells were co-transfected with circ-PITX1 wt or circ-PITX1 mut and miR-NC or miR-615-5p, and the luciferase activity was measured through dual-luciferase reporter assay. (c) The enrichment of circ-PITX1 or miR-615-5p was measured by RIP assay in A549 and H1581 cells. (d and e) The expression of miR-615-5p was measured by qRT-PCR in NSCLC tissues and cells and corresponding normal tissues and cells. (f) The correlation between miR-615-5p and circ-PITX1 expression was analyzed in NSCLC tissues. (g) Overexpression efficiency of circ-PITX1 was determined by qRT-PCR in A549 and H1581 cells transfected with pcDNA or circ-PITX1. (h) The expression level of miR-615-5p was detected by qRT-PCR in A549 and H1581 cells transfected with pcDNA, circ-PITX1, sh-NC, or sh-circ-PITX1. ****$p < 0.0001$.

Circ-PITX1 knockdown inhibited NSCLC cell malignancy through interaction with miR-615-5p

As presented in Figure 4a, anti-miR-615-5p transfection markedly decreased miR-615-5p expression in A549 and H1581 cells. The inhibitory effects of circ-PITX1 knockdown on cell proliferation were reversed by inhibiting miR-615-5p (Figure 4b–d). Inhibition of miR-615-5p abated sh-circ-PITX1-induced promoting effect on cell apoptosis and inhibitory effects on cell migration and invasion (Figure 4e–g). In addition, sh-circ-PITX1-induced inhibition of glutamine consumption, α-KG production, ATP production, and GLS1 expression were abolished by knockdown of miR-615-5p (Figure 4h–k).

miR-615-5p targeted ETS1 in NSCLC cells

TargetScan online database was used to predict mRNAs with miR-615-5p-binding sites. The predicted genes that have been shown to play a role in NSCLC were selected for the study, including IGF1R, KLF12, CXCR5, FZD4, THBS2, ETS1, E2F2, and EGFR. As shown in Figure S2b, the transfection with miR-615-5p mimics significantly downregulated ETS1 and E2F2 expression, especially downregulated ETS1 expression. Thus, ETS1 was employed for the following study. ETS1 had binding sequence with miR-615-5p (Figure 5a). Next, miR-615-5p overexpression suppressed the luciferase activity of ETS1 3′UTR wt, but it did not affect the luciferase activity of ETS1 3′UTR mut in A549 and H1581 cells (Figure 5b). Meanwhile, ETS1 and miR-615-5p were more abundant in Ago2 pellet than in IgG pellet (Figure 5c). Moreover, ETS1 was upregulated in NSCLC tissues and cells (A549 and H1581) (Figure 5d,e). Additionally, it was observed that miR-615-5p expression was negatively correlated with ETS1 mRNA expression in NSCLC tissues (Figure 5f). Similarly, ETS1 protein expression was also increased in NSCLC tissues and cells (A549 and H1581) (Figure 5g,h). The high efficiency of miR-615-5p overexpression was shown in Figure 5i. Next, the influence of miR-615-5p on expression of ETS1 was investigated. We discovered that miR-615-5p overexpression inhibited ETS1 expression, while miR-615-5p downregulation presented an opposite effect (Figure 5j).

MiR-615-5p overexpression repressed NSCLC cell malignancy by targeting ETS1 in NSCLC cells

The study analyzed the effect of ETS1 silencing on the malignant phenotypes of both A549 and H1581 cells. The high efficiency of ETS1 siRNA in downregulating ETS1

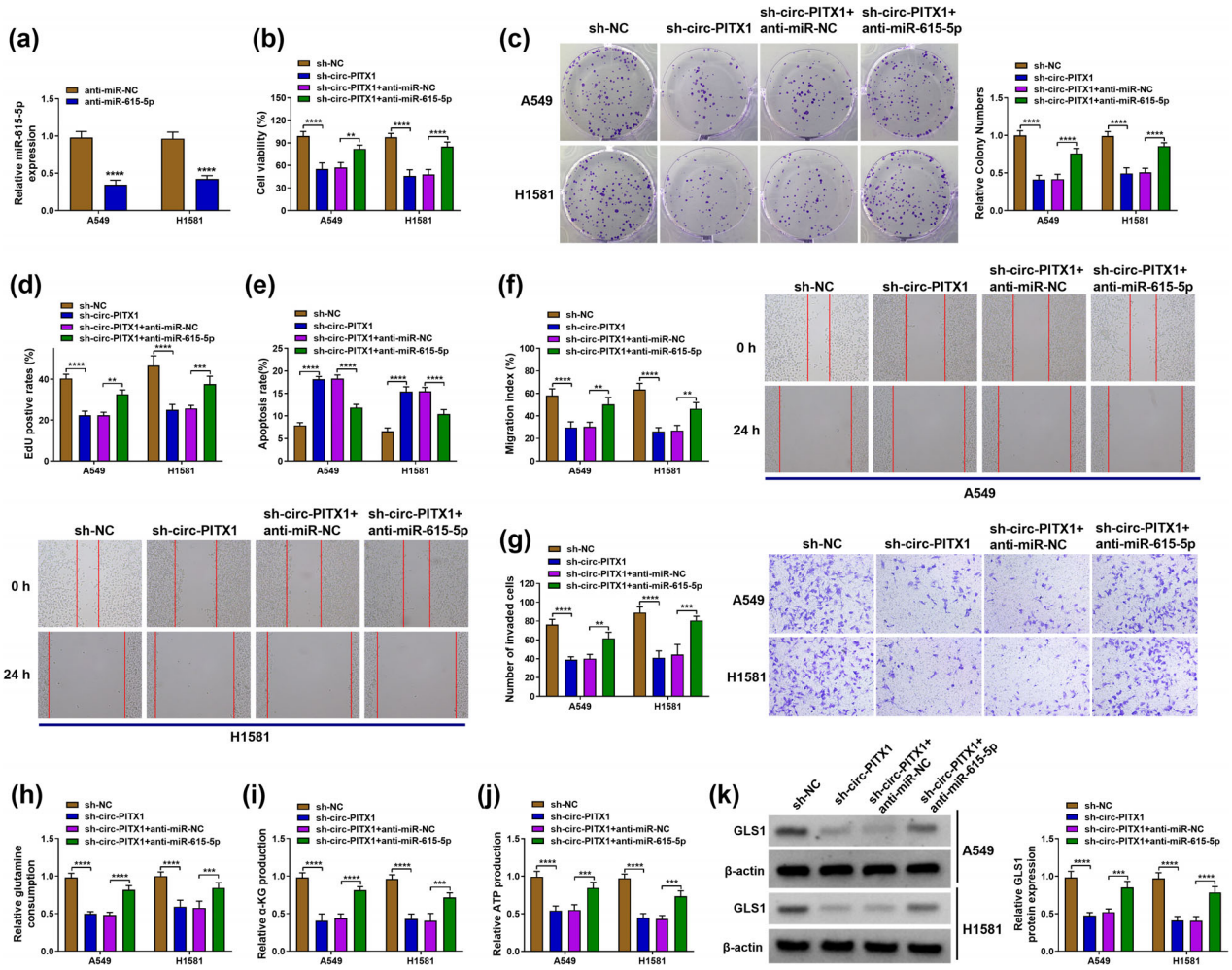

FIGURE 4 Inhibition of miR-615-5p reversed the effects of sh-circ-PITX1 on NSCLC cell proliferation, apoptosis, migration, invasion, and glutamine metabolism. (a) The expression of miR-615-5p was measured by qRT-PCR in A549 and H1581 cells transfected anti-miR-NC or anti-miR-615-5p. (B-K) A549 and H1581 cells were transfected with sh-NC, sh-circ-PITX1, shi-circ-PITX1 + anti-miR-NC, or sh-circ-PITX1 + anti-miR-615-5p. (b) Cell viability was evaluated by CCK-8 assay. (c) Cell colony formation ability was examined by colony formation assay. (d) DNA synthesis was evaluated by EdU assay. (e) Flow cytometry analysis was employed to determine the apoptosis rate. (f and g) Wound healing assay and transwell assay were used to evaluate cell migration and invasion abilities, respectively. (h–j) Glutamate Assay Kit, α-KG Assay Kit, and ATP Detection Assay Kit were used to measure the glutamine consumption, α-KG production, and ATP production, respectively. (k) Western blot assay was performed to analyze the protein expression of GLS1. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

expression is shown in Figure S1a. ETS1 knockdown inhibited cell viability, cell proliferation, migration, and invasion (Figure S1b–g). In addition, ETS1 silencing inhibited glutamine consumption, α-KG production, ATP production, and GLS1 protein expression (Figure S1h–k). Transfection of ETS1 enhanced ETS1 expression (Figure 6a,b). Subsequently, miR-615-5p overexpression inhibited cell proliferation and induced apoptosis in A549 and H1581 cells, which was reversed by upregulating ETS1 (Figure 6c–f). Moreover, miR-615-5p upregulation suppressed A549 and H1581 cell migration and invasion, while this inhibitory effect was attenuated by overexpression of ETS1 (Figure 6g,h). Meanwhile, enforced expression of miR-615-5p repressed glutamine consumption, α-KG production, ATP production, and GLS1 protein expression, which could be reversed by co-transfection of ETS1 (Figure 6i–l).

Circ-PITX1 regulated ETS1 expression via sponging miR-615-5p in NSCLC cells

We then found that circ-PITX1 knockdown inhibited ETS1 production, which was rescued by downregulation of miR-615-5p (Figure 7a,b), suggesting that circ-PITX1 positively modulated ETS1 expression by acting as a sponge of miR-615-5p.

Circ-PITX1 silence inhibited tumor growth

The data showed that transfection with sh-circ-PITX1 notably suppressed the expression of circ-PITX1 (Figure 8a). The images of tumor tissues were presented in Figure 8b. Circ-PITX1 knockdown decreased tumor volume and

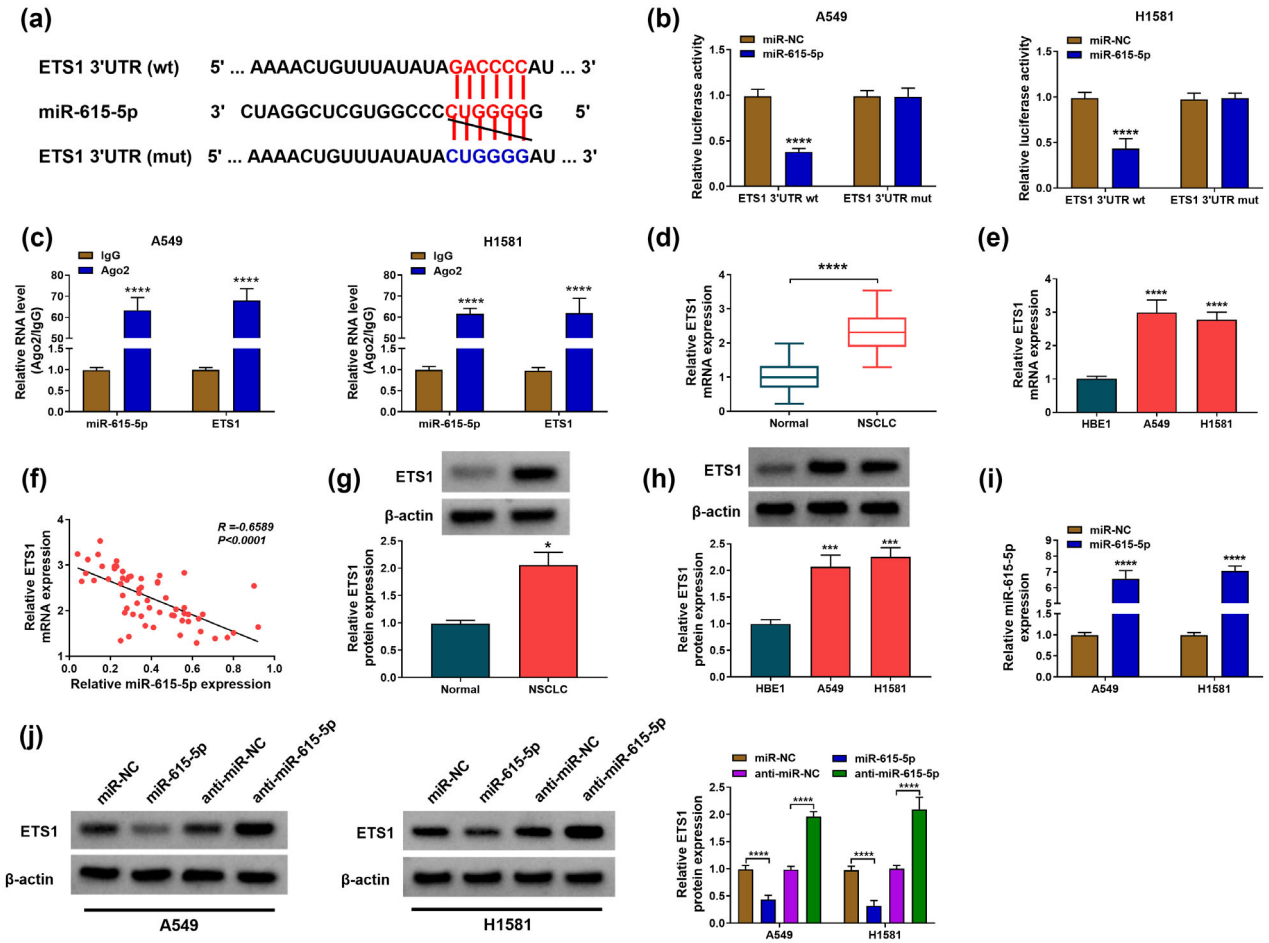

FIGURE 5 ETS1 could bind to miR-615-5p in NSCLC cells. (a) The potential binding sites between ETS1 and miR-615-5p were predicted by TargetScan. (b) The combination of ETS1 and miR-615-5p was investigated by dual-luciferase reporter assay. (c) The enrichment of ETS1 or miR-615-5p was measured by RIP assay in A549 and H1581 cells. (d and e) The mRNA expression of ETS1 was analyzed by qRT-PCR in NSCLC tissues and cells and corresponding normal tissues and cells. (f) The association between ETS1 mRNA level and miR-615-5p abundance was analyzed in NSCLC tissues. (g and h) ETS1 protein expression was detected by western blot assay in NSCLC tissues and cells and matched normal tissues and cells. (i) The expression of miR-615-5p was examined by qRT-PCR in A549 and H1581 cells transfected with miR-NC or miR-615-5p. (j) The protein expression of ETS1 was determined by western blot analysis in A549 and H1581 cells transfected with miR-NC, miR-615-5p, anti-miR-NC, or anti-miR-615-5p. $^{**}p < 0.01$, $^{****}p < 0.0001$.

weight (Figure 8c,d). Moreover, circ-PITX1 and ETS1 expression were decreased and miR-615-5p expression was increased in circ-PITX1-silenced tumor tissues (Figure 8e). Meanwhile, ETS1 protein expression was reduced after circ-PITX1 knockdown (Figure 8f). Immunohistochemistry analysis showed that deficiency of circ-PITX1 decreased the expression Ki67 (a proliferation marker) in tumor tissues (Figure 8g).

DISCUSSION

Lung cancer is a malignant cancer that seriously threatens human health.[17] It is believed that ncRNAs regulate diverse pathological and physiological processes.[18] Increasing evidence has revealed that dysregulation of circRNAs is related to human malignancies.[19,20] In recent years, more and more research has analyzed the association of circRNAs with the prognosis of NSCLC patients.[21,22] Despite these advances, the potential use of circRNAs in NSCLC treatment remains largely unknown. In the current research, the regulatory mechanisms of circ-PITX1 were explored in NSCLC.

Circ-PITX1 is a tumor promoter in glioma.[23] More importantly, its high expression in NSCLC cell and tissue samples, and its silence could limit NSCLC cell growth and increase apoptotic rate through the modulation of miR-335-5p/CUL4B axis.[24] In addition, it was overexpressed in NSCLC, and circ-PITX1 enhancement increased tumor cell mobility and suppressed apoptosis.[8] This study explains how it regulates the miR-185-3p/BRD4/MADD pathway to mediate tumor cell phenotypes. Consistently, we discovered circ-PITX1 was increased in NSCLC cells and tissues. Circ-PITX1 silencing repressed NSCLC cell growth, migration, and invasion, whilst accelerating the cellular apoptotic rate in vitro. Glutamine metabolism is considered to be an

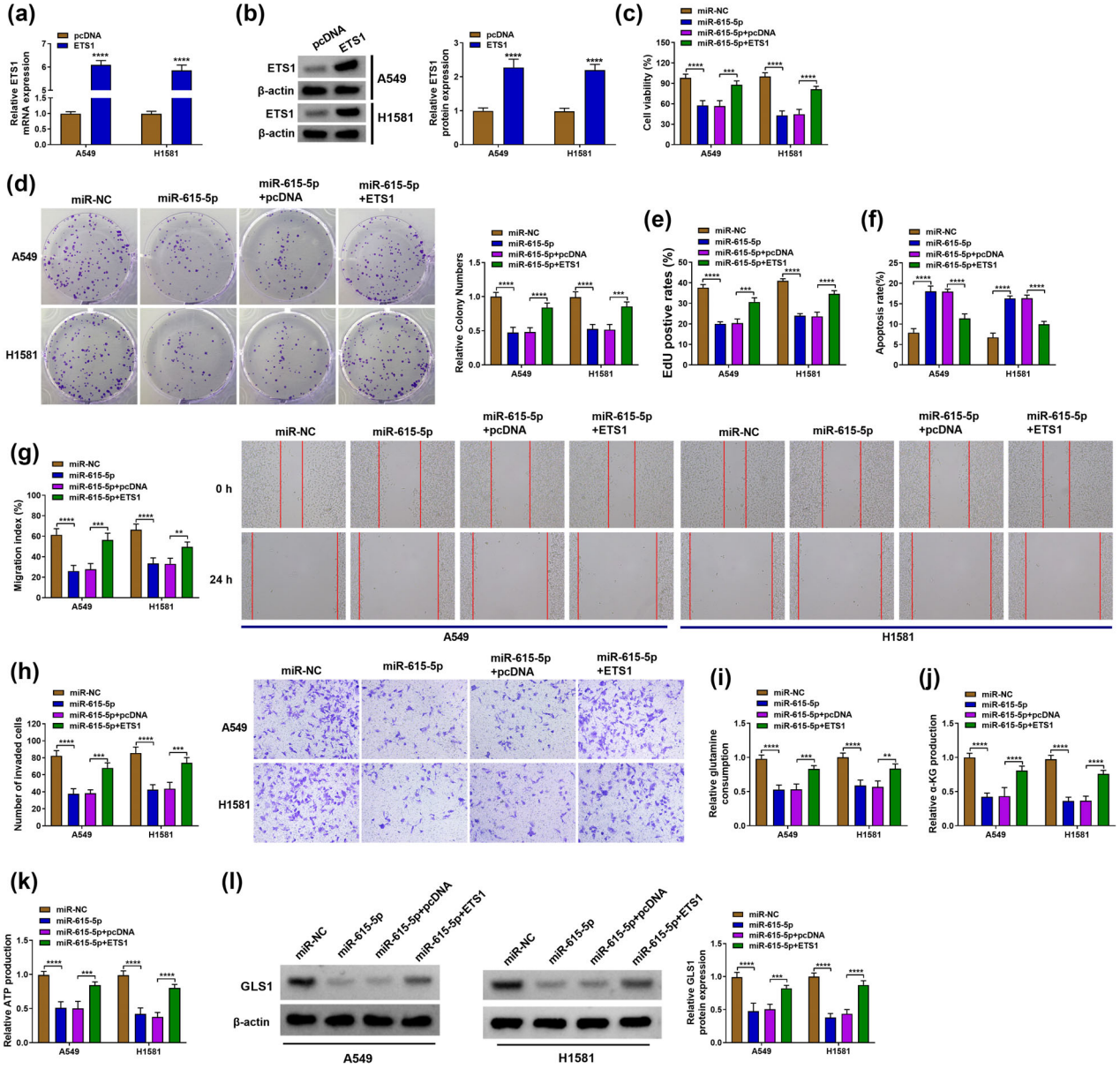

FIGURE 6 MiR-615-5p regulated NSCLC cell proliferation, apoptosis, migration, invasion, and glutamine metabolism by targeting ETS1. (a and b) The mRNA and protein levels of ETS1 in A549 and H1581 cells transfected with pcDNA or ETS1 were measured by qRT-PCR and western blot analyses, respectively. (c–l) A549 and H1581 cells were transfected with miR-NC, miR-615-5p, miR-615-5p + pcDNA, miR-615-5p + ETS1. (c–e) CCK-8 assay, colony formation assay and EdU assay were adopted to determine cell proliferation ability. (f) Flow cytometry analysis was used to detect cell apoptosis rate. (g and h) Wound healing assay and transwell assay were applied to evaluate cell migration and invasion abilities, respectively. (i–k) Glutamine consumption, α-KG production, and ATP production were evaluated by Glutamate Assay Kit, α-KG Assay Kit, and ATP Detection Assay Kit, respectively. (l) Western blot assay was conducted to detect the protein expression of GLS1. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

important metabolic process that supports cancer progression.[8] Recently, many studies have shown that glutamine metabolism restriction can effectively suppress the progression of multiple cancers.[25,26] Herein, we found the circ-PITX1 knockdown inhibits glutamine metabolism. Our results suggest that circ-PITX1 facilitates glutamine metabolism to promote NSCLC progression.

According to competing endogenous RNA hypothesis that circRNAs are able to function as sponges for miRNAs to bind with miRNAs in many cancers, thus regulation of the expression of target mRNAs.[27,28] We proved that circ-PITX1 is bound to miR-615-5p. Previous research has shown that miR-615-5p inhibited NSCLC progression. Also, miR-615-5p was declined in NSCLC cell samples and abated Gm15290-mediated NSCLC cell growth and invasion. Another article revealed that circRNA 100146 functioned as an oncogene via targeting miR-615-5p in NSCLC.[29] Similarly, a decrease of miR-615-5p in NSCLC cells and tissues

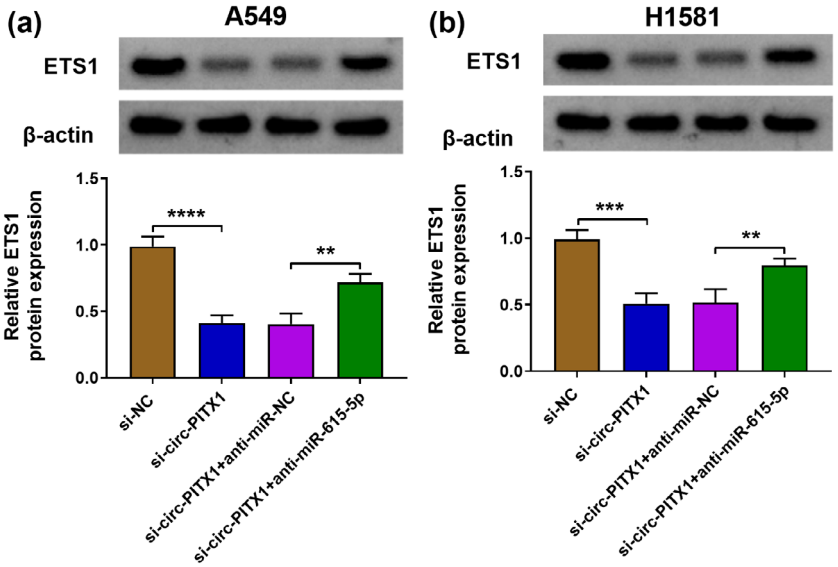

FIGURE 7 Circ-PITX1 modulated ETS1 expression by targeting miR-615-5p in NSCLC cells. (a and b) ETS1 protein expression was determined by western blot assay in A549 and H1581 cells transfected with sh-NC, sh-circ-PITX1, sh-circ-PITX1 + anti-miR-NC, or sh-circ-PITX1 + anti-miR-615-5p. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

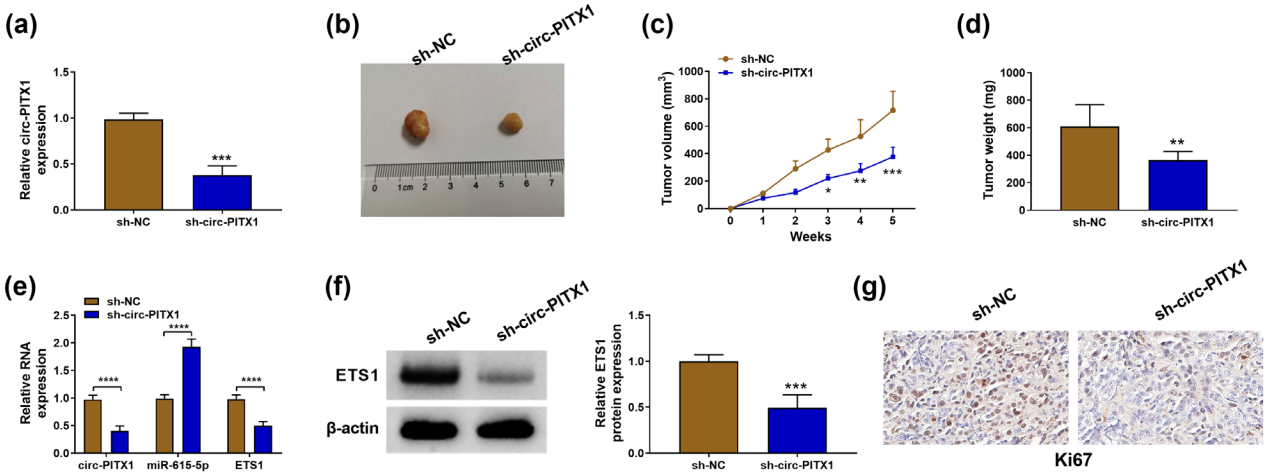

FIGURE 8 Knockdown of circ-PITX1 repressed tumor growth in vivo. (a) The expression of circ-PITX1 was measured by qRT-PCR in A549 cells transfected with sh-NC or sh-circ-PITX1. (b–f) A549 cells introduced with sh-NC or sh-circ-PITX1 were inoculated subcutaneously into the nude mice to establish mice xenograft model. (b) The images of the tumors were shown. (c) Tumor volume was monitored every week. (d) Tumor weight was measured 5 weeks later. (e) The levels of circ-PITX1 and miR-615-5p in the collected tissues were detected by qRT-PCR. (f) ETS1 protein level in the collected tissues was detected by Western blot assay. (g) The expression of Ki67 was determined by immunohistochemistry analysis in the collected tissues. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

was observed by the authors. In addition, we observed the influence of circ-PITX1 deficiency on NSCLC cell proliferation, apoptosis, invasion, migration, and glutamine metabolism was neutralized after miR-615-5p expression was reduced. These data suggest that circ-PITX1/miR-615-5p axis participates in NSCLC development.

MiRNAs can affect target mRNA expression to exert their functions.[30] In our research, ETS1 was identified as a target of miR-615-5p. As reported in 2019, upregulation of miR-512-5p suppressed proliferation and metastasis and accelerated apoptosis of NSCLC cells via targeting ETS1.[31] ETS1 was targeted by miR-300 to participate in NSCLC progression.[32] Herein, ETS1 was highly expressed in NSCLC. Rescue assays showed that ETS1 upregulation reversed miR-615-5p-induced effects in NSCLC cells, which indicates that ETS1 interacts with miR-615-5p to exert its functions in NSCLC. Moreover, circ-PITX1/miR-615-5p axis could regulate ETS1 expression. Additionally, circ-PITX1 interference also delayed tumor tumorigenesis in vivo.

Collectively, we demonstrated that circ-PITX1 deficiency inhibits the malignant growth of NSCLC cells by reducing ETS1 expression through miR-615-5p (Figure 9). The clinical significance of the conclusion is that the reduction in circ-PITX1 levels can impede the aggressive growth of NSCLC by reducing the expression of ETS1, a process

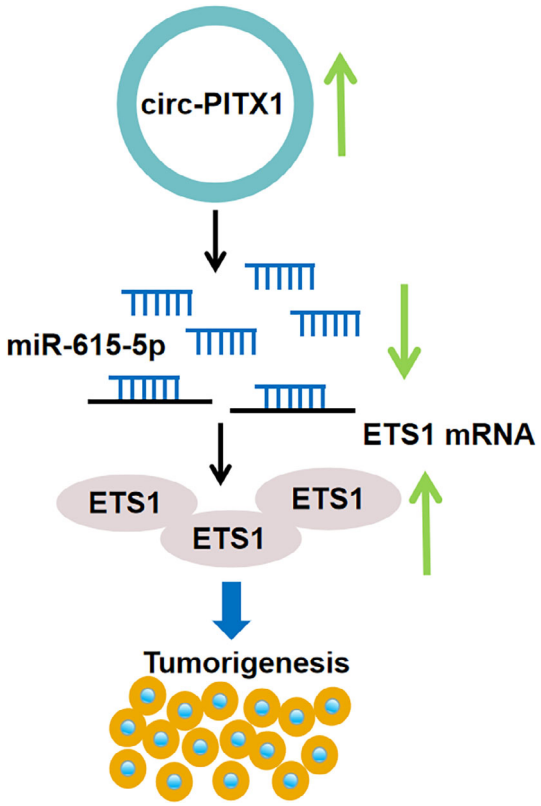

FIGURE 9 The schematic diagram showing the mechanism underlying circ-PITX1 in regulating NSCLC cell malignancy.

mediated by miR-615-5p. This finding suggests that targeting circ-PITX1 can serve as a novel therapeutic approach for NSCLC.

AUTHOR CONTRIBUTION

Yang Guo performed experiments and drafted the manuscript. Jianfang Pan collected and analyzed the data. Xiaofei Gao contributed the methodology, operated the software and edited the manuscript. Yan Zheng designed and supervised the study.

CONFLICT OF INTEREST STATEMENT

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

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Yan Zheng https://orcid.org/0009-0002-1015-7047

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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