

# The circ-PITX1 promotes non-small cell lung cancer development via the miR-30e-5p/ITGA6 axis

Wei Li<sup>a</sup>, Pan Yang<sup>a</sup>, Chucheng Zhong<sup>b</sup>, Xiaozhen Shen<sup>b</sup>, Xingyuan Shi<sup>c</sup>, and Xiaoping Li<sup>a</sup>

<sup>a</sup>Department of Thoracic Surgery, Tianjin First Central Hospital, School of Medicine, Nankai University, Tianjin, China; <sup>b</sup>Department of Thoracic Surgery, The Fifth Affiliated Hospital of Guangzhou Medical University, China; <sup>c</sup>Department of Radiology, The Fifth Affiliated Hospital of Guangzhou Medical University, China

## ABSTRACT

Non-small cell lung cancer (NSCLC) is one of the most prevalent tumors with high incidence and mortality across the globe. Recently, increasing studies have demonstrated that circular RNAs (circRNAs) exert outstanding functions in NSCLC progression. Notwithstanding, we are still in the dark about the function and exact mechanism of circ-PITX1, a newly discovered circRNA. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) confirmed the profile of circ-PITX1 in NSCLC tissues and adjacent normal tissues. Gain- and loss- of function assay verified the impact of circ-PITX1 and miR-30e-5p on the proliferation, invasion, and migration of NSCLC cells (H1975 and A549). Bioinformatics analysis corroborated the downstream mechanisms of circ-PITX1. Dual-luciferase reporter gene assay and RNA immunoprecipitation (RIP) examined the interactions between circ-PITX1 and miR-30e-5p, miR-30e-5p and ITGA6. The protein levels of ITGA6, PI3K, AKT were determined by Western blot. circ-PITX1 was substantially up-regulated in NSCLC tissues and cells, and circ-PITX1 up-regulation was correlated with NSCLC patients' poor survival. Functionally, circ-PITX1 overexpression or miR-30e-5p inhibition markedly facilitated proliferation, migration, invasion, epithelial-mesenchymal transition (EMT), reduced apoptosis, and enhanced ITGA6/PI3K/AKT expression in NSCLC cells, whereas circ-PITX1 knockdown or miR-30e-5p up-regulation resulted in the opposite results. Mechanistically, circ-PITX1 acted as a sponge of miR-30e-5p, which targeted the 3'untranslated region (UTR) of ITGA6. Knockdown of circ-PITX1 or overexpressing miR-30e-5p reduced ITGA6/PI3K/AKT axis. circ-PITX1 modulates the miR-30e-5p/ITGA6 axis to boost NSCLC progression, hence functioning as an oncogene.

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## KEYWORDS

NSCLC; progression; circ-PITX1; miR-30e-5p; ITGA6

## 1. Introduction

Non-small cell lung cancer (NSCLC), the most prevailing lung cancer that accounts for around 80% of all relevant cases, is a leading contributor to cancer-related death across the world [1]. Death caused by NSCLC can be largely attributed to recurrent metastasis, while there is still a lack of accurate indicators to evaluate NSCLC progression or effective targeted drugs for NSCLC treatment [2,3]. Therefore, probing the mechanisms of NSCLC is critical to identifying new therapeutic targets and developing more effective therapies.

Circular RNAs (circRNAs) are a new type of non-coding RNAs that are abundant in eukaryotes [4]. Also, circRNAs are a class of trashy by-products in the course of gene transcription. Nevertheless, circRNAs can act as a decoy molecule, a scaffold

molecule, or a guide molecule to modulate gene expression, thereby influencing the pathophysiological processes of diverse diseases [5–8]. Emerging circRNAs have been uncovered to present aberrant expression in tumors and affect tumor development. For instance, hsa-circRNA-103809 exhibits pronounced expression in lung cancer tissues and bolsters lung cancer cell proliferation and invasion [9]. Circ-0026344, down-regulated in colorectal cancer, can suppress colorectal cancer progression when it gets overexpressed [10]. Various circRNAs have been studied in NSCLC. For instance, circSLC8A1 and circFARSA are up-regulated in NSCLC tissues and function as prospective prognostic biomarkers and therapeutic targets of NSCLC [11,12]. Additionally, an increasing number of circRNAs have been confirmed to regulate NSCLC

**CONTACT** Xiaoping Li  [horaceli@nankai.edu.cn](mailto:horaceli@nankai.edu.cn)  Department of Thoracic Surgery, Tianjin First Central Hospital, School of Medicine, Nankai University, 24 Fukang Road, Nankai District, 300074 Tianjin, China

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development [13]. circ-PITX1, situated at chr5:134363423–134369964, was first identified by Julia Salzman, et al. in A549 cells [14]. Nonetheless, the function of circ-PITX1 in NSCLC remains poorly understood.

MicroRNAs (miRNAs) are small non-coding molecules that modulate the expression of over 60% of human genes. Abnormally expressed miRNAs regulate tumor proliferation, apoptosis, migration, and metastasis, pertaining to human cancer pathogenesis [15]. Fundamentally, miRNAs regulate gene expression by specifically combining with the 3'-untranslated region (3'-UTR) of target mRNAs, thus modulating tumor cells' biological behaviors [16]. For instance, miR-224-5p targets the PIK3R3/AKT3 pathway to participate in uveal melanoma cell proliferation, invasion, and migration [17]. miR-30e-5p, a member of the miR-30 family, regulates the Sirt1/JAK/STAT3 signaling mediated by USP22, serving as an inhibitor in NSCLC [18]. These reports hint that miR-30e-5p has the potential to mediate NSCLC treatment.

Integrin subunit alpha 6 (ITGA6), an integrin receptor, contributes to cell-to-cell adhesion [19] and exerts a function in inflammation and fibrosis [20]. ITGA6 has been revealed as a modulator to influence tumor cell proliferation, migration, and invasion. For instance, Twist2, a member of the basic helix-loop-helix (bHLH) family, modulates the profiles of ITGA6 and CD44 to exacerbate kidney cancer cells' proliferation, migration, and invasion and cramp their apoptosis [21]. What's more, higher ITGA6 expression predicts a poor prognosis of pancreatic cancer, while ITGA6 down-regulation attenuates cancer cell invasion and metastasis [22]. Interestingly, p53-elicited miR-30e-5p targets ITGA6 and ITGB1 to repress invasion and metastasis, displaying its anti-tumor function in colorectal cancer [23]. Nonetheless, the function of miR-30e-5p-mediated ITGA6 in NSCLC has not been investigated.

Here, we examined the interaction among circ-PITX1, miR-30e-5p, and ITGA6 in NSCLC cells so as to reveal their underlying mechanisms in NSCLC development. We discovered that circ-PITX1 was up-regulated in NSCLC tissues and cell lines. Moreover, circ-PITX1 accelerated the malignant phenotypes of NSCLC cells.

Mechanistically, circ-PITX1 sponged miR-30e-5p as a competitive endogenous RNA (ceRNA), which then targeted ITGA6. Overall, this work has disclosed a novel molecular mechanism of the circ-PITX1-miR-30e-5p-ITGA6 axis in NSCLC and provided new theoretical references for treating NSCLC.

## 2 Materials and methods

### 2.1 Clinical sample collection

Tumor tissues and adjacent non-tumor tissues of 40 patients with primary NSCLC who underwent surgical resections in the Tianjin First Central Hospital from January 2014 to January 2015 were collected. All patients were diagnosed with NSCLC by pathologists. None of the patients had been subjected to any chemotherapy or radiotherapy prior to the surgery. The adjacent non-tumor tissues were at least 3 cm away from the tumor margin, with no cancer cells discovered in them. All the specimens were removed and immediately stored in liquid nitrogen at  $-196^{\circ}\text{C}$  for subsequent analysis. This study had received the green light from the ethics committee of Tianjin First Central Hospital, and all patients involved signed the informed consent.

### 2.2 Cell culture

Human NSCLC cell lines (H1975 and A549) and the normal lung epithelial cell line BEAS-2B were ordered from the Chinese Academy of Sciences (Shanghai, China). The cells were cultured with a DMEM-F12 medium (Thermo Fisher HyClone, Utah, USA) incorporating 10% FBS (Thermo Fisher Scientific, MA, USA) in an incubator with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . The medium was substituted every 2 days, and the cells were passed every 2 or 3 days. The following experiments were implemented when the cells achieved about 70–90% abundance.

### 2.3 Cell transfection

H1975 and A549 cells in the logarithmic growth phase were inoculated onto 60-mm culture plates at  $1 \times 10^7$  cells/well and incubated at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  for 24 hours prior to transfection.

GenePharma (Shanghai, China) was responsible for the design of the siRNA specifically targeting circ-PITX1 (si-circ-PITX1), siRNA specifically targeting ITGA6 (si-ITGA6), circ-PITX1 overexpression plasmids, and circ-PITX1 negative controls (vector or si-NC). miR-30e-5p mimics, miR-30e-5p inhibitors, and their negative controls (miR-NC and NC-in) were supplied by RiboBio (Guangzhou, China). Lipofectamine 3000 (Thermo Fisher Scientific, IL, USA) was adopted to transfect the above expressing vectors into NSCLC cells in line with the supplier's instructions. The transfection validity was examined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) 48 hours following the transfection.

#### 2.4 RNA extraction and qRT-PCR analysis

Total RNA was extracted out of tissues or cultured cells with the use of the TRIzol reagent (Invitrogen, Shanghai, China). The Cytoplasmic and Nuclear RNA Purification Kit (Norgen Biotek, Cat. 21000) was employed for the purification of the cytoplasmic and nuclear RNA obtained from the cultured NSCLC cells. The First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) was taken to reversely transcribe the total RNA into cDNA. The LightCycler FastStart DNA MasterPlus SYBR Green I kit (Roche Diagnostics, Burgess Hill, UK) was applied for qRT-PCR as instructed by the manufacturer. The  $2^{-\Delta\Delta CT}$  method was utilized to calculate the relevant profiles of circ-PITX1, miR-30e-5p, and ITGA6. Primers for qRT-PCR are as follows: circ-PITX1 Forward: 5'-GTGCCAGT AGCAATAACTGCC-3', Reverse: 5'-CAGAA GATGGCACTGGTAGGT-3'; miR-30e-5p Forward: 5'-GTCTCCCAGAUAAUGCG-3', Reverse: 5'-CGTTCAGGGTCC-3'; ITGA6 Forward: 5'-TCATGGATCTGCAAATGGAA-3', Reverse: 5'-GGCGGAGGTCAATTCTGTTA-3'; GAPDH Forward: 5'-AGGTCGGTGTGAACGG ATTTG; Reverse: 5'-GGGGTCGTTGATGG CAACA-3'; U6 Forward: 5'-TCTTCGTCA TCACATATACTAAAAT-3', Reverse: 5'-CTCTTCACGAATTTTCGTGTCAT -3'.

#### 2.5 Cell counting kit-8 (CCK-8) assay

H1975 and A549 cells in the logarithmic phase of each group were counted following trypsinization and centrifugation. Then the cells were inoculated into 96-well plates, with 100  $\mu$ l of cell suspension ( $2 \times 10^4$  cells/mL) administered to each well. Twenty-four hours after incubation, the culture medium was discarded and substituted by a 90  $\mu$ L complete medium in company with 10  $\mu$ L of CCK-8 solution (Abcam, Shanghai, China). Subsequent to one hour's incubation, a microplate reader was adopted to check the absorbance value of each well at 450 nm.

#### 2.6 Transwell assay

Before the Transwell assay was conducted, the bottoms of the Transwell chambers were coated with Matrigel (Becton, Dickinson and Company) overnight. H1975 and A549 cells in the logarithmic growth phase were harvested, with the cell density adjusted to  $1 \times 10^5$ /mL using an FBS-free DMEM-F12 medium. The cell suspension was given to the upper Transwell compartment, with 500  $\mu$ L of a complete medium supplemented with 20% FBS administered to the lower chamber of the 24-well plate, for 24 hours' incubation at 37°C with 5% CO<sub>2</sub>. As the Transwell compartments were taken out, the cells on the bottoms of them were cleaned using cotton swabs. The cells in the plates were immobilized with 95% ethanol at room temperature for 30 minutes, dyed with crystal violet for 20 minutes, and flushed in water 3 times. An inverted microscope was introduced to observe the cells and capture their images. In the invasion experiment, the steps were the same as those in the migration assay barring the use of Matrigel for coating the bottoms of Transwell chambers. The experiment of each group was implemented in triplicate.

#### 2.7 Western blot

The tissues and cells were lysed employing RIPA lysis buffer (Beyotime, Wuhan, China) on ice for 20 minutes and then centrifuged at 14000 rpm and 4°C for 20 minutes. Following centrifugation, the supernatant was harvested, and the total protein

was quantified with the BCA protein quantitative kit (Beyotime, Wuhan, China). With the concentration adjusted, the protein samples were boiled for 5 minutes for denaturation. Subsequent to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the protein was electrically transferred onto PVDF membranes. After that, TBST solution containing 3% BSA was applied to seal the membranes at room temperature for 1 hour, which were then incubated along with the diluted primary antibodies at 4°C overnight. The next morning, the membranes were flushed with TBST 5 times (3 minutes each) and incubated along with the diluted horseradish peroxidase (HRP)-tagged anti-rabbit secondary antibody (concentration 1:300) for an hour. They were then rinsed in TBST another 3 times, 10 minutes each. Western blot reagent (Invitrogen) was exploited for color development. All the primary antibodies utilized in this study were bought from Abcam, covering the Anti-ITGA6 antibody (ab181551, 1:1500), anti-PI3K(ab32089, 1:1000), anti-PI3K (phospho Y458) (ab278545, 1:1000), anti-AKT(ab8805, 1:500), anti-AKT (phospho T308) (ab38449, 1:1000), Anti-E-cadherin antibody (ab40772, 1:1000), Anti-Bax antibody (ab32503, 1:1000), Anti-Bcl-2 antibody (ab32124, 1:1000), Anti-C-caspase-3 antibody (ab32042, 1:1000), Anti-GAPDH antibody (ab9485, 1:1000), Anti-N-cadherin antibody (ab207608, 1:1000), Anti-Vimentin antibody (ab16700, 1:1000), and anti-GAPDH (ab9485, 1:500).

## 2.8 Colony formation assay

Colony formation assay evaluated NSCLC cell proliferation. NSCLC cells were collected, and 500 cells were seeded onto a 6-well plate. Following ten days of incubation, the cells in the plate were flushed with PBS three times, immobilized with ethyl alcohol for 30 seconds, and dyed using crystal violet. Finally, the number of colonies was calculated with the naked eye after PBS washing.

## 2.9 RNA immunoprecipitation (RIP)

The Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was taken for RIP. NSCLC cells

H1975 and A549, transfected along with miR-30e-5p or miR-NC, were lysed with RIP Lysis buffer incorporating the protease inhibitor cocktail and RNase inhibitor. Cell lysates were extracted and incubated along with the antibodies against Ago2 (Millipore) or rabbit IgG-coated beads at 4°C overnight. After the use of proteinase K buffer for protein removal, the immunoprecipitated RNA was extracted using TRIzol. qRT-PCR examined circ-PITX1 and ITGA6 enrichment.

## 2.10 Dual-luciferase reporter assay

The targeting correlation between miR-30e-5p and circ-PITX1, miR-30e-5p and ITGA6 was verified by the dual-luciferase reporter assay. The sequences of wild-type circ-PITX1 (circ-PITX1-WT) and ITGA6 (ITGA6-WT) or Mutant circ-PITX1 (circ-PITX1-MT) and ITGA6 (ITGA6-MT) without the binding sites of miR-30e-5p were amplified and slotted into pmirGLO dual-luciferase vectors. Next, H1975 and A549 cells were transfected together with the above vectors and miR-30e-5p or miR-NC for 48 hours' incubation. The Dual-Luciferase Reporter Assay System (Promega Corp.) was introduced to examine the dual-luciferase activity as instructed by the supplier. The experiment of each group was done in triplicate.

## 2.11 In-vivo experiments

Twenty female nude mice on a BALB/c background, 4 to 5 weeks of age, were supplied by Shanghai Jiesijie experimental animal Co., Ltd. The mice were reared under specific pathogen-free (SPF) housing. H1975 cells were transfected along with circ-PITX1 overexpression plasmids and their negative controls (Vector). Then,  $1 \times 10^7$  cells suspended in 100  $\mu$ L of PBS buffer were subcutaneously administered to the animals, which were further kept under SPF conditions. Within the following 5 weeks, the tumor volume *in vivo* was gauged every week employing a digital caliper as per the formula  $V = 0.5 \times (W^2 \times L)$ , where "V" is defined as the volume, "W" as the minor axis, and L as the major axis of the measurement. Five weeks later, the mice were sacrificed, with their tumors resected, weighed, and photographed. The expression of in the tumor tissues ITGA6 was detected by immunohistochemistry using

Anti-ITGA6 antibody (ab181551, 1:100) as primary antibody [24]. This experiment had received the imprimatur from the Animal Ethics Committee of Tianjin First Central Hospital.

## 2.12 Statistical analysis

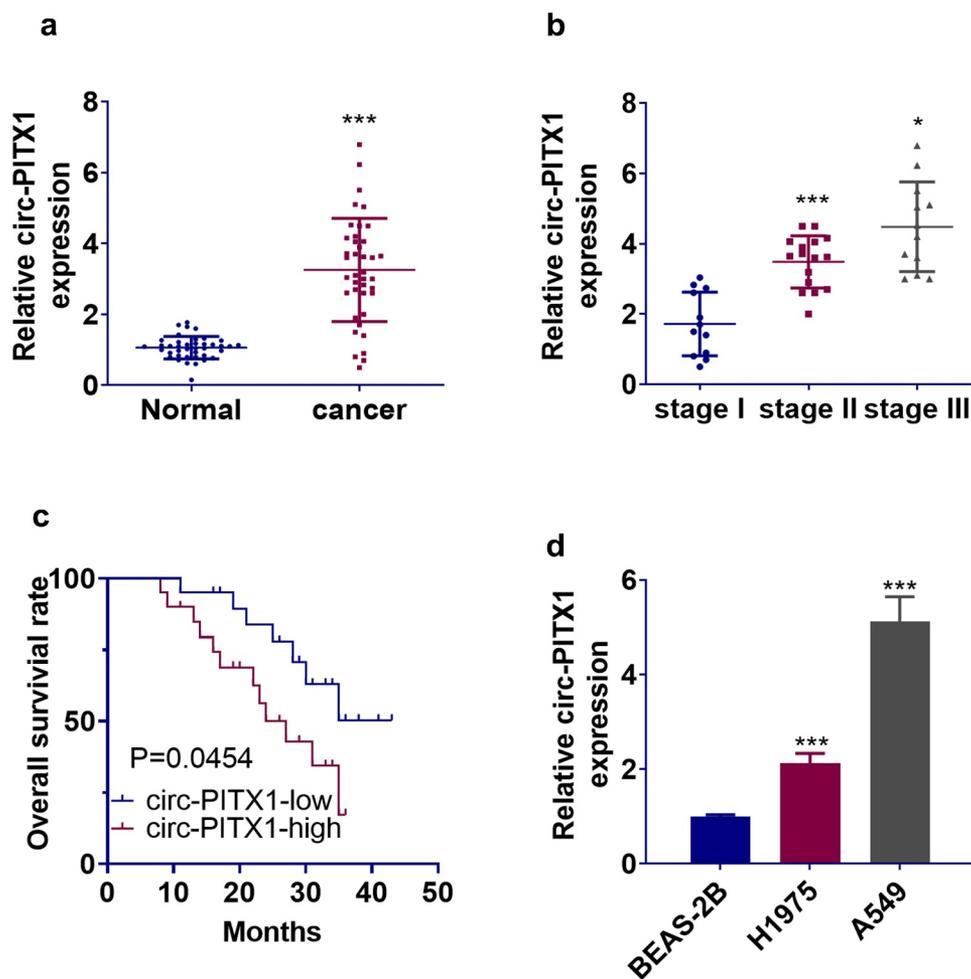
The statistical software SPSS17.0 (SPSS Inc., Chicago, IL, USA) was introduced for analysis, with the measurement statistics presented as mean  $\pm$  standard deviation ( $\bar{x} \pm s$ ). T test was taken for comparison between two groups, and the analysis of variance was adopted to compare multiple means. The Chi-square test was done for fourfold table data analysis. The Kaplan-Meier

method and log-rank test analyzed the overall survival difference between patients with a high or low profile of circ-PITX1.  $P < 0.05$  was regarded statistically meaningful.

## 3 Results

### 3.1 The profile and significance of circ-PITX1 in NSCLC tissues and cells

To understand the function of circ-PITX1 in NSCLC, we implemented qRT-PCR to check circ-PITX1 expression in NSCLC tissues and cell lines. It turned out that circ-PITX1 expression was dramatically elevated in NSCLC tissues as opposed to normal non-tumor tissues (Figure 1a,  $p < 0.001$ ).

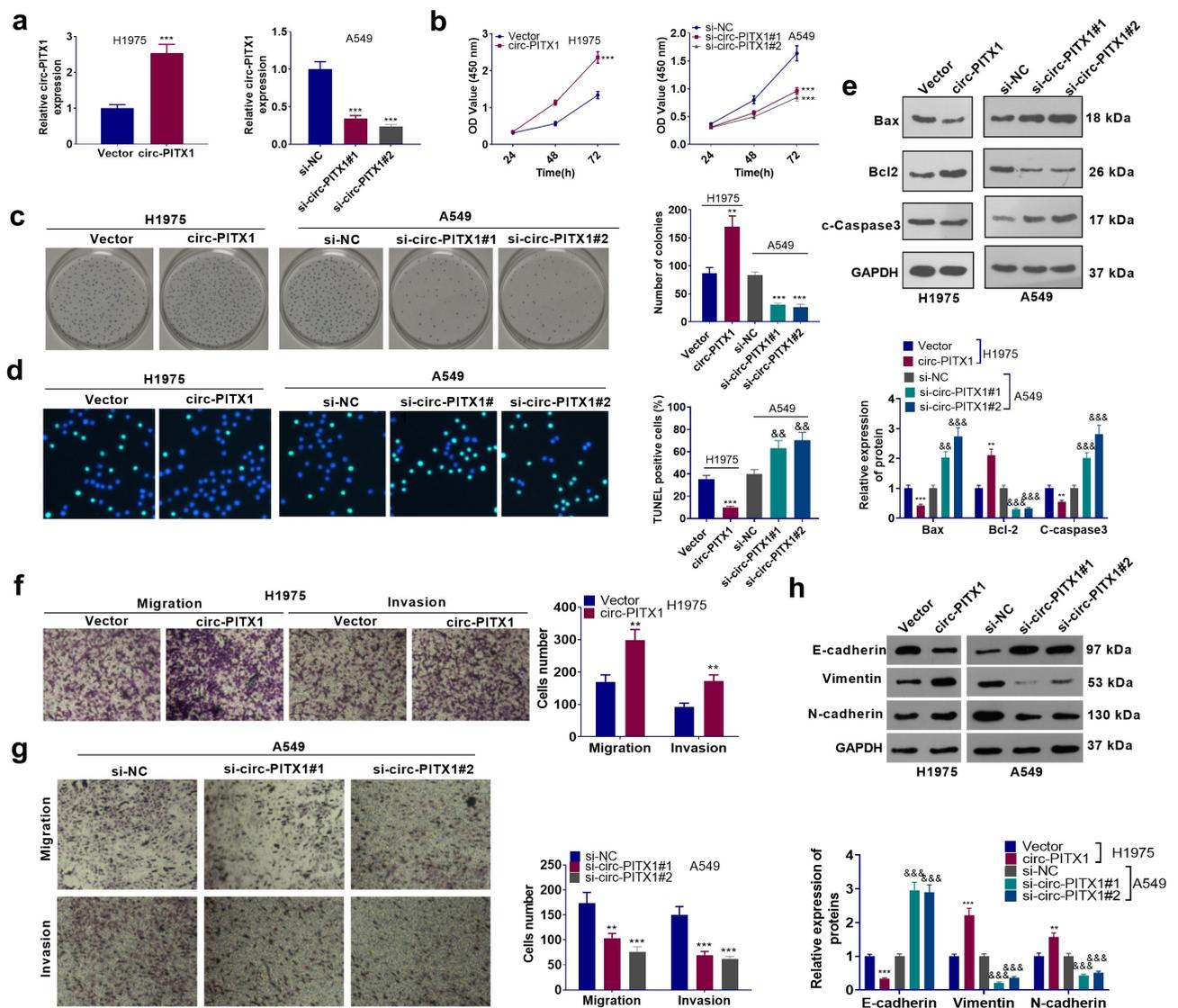


**Figure 1.** The expression and significance of circ-PITX1 in NSCLC tissues and cells. (a) qRT-PCR determined circ-PITX1 expression in 40 NSCLC tissues and in the 40 paired normal non-tumor tissues, \*\*\* $P < 0.001$ . (b) The level of circ-PITX1 in NSCLC tissues with different TNM stages, \* $P < 0.05$ , \*\* $P < 0.01$ . (c) The Kaplan-Meier method and log-rank test evaluated the overall survival differences between NSCLC patients with high or low levels of circ-PITX1 expression. (d) qRT-PCR checked the profile of circ-PITX1 in human NSCLC cell lines (H1975 and A549) and normal lung epithelial cells BASE-2B, \*\*\* $P < 0.001$  vs. the BASE-2B group.

With the increase in the TNM stage, there was also an uplift in circ-PITX1's levels (Figure 1b). NSCLC patients with up-regulated circ-PITX1 manifested poorer overall survival (Figure 1c), higher TNM stages, and earlier local lymph node metastasis (Table 1). More interestingly, circ-PITX1's level was substantially heightened in human NSCLC cells (H1975 and A549) as compared with normal lung epithelial cells BEAS-2B (Figure 1d). These findings revealed that circ-PITX1 functioned as an oncogene in NSCLC.

### 3.2 The impact of circ-PITX1 on the malignant phenotypes of NSCLC cells

To dig deeper into the function of circ-PITX1 in NSCLC, we performed gain- and loss- of function assays to examine circ-PITX1 expression in H1975 and A549 cell lines, respectively. CCK-8 and colony formation assays measured cell proliferation, indicating that circ-PITX1 overexpression facilitated NSCLC cell proliferation, whereas circ-PITX1 down-regulation led to proliferation inhibition (Figure 2a-c). As



**Figure 2.** The influence of circ-PITX1 on the malignant phenotypes of NSCLC cells. (a) H1975 and A549 cell lines were taken for the construction of the circ-PITX1 overexpression and down-regulation cell models. B and C. CCK-8 (b) and colony formation assay (c) examined cell proliferation, NS  $P > 0.05$ , \*\* $P < 0.01$  \*\*\* $P < 0.001$  vs. the vector group or the si-vector group. (d) TUNEL gauged cell apoptosis. (e) Western blot tested the expression of apoptosis-related proteins (Bax, Bcl-2, and c-Caspase3). f and g. Transwell tracked cell migration (f) and invasion (g). (h) Western blot determined the protein levels of EMT markers (E-cadherin, Vimentin, and N-cadherin). \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (vs. Vector group), && $P < 0.01$ , &&& $P < 0.001$  (vs. si-NC group).  $n = 3$ .

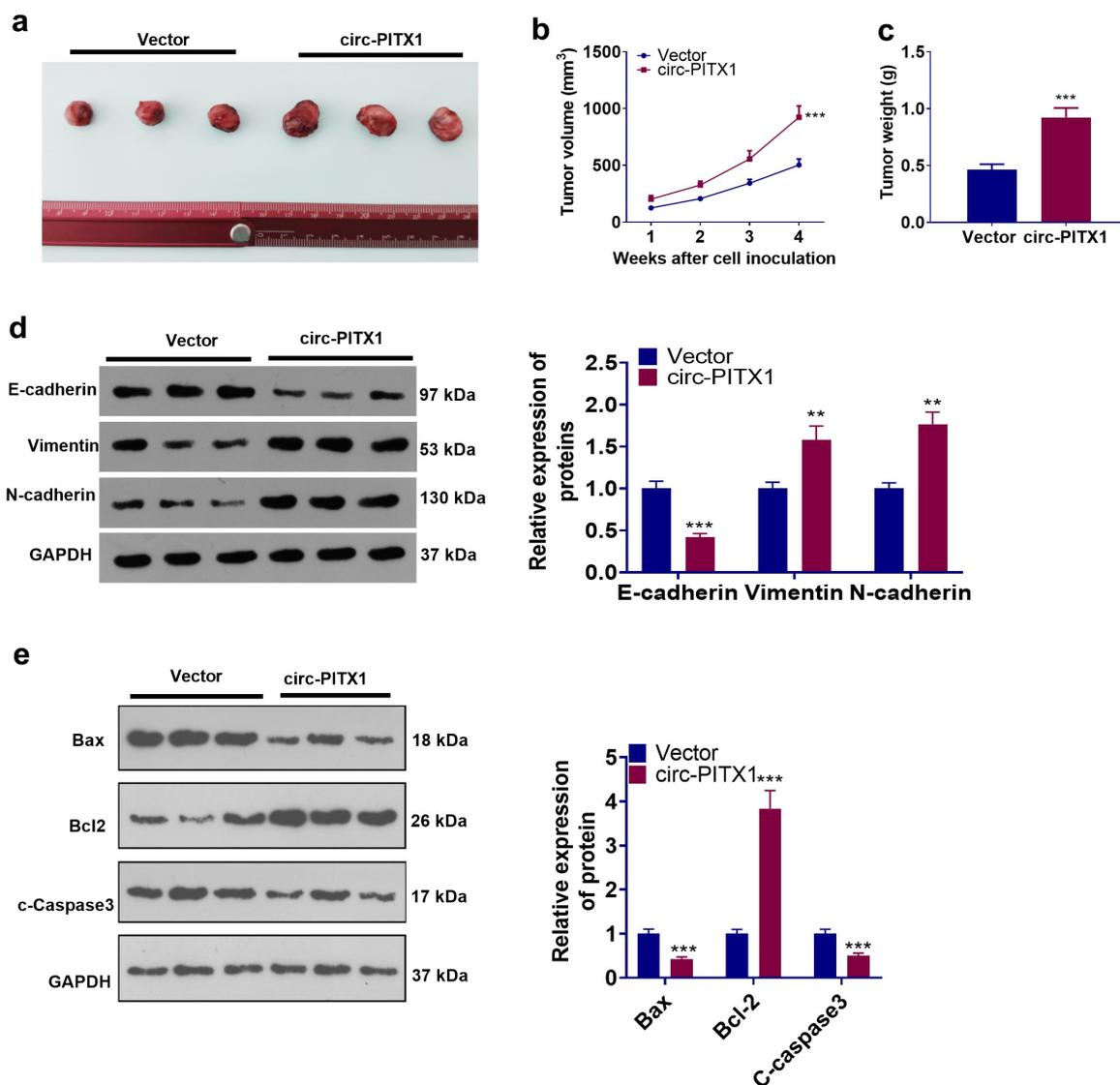
testified by TUNEL outcomes, circ-PITX1 overexpression restrained TUNEL-positive cell numbers, whereas down-regulation of circ-PITX1 raised the number of TUNEL-positive cells in H1975 and A549 cells (Figure 2d). WB examined the expression of apoptosis-related proteins (Bax, Bcl-2, and c-Caspase3) in H1975 and A549 cells. As a result, circ-PITX1 overexpression blocked the expression of the proapoptotic proteins Bax and c-Caspase3 and facilitated the expression of the apoptosis-inhibitory protein Bcl-2 in H1975 and A549 cells, while down-regulation of circ-PITX1 led to the opposite result (Figure 2e). Tranwell assay was adopted to monitor cell migration and invasion, while Western blot was utilized to confirm the protein profiles of EMT markers (E-cadherin, Vimentin, and N-cadherin). Notably, circ-PITX1 overexpression enhanced cell migration and invasion, down-regulated the epithelial marker E-cadherin, and up-regulated interstitial markers Vimentin and N-cadherin (Figure 2f–h). On the contrary, circ-PITX1 down-regulation markedly reduced NSCLC cells' migration, invasion, and EMT. Therefore, circ-PITX1 was involved in NSCLC development via boosting cell proliferation and metastasis.

### 3.3 The influence of circ-PITX1 on NSCLC cells *in vivo*

To grasp the function of circ-PITX1 in NSCLC progression, we resorted to *in-vivo* experiments. It was discovered that circ-PITX1 overexpression considerably accelerated NSCLC cell growth (Figure 3(a–c)). We also determined the profiles of EMT markers in the *in-vivo* tumors and uncovered that E-cadherin was down-regulated, whereas Vimentin and N-cadherin were up-regulated in the circ-PITX1 overexpression group (vs. the vector group) (Figure 3d). Additionally, WB data identified that up-regulation of circ-PITX1 curbed the expression of Bax and c-Caspase3 and heightened the Bcl-2 expression in tumor tissues versus the Vector group (Figure 3e). These discoveries supported that circ-PITX1 was an oncogene in NSCLC.

### 3.4 Circ-PITX1 bolstered ITGA6 expression

We analyzed ITGA family members expression in LUSC (Lung squamous cell carcinoma) through the online GEPIA database (<http://gepia.cancer-pku.cn/>) [25]. The data showed that ITGA6 and ITGA11 was significantly upregulated in LUSC tissues compared with those in normal tissues (sup Figure 1). As indicated by the analysis result from Kaplan-Meier Plotter (<http://kmplot.com/analysis/>) [26], higher level of ITGA5 and ITGA6 predict poorer overall survival of lung cancer patients (sup Figure 2). The data from GEPIA and the Human Protein atlas (<http://gepia.cancer-pku.cn/detail.php>) unveiled that ITGA6 presented a negative expression in normal lung tissues but exhibited moderate-to-strong expression in lung tissues, mainly situated at the cytomembrane of lung cancer cells (Figure 4(a–b)). A higher level of ITGA6 was correlated with the poorer overall survival and first progression of lung cancer patients (<http://kmplot.com/analysis/>) (Figure 4c). Those statistics further demonstrated that ITGA6 exerted an oncogenic function in NSCLC. Next, the mRNA and protein levels of ITGA6 in NSCLC cells with up-regulated circ-PITX1 or down-regulated circ-PITX1 were determined. The outcomes denoted that circ-PITX1 boosted ITGA6 expression at both the mRNA and protein levels (Figure 4d–e). For investigating the downstream mechanism of ITGA6, we analyzed ITGA6-related genes in lung cancer through LinkedOmics (<http://linkedomics.org/login.php>) [27]. Using its online Gene Set Enrichment Analysis (GSEA) tool, we found that ITGA6 positively regulates PI3K/AKT pathway (sup Figure 3a–b). Moreover, PIK3CA and AKT1 have positive relationship with ITGA6 in terms of their expression in LUSC (<http://gepia.cancer-pku.cn/>) (sup Figure 3c). We implemented WB, which corroborated that up-regulating circ-PITX1 boosted the phosphorylation of PI3K and Akt, while down-regulating circ-PITX1 choked the phosphorylation of PI3K and Akt in NSCLC cells (Figure 4f). Finally, we employed IHC and uncovered that up-regulating circ-PITX1 elevated the ITGA6 expression in tumor tissues (compared with vector group) (Figure 4g). Hence, we substantiated that circ-PITX1 might boost NSCLC via enhancing ITGA6 expression.

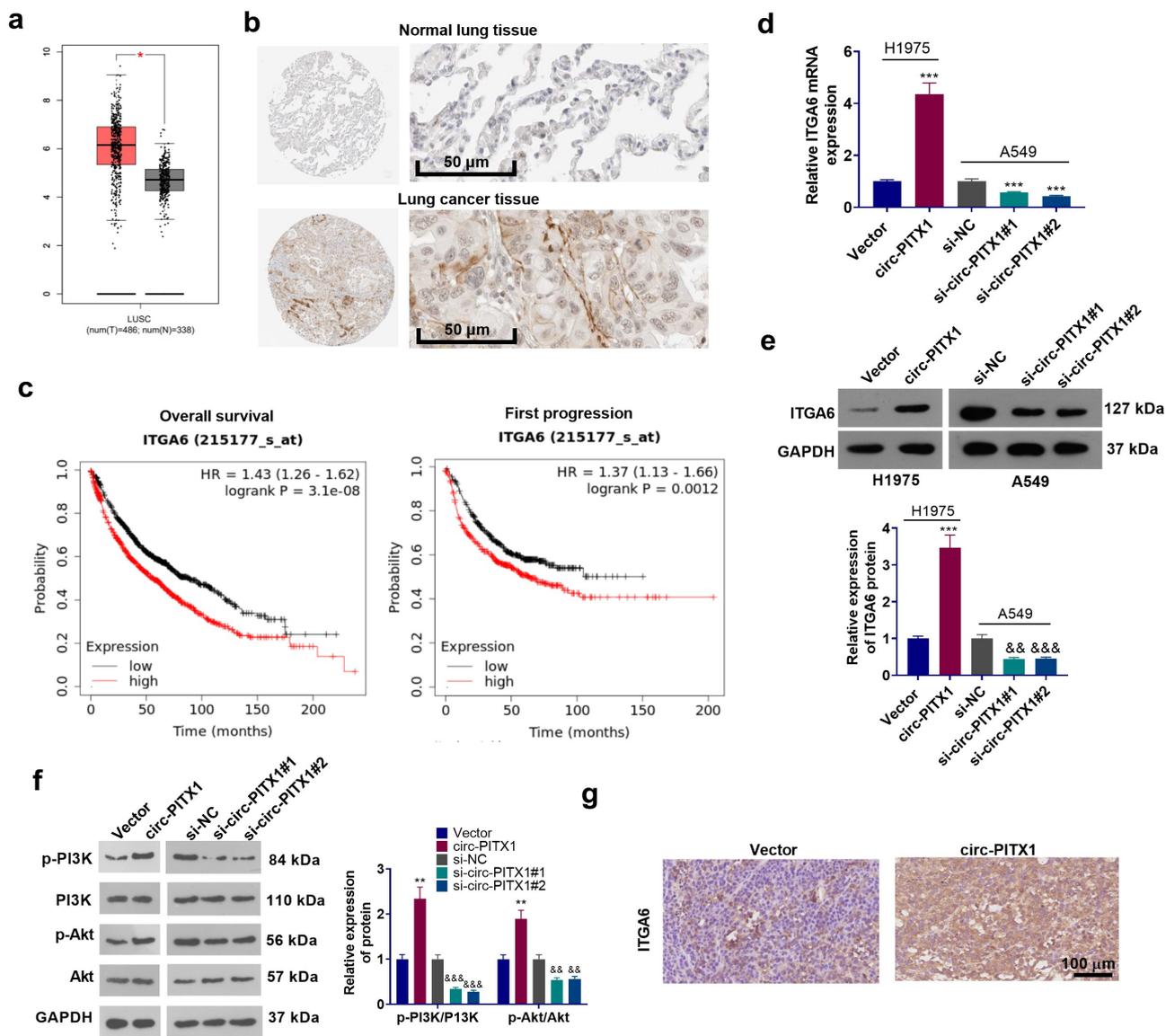


**Figure 3.** The impact of circ-PITX1 on NSCLC cells in vivo. H1975 cells were transfected along with circ-PITX1 overexpression plasmid or the vector (NC) and then subjected to *in-vivo* experiments. (a) The images of tumors in the two groups. b and c. The tumor volume (b) and weight (c) were calculated, \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. the vector group. d and e. Western blot verified the protein levels of EMT markers (E-cadherin, Vimentin, and N-cadherin) and apoptosis-related proteins (Bax, Bcl-2, and c-Caspase3). \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (vs. Vector group).  $n = 3$ .

### 3.5 Circ-PITX1 sponged miR-30e-5p

To probe the molecular mechanism between circ-PITX1 and ITGA6, we predicted the underlying miRNA of circ-PITX1 or ITGA6 with the help of Starbase (<http://starbase.sysu.edu.cn/>). Venn's diagram displayed that eight miRNAs were discovered. miR-30e-5p has been substantiated to target ITGA6 [23]. To further examine the binding correlation between circ-PITX1 and miR-30e-5p, ITGA6 and miR-30e-5p, we

implemented the dual-luciferase reporter gene assay and disclosed that miR-30e-5p mimics vigorously dampened the luciferase activity of H1975 cells transfected along with the circ-PITX1-WT or ITGA6-WT luciferase vectors (Figure 5b–c) but exerted no remarkable influence on H1975 cells transfected with the circ-PITX1-MT or ITGA6-MT luciferase vectors (Figure 5d). RIP assay confirmed that circ-PITX1 and ITGA6 were more enriched by miR-30e-5p in the anti-Ago2 group

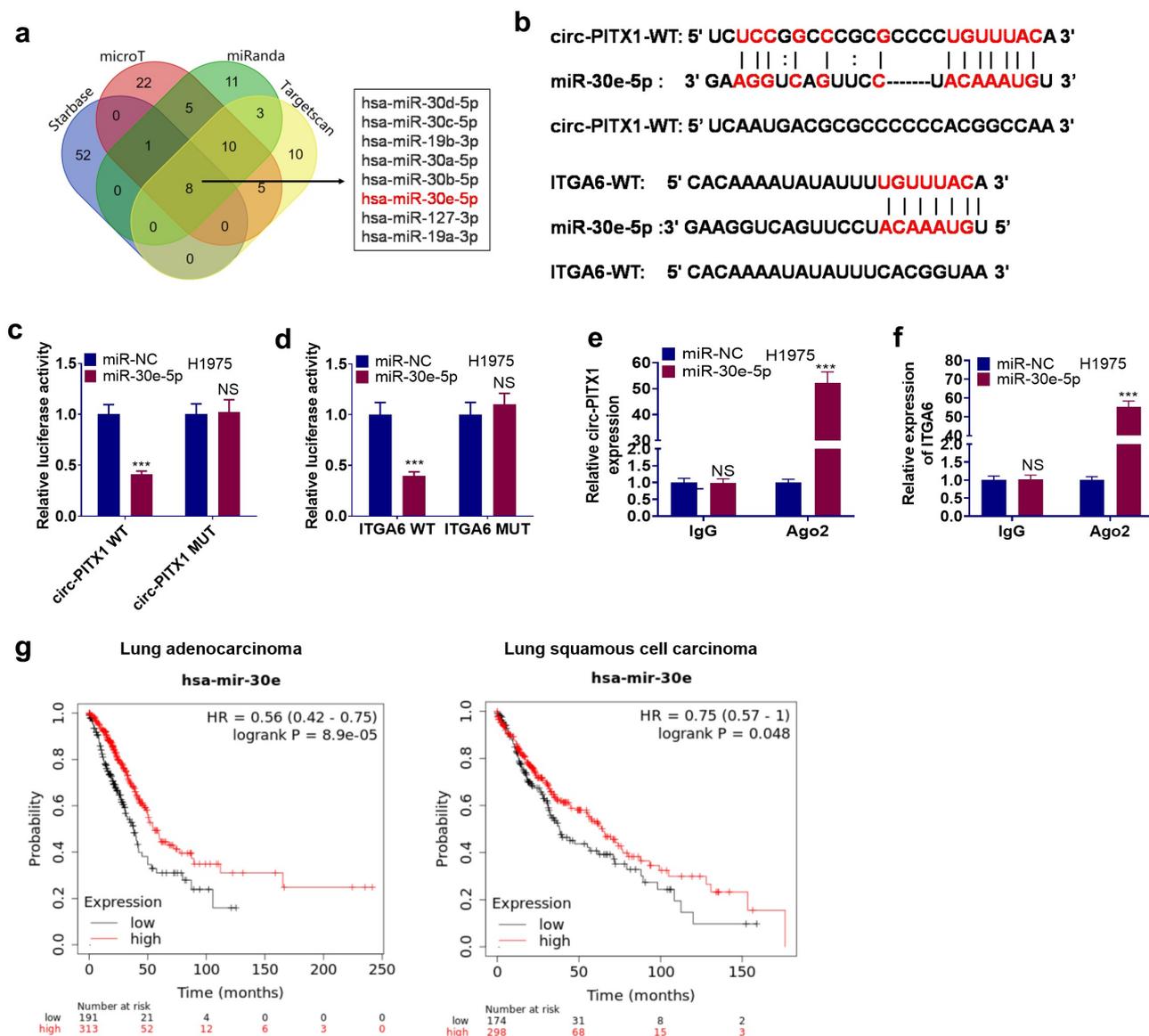


**Figure 4.** Circ-PITX1 enhanced ITGA6's expression. (a) The GEPIA (<http://gepia.cancer-pku.cn/detail.php>) database determined ITGA6's expression in lung squamous cell carcinoma (LUSC). b–c. The Human Protein atlas (<http://gepia.cancer-pku.cn/detail.php>) confirmed ITGA6 expression in normal lung tissues or lung cancer tissues. A higher level of ITGA6 intricately pertained to the poorer overall survival of lung cancer patients. D–F. RT-PCR and Western blot measured the mRNA and protein levels of ITGA6, p-PI3K and p-Akt in NSCLC cells up-regulating circ-PITX1 or down-regulating circ-PITX1. G: IHC testified ITGA6 expression in tumor tissues.  $^{***}P < 0.01$  (vs.Vector group).  $^{&&}P < 0.01$ ,  $^{&&&}P < 0.001$  (vs.si-NC group),  $n = 3$ .

(Figure 5e–f). The affinities between miR-30e-5p's level and the overall survival status of lung cancer patients were examined. As a result, a lower level of miR-30e-5p correlated with the worse survival of lung cancer patients (Figure 5g). In short, miR-30e-5p was a sponge of circ-PITX1 and targeted ITGA6, and miR-30e-5p might play a potential part in NSCLC development.

### 3.6 miR-30e-5p regulated NSCLC development

To investigate the function of miR-30e-5p in NSCLC development, we transfected H1975 cells along with miR-30e-5p mimics and transfected A549 cells with miR-30e-5p inhibitors (Figure 6a). CCK8, colony formation assay, TUNEL staining, Transwell assay, and Western blot evaluated the function of miR-30e-5p in



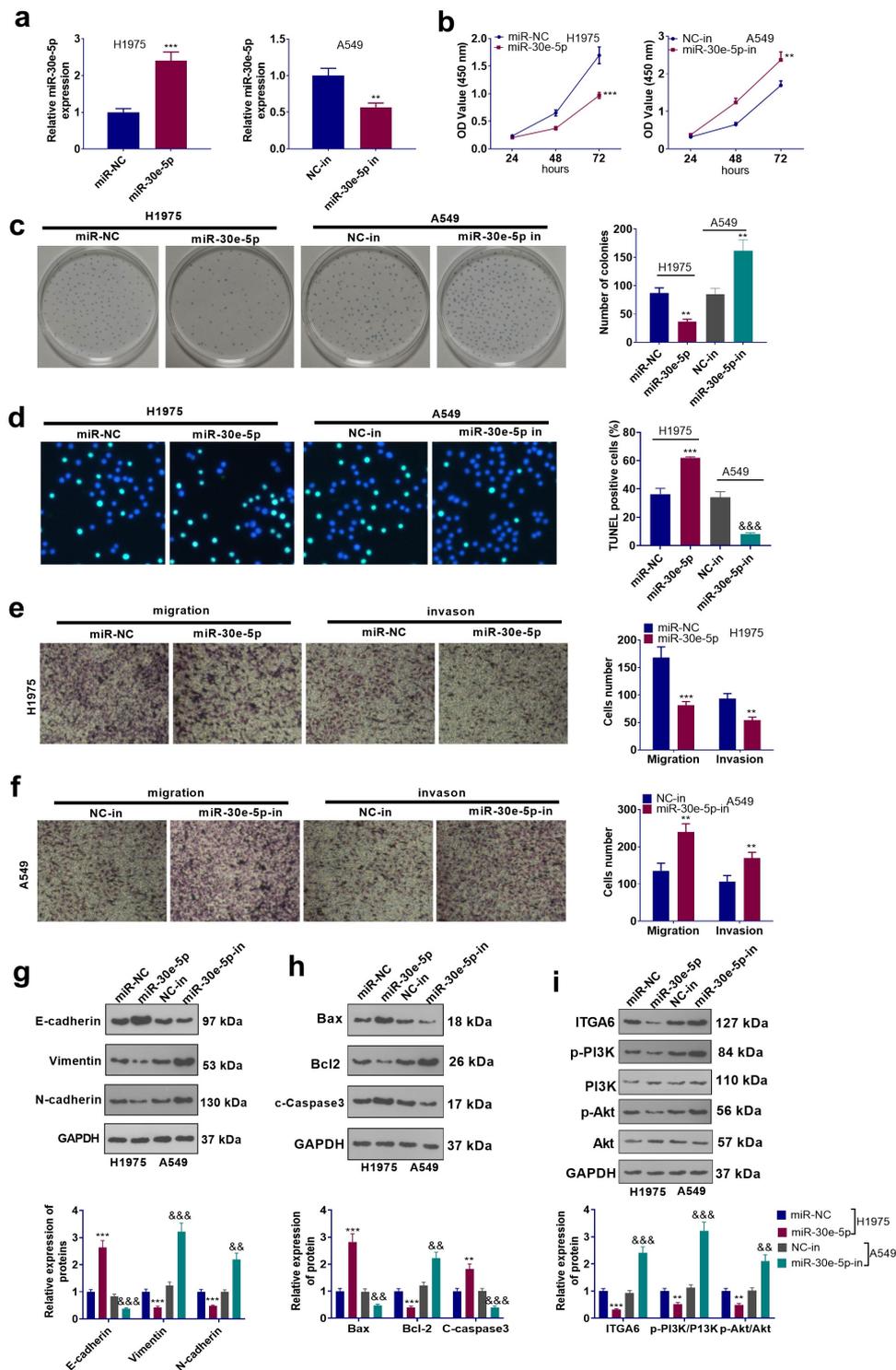
**Figure 5.** Circ-PITX1 sponged miR-30e-5p. (a) miR-30e-5p was a candidate miRNA target of circ-PITX1 and ITGA6 as predicted by Starbase (<http://starbase.sysu.edu.cn/>). (b) The binding sites between miR-30e-5p and circ-PITX1, miR-30e-5p and ITGA6. c–f. Dual-luciferase reporter gene assay (c–d) and RIP assay (e–f) examined the binding correlation between miR-30e-5p and circ-PITX1, miR-30e-5p and ITGA6. (g). K-M plotter (<http://kmplot.com/analysis/>) revealed that a lower level of miR-30e-5p was relevant to the poorer survival of lung cancer patients. NS  $P > 0.05$ , \*\*\* $P < 0.001$ .

NSCLC cells' proliferation, apoptosis, invasion, migration, and EMT. As a result, miR-30e-5p up-regulation mitigated H1975 cells' proliferation, intensified apoptosis, curbed cell migration, invasion, and EMT. In contrast, miR-30e-5p inhibition contributed to opposites results (Figure 6b–g). Finally, we evaluated the expression of Bax, Bcl-2, C-caspase2 and the ITGA6/PI3K/Akt pathways in the cells using WB. The outcome displayed that up-regulating miR-30e-5p enhanced the expression of Bax and c-Caspase3 and declined the expression of Bcl-2, ITGA6 and phosphorylation

of PI3K and Akt in cells, while inhibition of miR-30e-5p exerted the opposite effect (Figure 6h,i). These findings signified that miR-30e-5p attenuated the profile of ITGA6 to display its tumor-suppressing function in NSCLC.

### 3.7 Circ-PITX1 modulated NSCLC cell proliferation and metastasis by sponging miR-30e-5p

Considering the correlation between miR-30e-5p and circ-PITX1 in NSCLC cells, we performed



**Figure 6.** miR-30e-5p exerted an inhibitory function in NSCLC development. (a) H1975 and A549 cells were transfected along with miR-30e-5p mimics or the miR-30e-5p inhibitor, with miR-30e-5p's level confirmed by RT-PCR. B and C. CCK-8 (b) and colony formation assay (c) examined cell proliferation, NS  $P > 0.05$ , \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. the miR-NC group or the si-NC group. (d) TUNEL assay checked cell apoptosis. E and F. Transwell tracked cell migration (e) and invasion (f). \*  $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . g–i. Western blot measured the protein levels of EMT markers (g), apoptosis-related proteins (h), ITGA6, p-PI3K and p-Akt (i). \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (vs. miR-NC group). && $P < 0.01$ , &&& $P < 0.001$  (vs. NC-in group).  $n = 3$ .

rescue experiments to better understand the function of the circ-PITX1/miR-30e-5p axis in NSCLC. By contrast to the circ-PITX1 group, miR-30e-5p mimics barely influenced circ-PITX1's level, whereas circ-PITX1 overexpression hampered miR-30e-5p's level (Figure 7a–b). miR-30e-5p up-regulation repressed ITGA6's level and curbed the phosphorylation of PI3K and Akt, while the expression of these molecules was enhanced by circ-PITX1 (Figure 7c). Furthermore, NSCLC cells' malignant behaviors were monitored. It turned out that overexpression of circ-PITX1 exacerbated NSCLC cell proliferation, curbed apoptosis, and heightened migration, invasion, and EMT. Surprisingly, miR-30e-5p impeded cells' proliferation, amplified apoptosis, and hindered migration, invasion, and EMT in the circ-PITX1 group, which were all elicited by circ-PITX1 overexpression (Figure 7d–i). These outcomes disclosed that circ-PITX1 sponged miR-30e-5p to modulate NSCLC cell proliferation and metastasis.

### 3.8 Circ-PITX1 exerted oncogenic effects via the ITGA6/PI3K/Akt pathway

To further assay the function of the ITGA6/PI3K/Akt pathway in NSCLC, we administered si-ITGA6 and PI3K inhibitor (Wortmannin) to H1975 cells overexpressing circ-PITX1 or knocking down miR-30e-5p, respectively, for 24 hours. The ITGA6/PI3K/Akt pathway expression in H1975 cells was tested using WB. The data displayed that inhibition of ITGA6 choked ITGA6 expression and abated phosphorylation of PI3K and Akt in H1975 cells versus the circ-PITX1 group or the miR-30e-5p-in group. In parallel, the application of PI3K inhibitors resulted in the attenuation of PI3K and Akt phosphorylation in H1975 cells versus the circ-PITX1 group or the miR-30e-5p-in group, but it had little impact on ITGA6 expression (Figure 8a,b).

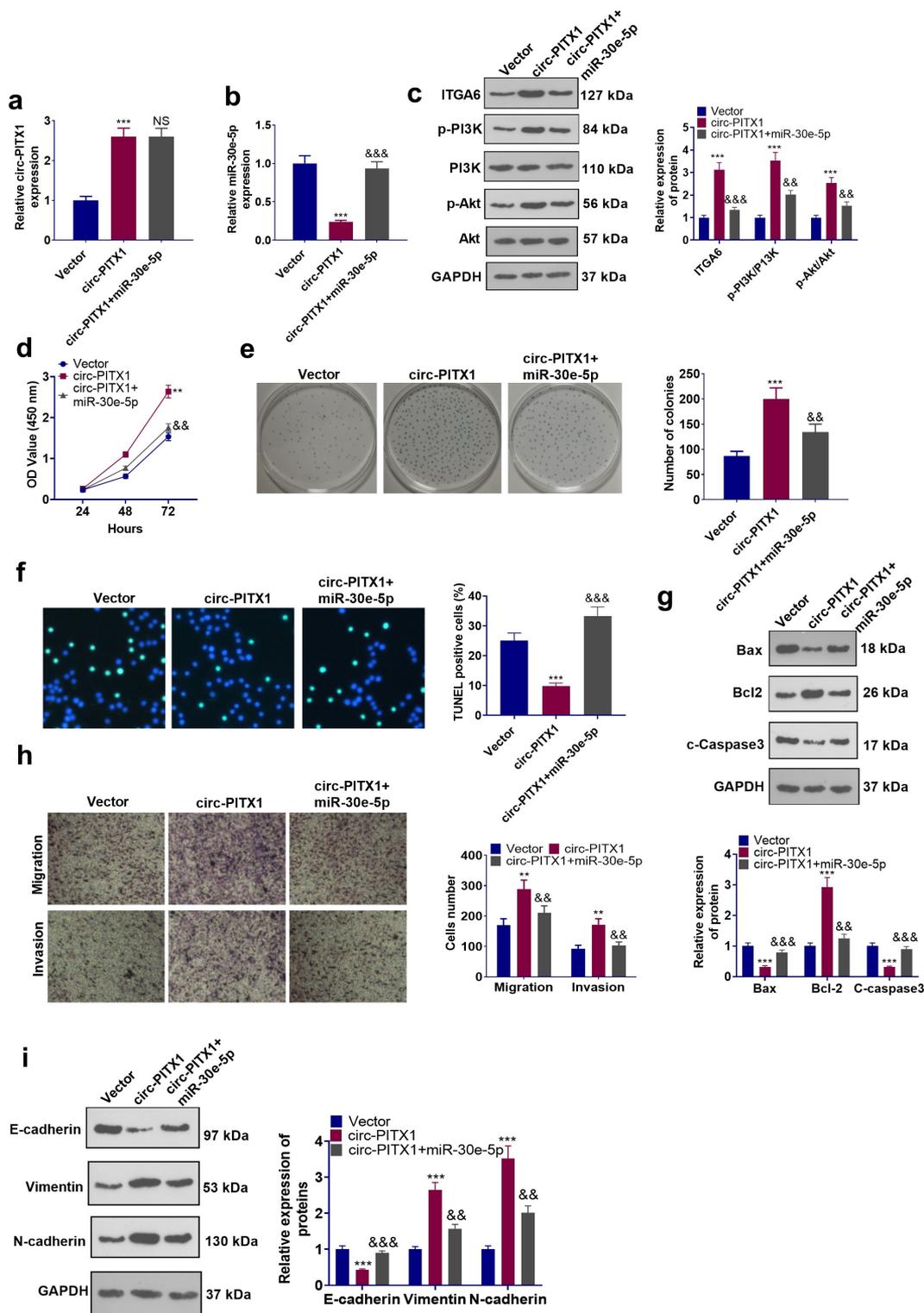
## 4 Discussion

With continuous explorations in NSCLC growth and metastasis, many circRNAs are discovered to be associated with NSCLC development [28]. Here, we uncovered that circ-PITX1 was notably up-regulated in NSCLC tissues and cells. Further

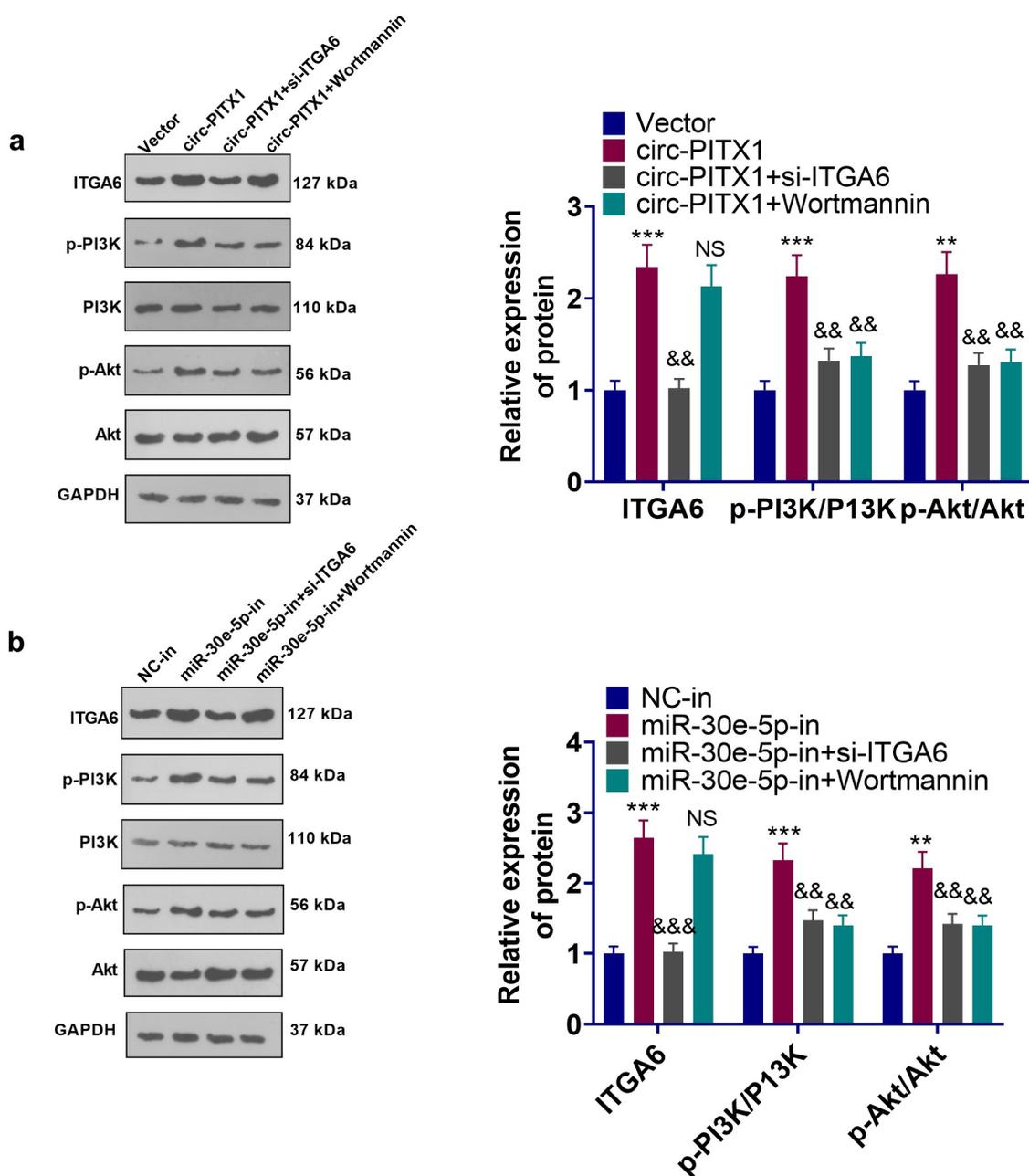
experiments confirmed the novel circ-PITX1/miR-30e-5p/ITGA6 axis in NSCLC development.

circRNAs, a type of non-coding RNAs widely consisting in human cells, and emerging studies have demonstrated that circRNAs present an aberrant expression in tumors and are entangled in tumor occurrence and development [29]. Abnormally expressed circRNAs can be used as significant indicators for the early diagnosis of tumors, including lung cancer [30,31]. Moreover, the exploration of the circRNA mechanism in tumors can offer new insights into NSCLC treatment by inhibiting tumor growth and chemoresistance. For instance, circARHGAP10 presents a higher expression and bears a relation to the underwhelming prognosis of NSCLC patients, and circARHGAP10 down-regulation abates GLUT1 to suppress glycometabolism in NSCLC cells [32]. circRNA\_103762 down-regulation potentiates NSCLC cells to chemotherapeutic drugs via enhancing CHOP's expression [33]. circ-PITX1, a novel circRNA up-regulated in glioma, facilitates glioma cells' growth, migration, and invasion and curbs their apoptosis to exert an outstanding function in glioma development regulation [34,35]. Here, we discovered that circ-PITX1 was also up-regulated in NSCLC and markedly associated with the poorer outcomes of NSCLC patients. circ-PITX1 functioned as an oncogene via boosting NSCLC cells' proliferation, migration, invasion, and EMT, and inhibiting cell apoptosis, indicating that circ-PITX1 could serve as an efficient diagnosis index and treatment target for NSCLC.

With the advances of deep sequencing technologies, more miRNAs have been discovered, and their important functions in tumor progression have also been corroborated [36,37]. Mechanistically, altered miRNAs in tumors affect gene expression and induce abnormal activation of tumor-associated signaling pathways by targeting specific genes at the transcription or post-transcriptional level [38,39]. Many miRNAs present an abnormal expression in NSCLC and exert a significant function [40]. miR-30a, b, c, d, e-5p, five miRNAs belonging to the miR-30 sub-family, all play a part in NSCLC by modulating cell proliferation, drug resistance, migration, and invasion [41–45]. Here, our statistics displayed that miR-



**Figure 7.** Circ-PITX1 sponged miR-30e-5p to modulate NSCLC cell proliferation and metastasis. a and b. H1975 cells were transfected together with miR-30e-5p mimics and/or circ-PITX1 overexpression plasmid. RT-PCR confirmed the levels of circ-PITX1 (a) and miR-30e-5p (b). (c). Western blot checked ITGA6, p-PI3K and p-Akt expression. d and e. CCK-8 (d) and colony formation assay (e) monitored cell proliferation. (f) TUNEL staining was adopted to examine the TUNEL-positive cell number. (g) WB determined the profiles of Bax, Bcl-2 and c-Caspase3. (h) Tranwell assay tracked cell migration and invasion. (i) Western blot determined the protein levels of EMT markers (E-cadherin, Vimentin, and N-cadherin). \*\* $P < 0.01$ , \*\*\* $P < 0.001$  (vs.Vector group), &&  $P < 0.01$ , &&&  $P < 0.001$  (vs.circ-PITX1 group),  $n = 3$ .



**Figure 8.** Circ-PITX1 exerted carcinogenic effects via the ITGA6/PI3K/Akt pathway. H1975 cells overexpressing circ-PITX1 or knocking down miR-30e-5p were handled with si-ITGA6 or dealt with the PI3K inhibitor (Wortmannin) for 24 hours, respectively. a and b: WB tested the expression of the ITGA6/PI3K/Akt pathway in H1975 cells. ns  $P > 0.05$ , \*\*  $P < 0.01$ , \*\*\* $P < 0.001$  (vs. Vector or NC-in group). &&  $P < 0.01$ , &&& $P < 0.001$  (vs. circ-PITX1 or miR-30e-5p-in groups).  $n = 3$ .

30e-5p overexpression could considerably impede NSCLC cells' proliferation and metastasis, and miR-30e-5p knockdown was associated with the poorer survival of NSCLC patients, which further verified the anti-tumor function of miR-30e-5p in NSCLC.

Prior studies have exhibited that circRNAs interact with miRNAs as ceRNAs, thus influencing tumor progression [46]. For instance, hsa-circ

-001895 sponges miR-296-5p to modulate clear cell renal cell carcinoma progression [47]. circ-ARHGAP-10 sponges miR-150-5p to influence NSCLC proliferation, differentiation, and metastasis [32]. Here, we uncovered that circ-PITX1 manifested negative affinities with miR-30e-5p in NSCLC cells. Besides, circ-PITX1 could function as a miRNA sponge to hinder miR-30e-5p in NSCLC. The rescue experiments also

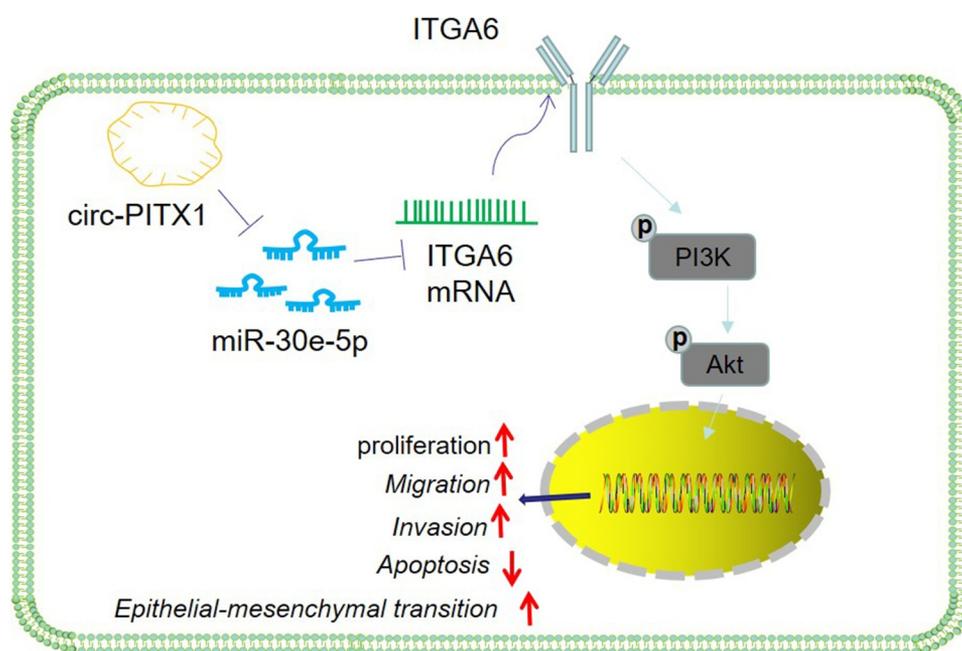
demonstrated miR-30e-5p up-regulation partly inverted the promoting function elicited by circ-PITX1 in NSCLC. Our work signified that circ-PITX1 dampened miR-30e-5p to modulate NSCLC progression.

Integrin subunit alpha family contains a group of genes, and their encoded proteins form a cell-surface receptor for collagen and laminin, thus getting involved in cell-cell adhesion [48]. ITGA proteins is abnormally expressed in several tumors, and function an oncogenic role in tumor progression by modulating cell cycle progression and cell differentiation, influencing tumor cell proliferation, invasion, migration, and apoptosis [49–51]. ITGA6, a member of ITGAs, is a valuable prognostic factor in the context of many cancers like ovarian cancer [52]. Downregulating ITGA6 is a promising method in treating tumors [53]. For example, microRNA-3940-5p in exosomes derived from mesenchymal stem cells targets ITGA6 to cramp colorectal cancer metastasis remarkably [54]. Here, we discovered that ITGA6, distinctly up-regulated in NSCLC tissues, predicted a poorer survival rate of NSCLC patients. circ-PITX1 drove up ITGA6's expression. Given that circ-PITX1 and miR-30e-5p were both marked mediators in NSCLC cells'

EMT, we believed that the circ-PITX1/miR-30E-5p axis could modulate cells' metastasis and EMT via ITGA6.

PI3K/AKT pathway activation is an outstanding characteristic in lung cancer, and this pathway plays a prominent role in controlling tumor cell proliferation, metastasis, stemness maintenance, chemoresistance [55,56]. Repressing PI3K/AKT pathway is a promising method in treating lung cancer [57]. Interestingly, ITGA6 has been confirmed to induce PI3K/AKT pathway activation [58]. Targeting ITGA6 by miR-143-3p represses tumor growth and angiogenesis via the PI3K/AKT pathway in gallbladder carcinoma [59]. Here, we also observed that circ-PITX1 activates PI3K/AKT pathway activation, accompanied with ITGA6 overexpression. Downregulating ITGA6 or inhibiting PI3K/AKT pathway reversed circPITX1-mediated or miR-30e-5p inhibitor-mediated PI3K/AKT pathway activation.

To conclude, our study has, for the first time, confirmed that circ-PITX1 could enhance NSCLC cells' proliferation, metastasis, and EMT via the miR-30E-5p/ITGA6/PI3K/AKT axis (Figure 9). Notwithstanding, the function of ITGA6 modulated by the circ-PITX1/miR-30e-5p axis in NSCLC development, particularly in regulating



**Figure 9.** The schematic diagram. circ-PITX1 is upregulated in NSCLC, and it targets miR-30e-5p and upregulates ITGA6/PI3K/AKT pathway, thus regulating the proliferation, invasion, migration, and apoptosis of NSCLC cells.

metastasis, requires more experiments. Collectively, these findings provide a better understanding of NSCLC and new substances for its diagnosis and treatment.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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## Ethics statement

Our study was approved by the Ethics Committee of Tianjin First Central Hospital.

## Data availability statement

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

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