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Original Article

Circ-PAX2 promotes proliferation and metastasis by absorbing miR-186 in lung cancer cells

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Abstract: Circ-RNAs are a type of non-coding-protein RNAs which act as an effector role in many physiological processes. However, the novel function of circ-PAX2 in lung carcinomas is still unidentified. The current study is to detect the expression of circ-PAX2 in lung squamous cell carcinoma (LSCC) tissues and the physiological functions of circ-PAX2. Circ-PAX2 was distinguished in LSCC samples and matched non-tumor samples by human circRNA microarray analysis and was validated to be up-regulated in 86 specimens of LSCC tissues and lung cancer cell lines by qRT-PCR. Functional validation experiments showed that knockdown of circ-PAX2 promoted apoptosis of lung carcinoma cells, and then suppressed proliferation and migration of tumor cells. Small interfering RNA (siRNA) to circ-PAX2 inhibited growth in lung tumor cells. Bioinformatics prediction and rescue experiments showed that circ-PAX2 was a target of microRNA-186, confirmed by qRT-PCR and double luciferase reporter assay. On the whole, our findings reveal that circ-PAX2 was up-regulated and may be an oncogene in lung cancer; its function was reducing apoptosis, promoting cell proliferation and migration in lung carcinoma cells, which might be a novel therapeutic targetgene in lung cancer.

Keywords: Lung carcinoma, circ-PAX2, proliferation, metastasis, miR-186

Introduction

Lung squamous cell carcinoma (LSCC) is the most common type of lung neoplasm; its incidence ranks first among human tumors in worldwide. In recent years, LSCC has had high morbidity and mortality [1], so the most effective prevention of LSCC is to detect out effective molecular markers in the early stage. However, the biological mechanisms of LSCC tumorigenesis and progression are not well illuminated. Therefore, we must discover unidentified pathogenesis of LSCC development and progression.

Circular ribonucleic acids (circ-RNAs) are a type of non-coding protein RNA, which contain a covalently closed loop without 5' to 3' polarity or adenosine tail [2]. Previous evidences indicate that circ-RNAs play many functional roles in tumorigenesis and progression [3]. For example, hsa_circ_0013958 is up-regulated in pulmonary adenocarcinoma compared to adjacent normal tissue and might be a novel gene-marker [4]. Circular RNA circMTO1 promotes p21 expression and suppresses progression of liver cancer cells by targeting miR-9 [5]. Hsa_circ_0092509 was detected as an oncogene through binding of miR-448, which promotes tumorigenesis in osteosarcoma [6].

In our study, the expression profile of circRNAs was screened in 3 pairs of LSCC specimens and matched normal tissue. The circRNA microarray analysis showed hsa_circ_0092400 (circ-PAX2) is up-regulated more than 2 fold in lung cancer tissue, based on 86 cases of lung cancer. So we selected hsa_circ_0092400 as the research object to analyze its role of proliferation and metastasis in lung carcinoma. In addition, to understand the mechanism of tumorigenesis in lung cancer, the circ-PAX2 target was further analyzed and investigated.

Materials and methods

LSCC tissue collection

A total of 86 paired LSCC and marched non-cancerous tissues were obtained at the Affiliated Hospital of Yangzhou University (Yangzhou, China) from January 2016 to May 2017. The LSCC and adjacent normal lung tissues were diagnosed by two pathologists. Samples were collected after surgical resection, snap-frozen in liquid nitrogen prior to use. Approval for this project was granted by the Medical Ethics Committee of the Affiliated Hospital of Yangzhou University. Informed consent was provided by patients for the 86 specimens.

Plasmid, cell culture and transfection

Circ-PAX2 siRNAs and miR-186 siRNAs were bought by RiboBio. The siRNAs were transfected according to the manufacturer's instructions by using Lipofectamine 2000 (Invitrogen, California, USA). The lung cancer cell lines (NCI-H2170, SPC-A-1, H460 and A549) and human normal lung cell line (16HBE) were purchased from ATCC (Rockville, USA). All these cells were cultured in DMEM nutrient solution (Gibco, Carlsbad, CA, USA), containing 10% fetal bovine serum (FBS, Gibco) and antibiotics. NCI-H2170, SPC-A-1, H460 and A549 cells were transfected with relative plasmid and control for 48 h before further experiments. The transfected cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. Cells were detached using 0.25% trypsin when they were approximately 80% confluent and consequently subcultured.

CirRNA microarray analysis

Total RNA was extracted from 3 pairs of lung squamous cell cancer samples and marched normal lung tissue, the expression profile of circRNAs in all samples detected by Arraystar Super RNA Labeling Kit according to Arraystar's standard protocols in Kangcheng Biological Engineering Co., LTD., Shanghai, China [6].

qRT-PCR

Total RNA was extracted by trizol reagent (Invitrogen, Carlsbad, Calif, USA). According to the manufacturer's protocol. Two micrograms of RNA were reverse-transcribed into cDNA using the Primescript RT reagent Kit with gDNA Eraser (Takara Bio Inc, Japan). qRT-PCR was performed on an Applied Biosystems 7500 Real Time PCR system (Applied Biosystems, White Plains, NY, USA). The U6 small RNA was used as internal controls for cDNA expression. Primers used for qPCR were as follows: circ-PAX2, 5'-CATCCGGACCAAAGTTCAGCA-3' (forward), 5'-CTATGGCTACAGTAGCACCAAG-3' (reverse); miR-186, 5'-ACACTCCAGCTGGGCAGCAGCACACT-3' (forward), CTCAACTGGTGTCGTGGA (reverse); U6, 5'-CGCTTCGGCAGCACATATAC-3' (forward), 5'-TTCACGAATTTGCGTGTCAT-3' (reverse). The relative expression of circ-PAX2 and miR-186 was normalized to the expression level of U6 small RNA using the 2-ΔCt method.

Colony formation and proliferation assay

In the colony formation assay, transfected lung cancer cells (1000 cells/well) were seeded into six-well plates and maintained in 10% FBS medium incubated at 37°C with 5% CO_2. After two weeks, the lung cancer colonies were fixed in methanol and stain 20 minutes with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA). The number of stained colonies was counted; triplicate wells were operated in each colony formation group. In the proliferation assay, transiently transfected cells were seeded into 96-well plates at a density of approximately 2000 cells per well, and cell proliferation was tested every 24 hours. After incubation with 10 μl of the CCK-8 reagent (Beyotime Institute of Biotechnology, Shanghai, China) for 2 or 4 hours, the 450 nm absorbance was measured for each well. The assay was performed in five replicate wells, and three parallel experiments were conducted for each sample.

Transwell invasion assay

Lung cancer cells were respectively harvested after trypsin digestion, and resuspended into serum-free medium, and inoculated into the upper chamber for transwell at 5×10^3 per well in 200 μL of serum-free medium, and 500 μl DMEM containing 10% FBS was added into the lower chamber. After 24 h incubation, the chambers were rinsed twice in PBS, and cells were fixed using 4% paraformaldehyde, and subsequently stained with 0.1% crystal violet. Afer gently wiping the upper chamber membrane with a cotton swab, the image was photographed under the microscope.

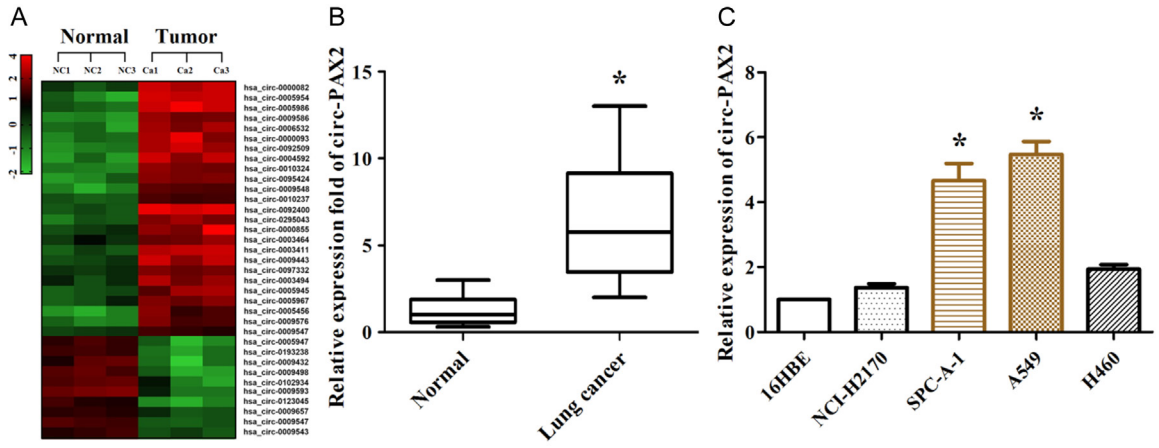

Figure 1. Circ-PAX2 was highly expressed in lung cancer tissue and cell lines. A. Heat map and hierarchical cluster analysis show the circular-PAX2 upregulation in 3 pairs of LSCC tissue and adjacent normal tissue. B. In 86 cases of lung cancer patients, circ-PAX2 was significantly up-regulated in tumor tissue compared to adjacent non-tumor tissue. C. Circ-PAX2 expression detected by RT-PCR in lung cancer cell lines (NCI-H2170, SPC-A-1, H460 and A549) and lung normal cell line (16HBE).

Cell apoptosis assay

Lung cancer cells were seeded in six-well plates and transiently transfected with circ-PAX2 and the control vector. After 2 days, the cells were washed with 4°C PBS buffer solution twice, then stained with 5 μl of Annexin V and 10 μl of propidium iodide (PI) using Alexa Fluor 488 annexin V Cell Apoptosis Kit (Invitrogen) to detect the tumor cell apoptosis according to the standard protocol and then analyzed by a BD flow cytometer (BD Biosciences, CA, USA).

Dual luciferase reporter assays

A549 were seeded onto a 24-well plate overnight. Cells were co-transfected with pGL3-circ-PAX2-wildtype/pGL3-circ-PAX2-mutant and miR-186 mimics or blank controls using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. After 48 h, luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to renilla luciferase activity for each transfected well. The results were obtained from three independent experiments and each one was performed in triplicate.

Xenograft study

Athymic BALB/c mice experiments were authorized by the YZU Institutional Animal Care and Use Committee. Healthy, the six-week-old mice were nursed in specific pathogen-free (SPF) conditions. The ten mice were randomly divided into an experimental group and negative controls (NC). A total of 2×10^6 SPC-A-1 cells transfected with each plasmid in 0.2 ml of PBS were injected into the back of nude mice. After injection, the tumor volumes and weight were examined every five days after the tumors appeared. After one month, the mice were sacrificed, and the tumor volumes and weights was measured. The tumors were fixed, collected, and prepared for IHC staining.

Statistical analysis

SPSS 16.0 software (SPSS 16.0, Chicago, IL) and GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA) were used for statistical analyses. The significance of the differences among groups was estimated by one-way ANOVA or Student's t-test. Pearson correlation analyses were performed to investigate the correlation between circ-PAX2 and miR-186. All tests performed were two-sided and statistical significance was set at $P<0.05$.

Results

Circ-PAX2 was highly expressed in lung carcinoma samples and lung cancer cell lines

The differential expression profile of circRNAs was screened in 3 pairs of LSCC specimens and matched adjacent normal tissue. Hierarchical cluster analysis and heat map results showed up-regulated circRNAs (including hsa_

Table 1. Circ-PAX2 expression and clinicopathologic parameters in patients with lung cancer

Characteristics	Circ-PAX2 high (n)	Circ-PAX2 unchanged (n)	*P*-value	χ^2
Gender			0.073	6.943
Male	30	3		
Female	49	4		
Age (years)			0.074	5.230
<50	23	2		
≥50	57	5		
Chemotherapy				
No	19	1	0.129	2.309
Yes	60	6		
Tumorsize (cm)			0.069	3.302
≥5	22	4		
<5	57	3		
Nodal status			0.008*	9.626
Positive	43	5		
Negative	36	2		
TNM stage			0.02*	9.183
I+II	41	3		
III+IV	28	4		
Recurrence			0.071	3.482
No	23	3		
Yes	57	3		

*P<0.05 represents statistical differences.

circ_0092400, circ-PAX2) and down-regulated circRNAs (**Figure 1A**). Circ-PAX2 (Hsa_circ_0092400) was more than 2 fold increased in lung cancer tissue and adjacent non-tumor tissue by this microarray analysis. In further validated tests, we detected circ-PAX2 high-expression by qRT-PCR in 86 pairs of lung cancer tissue compared with adjacent normal lung tissue. The clinicopathologic parameters of 86 patients are listed in **Table 1**. We found that the relative expression level of circ-PAX2 was dramatically up-regulated in lung LSCC samples (**Figure 1B**). Similarly, its RNA expression level was also significantly up-regulated by qRT-PCR in lung cancer cell lines, compared with normal lung 16HBE cells (**Figure 1C**). These data disclosed that circ-PAX2 was over-expressed in LSCC and acted as a novel oncogene, which might be a valuable marker for LSCC detection.

Circ-PAX2 siRNA suppressed lung cancer cells proliferation

Expression levels of circ-PAX2 had been shown to be increased in LSCC and lung cancer cell lines. Circ-PAX2 expression in A549 and SPC-A-1 cells is significantly higher than that in NCI-H2170 and H460. Thus, the A549 and SPC-A-1 cells were selected as the experimental cells for following functional research on circ-PAX2. Synthesized circ-PAX2 siRNAs (siRNA1 and siRNA2) dramatically decreased the circ-PAX2 expression in A549 and SPC-A-1 cell lines (Figure S1). To define the proliferative role of circ-PAX2 in lung cancer cells, CCK-8 assay (**Figure 2A**, **2B**) and colony formation assay (**Figure 2C**, **2D**) were performed using circ-PAX2-siRNA. In vitro results revealed circ-PAX2-siRNA inhibited cell proliferation in lung cancer cell lines.

Circ-PAX2 siRNA suppressed lung cancer cell progression

Apoptosis assay, transwell invasive assay, and xenograft mice assay were performed to further explore the function of circ-PAX2 on lung cancer cell progression. The result of apoptosis assay showed that circ-PAX2 siRNA led the apoptotic cell percentage to increase in A549 and SPC-A-1 cells compared to NC (**Figure 3A**, **3B**). Invasion assay revealed that circ-PAX2 siRNA suppressed the invasion of lung cancer cells compared to NC (**Figure 3C**, **3D**). In vivo nude mice assay indicated that knockdown of circ-PAX2 inhibited lung cancer growth in tumor volume and tumor weight (**Figure 3E-G**). These results above showed knockdown of circ-PAX2 could inhibit the progression of lung cancer cells not only in vitro, but also in vivo.

Circ-PAX2 was a sponge of miR-186

The experiments above showed circ-PAX2 had an oncogenic role in lung cancer progression. The prediction of bioinformatics (TargetScan, MicroRNA) indicated that hsa-miR-186, hsa-mir-365, and hsa-mir-129 might target circ-PAX2 because of the contrary binding sites on each other (**Figure 4A**). Dual-luciferase reporter assay indicated that luciferase activities decreased significantly in the cells transfected with microRNA-186 and circ-PAX2 wild-type reporter plasmids but not in those transfected with mutant-type plasmids while microRNA-365 and microRNA-129 did not have a difference in luciferase activities (**Figure 4B**). In lung cancer samples, qRT-PCR data showed that the relative expression levels of miR-186 were less low than that of marched non-tumor lung tissue

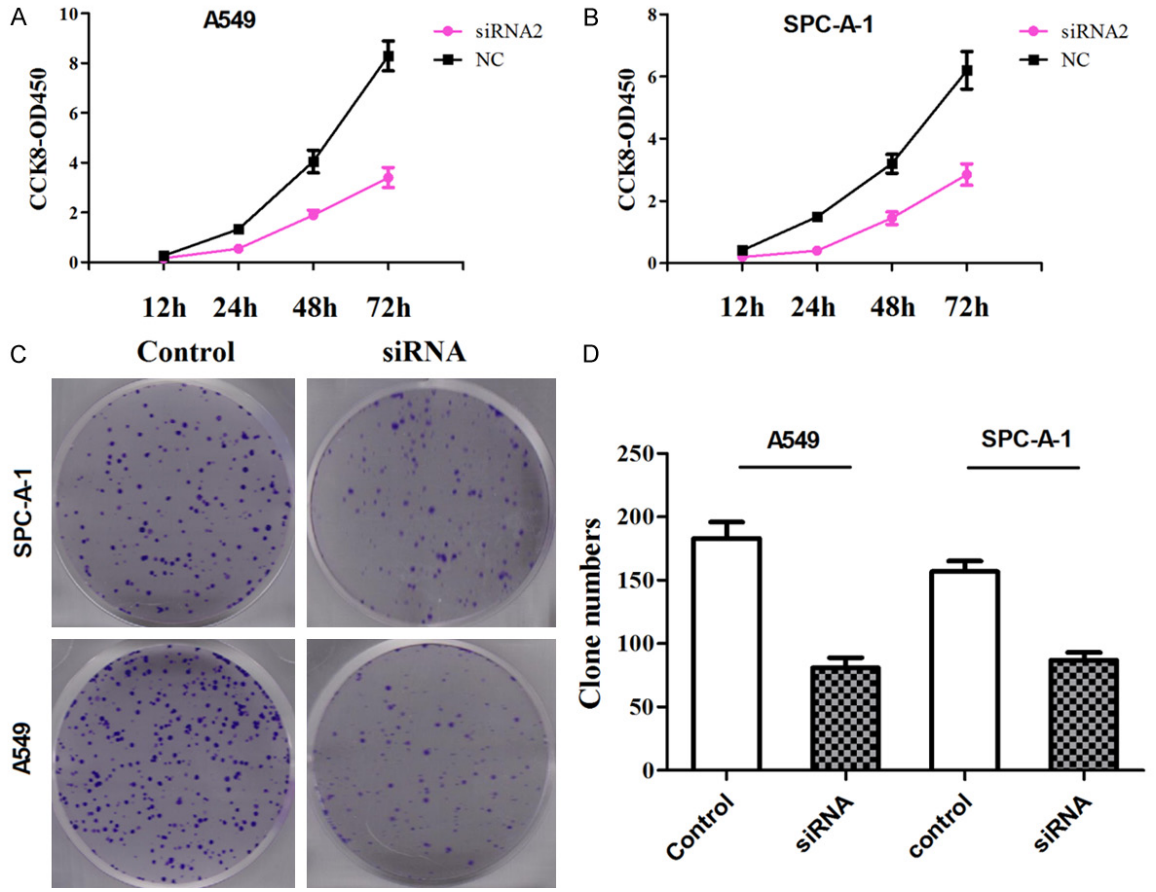

Figure 2. Circ-PAX2 siRNA suppressed lung cancer cell proliferation in vitro. A, B. CCK-8 assay showed the absorbency of proliferation of A549 and SPC-A-1 cells. C, D. Colony formation assay showed the clone number of A549 and SPC-A-1 cells. Data were expressed as mean ± SD. *P<0.05 represents significant difference.

(**Figure 4C**). Pearson's correlation analysis arrived at an inverse correlation between the RNA expression of circ-PAX2 and miR-186 (**Figure 4D**). In addition, cell proliferation assay revealed that miR-186 siRNA could counteract the proliferative effect of circ-PAX2 knockdown in lung cancer A549 cells (**Figure 4E**, **4F**). Overall, bioinformatics prediction and test results showed that circ-PAX2 binding to miR-186 served as a sponge of miR-186.

Discussion

Lung cancer occurred in about two million people and resulted in about 1.6 million deaths worldwide in 2016 [1]. Lung squamous cell carcinoma (LSCC) is the most common type of lung malignant tumor characterized by uncontrolled proliferation of cells in lung tissues. Numerous circular RNAs (circRNAs) have been disclosed by gene chip technology in recent years [7, 8]. Previous study showed circRNAs, a subtype of noncoding RNA, participated in series of physiological process involved in lung cancertumorgenesis and progression [9-12].

In the current study, we detected circ-PAX2 (hsa_circ_0092400) was more than 2 fold changed in lung cancer tissue and adjacent non-tumor tissue by human circRNAs microarray analysis. Hsa_circ_0092400 is located at chr10:102539254-102541122 with 1868 gene length and its symbol is PAX2 gene (circBase, www. circbase.org/), so it is also named circ-PAX2. In cases of lung cancer patient specimens and lung cancer cells, circ-PAX2 expression was validated to be over-expressed compared with matched noncancerous tissue and normal lung 16HBE cells. Our data reveal the different expression of circRNAs and confirm the up-regulated expression of circ-PAX2 in lung cancer samples, this means that circ-PAX2 might be an oncogene in lung cancer.

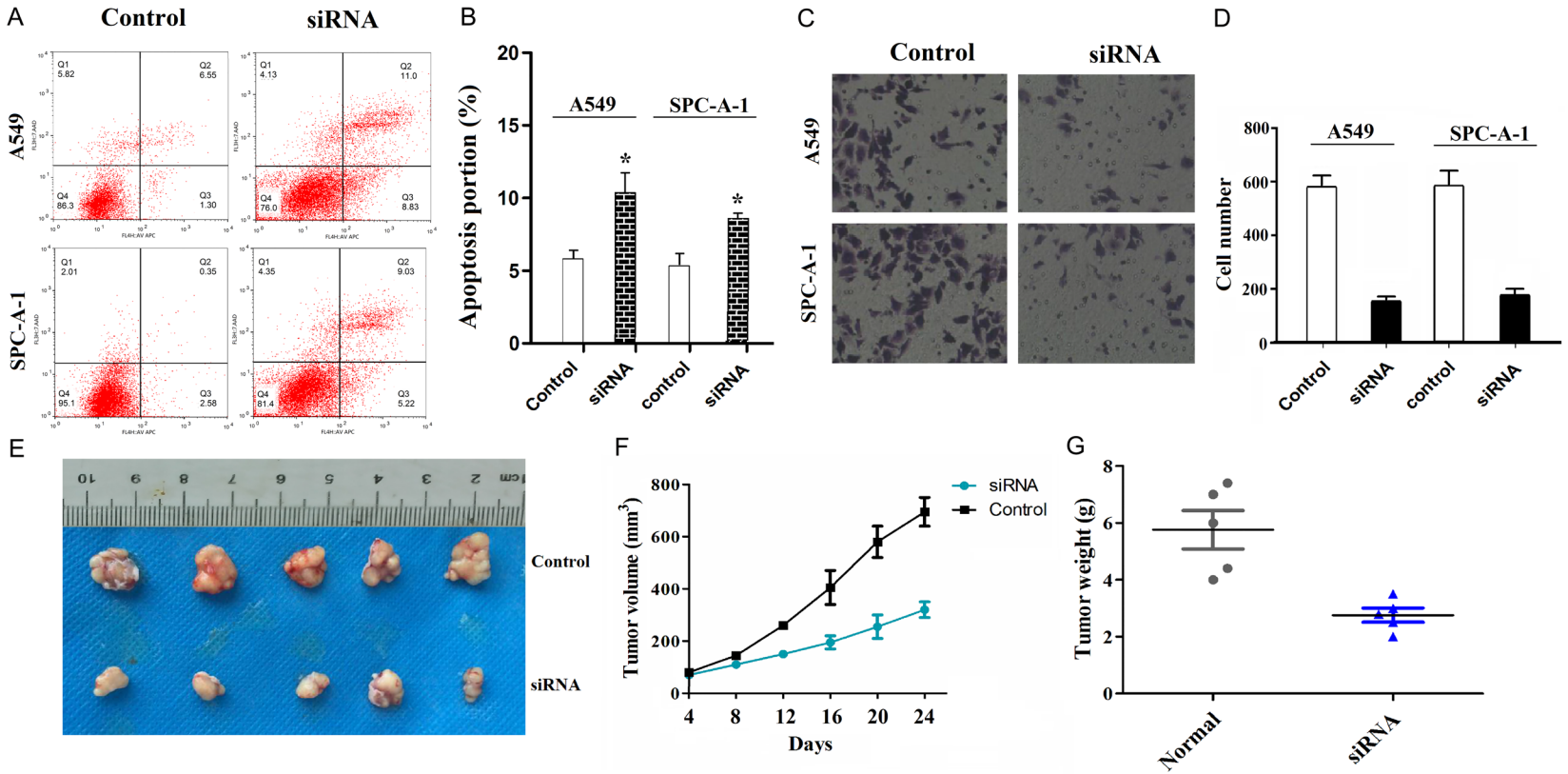

Figure 3. Circ-PAX2 siRNA promoted apoptosis and suppressed metastasis and growth. A, B. Apoptosis assay detected by flow cytometry shows the apoptotic cell rate in in A549 and SPC-A-1 cells transfected with circ-PAX2 siRNA and negative controls. C, D. Transwell invasive assay shows the invaded tumor cells. E-G. Xenograft mice assay shows the tumor volume and weight in nude mice injected A549 cells transfected with circ- PAX2 siRNA or negative controls. Data are expressed as mean ± SD. *P<0.05 represents significant difference.

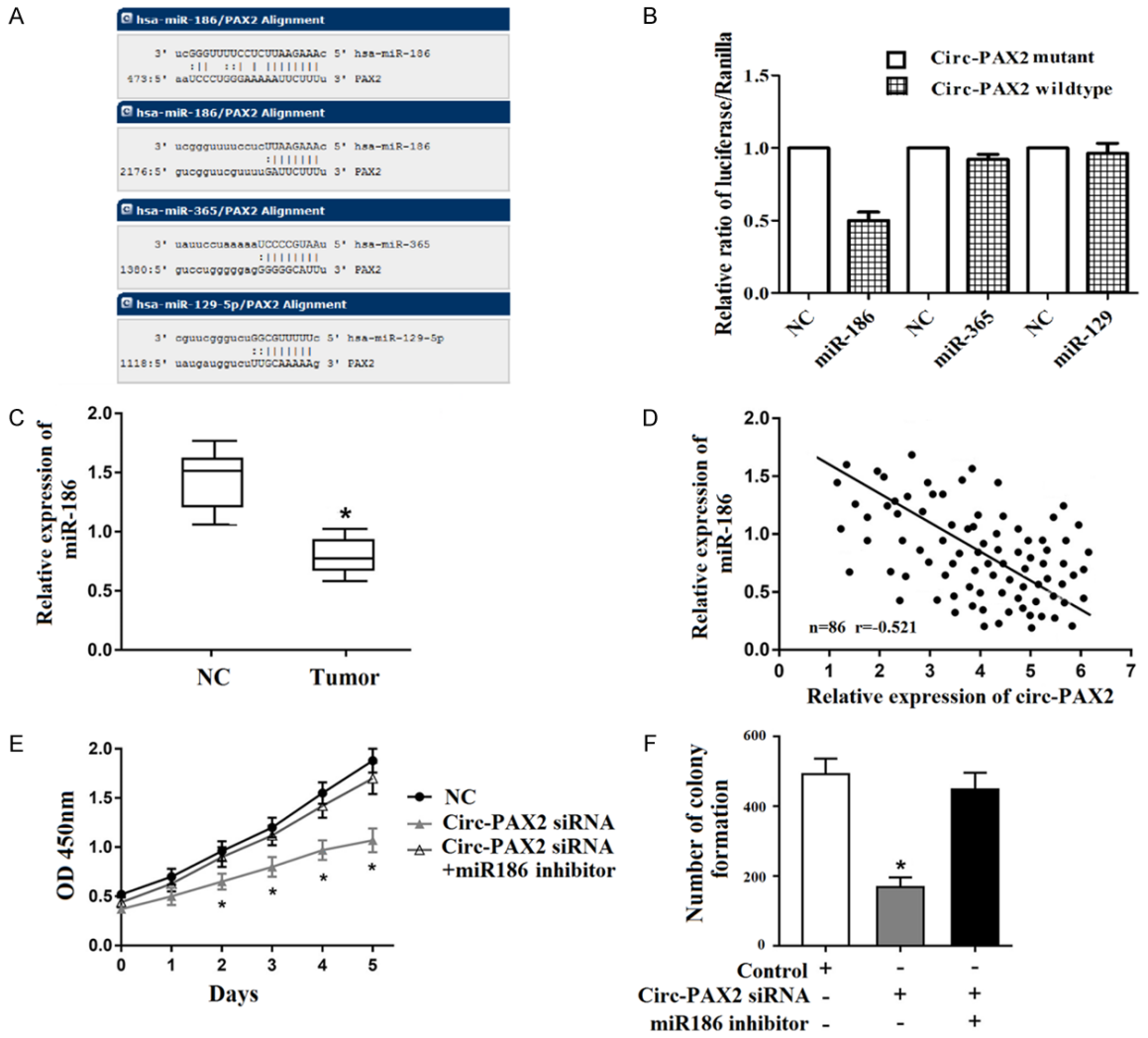

Figure 4. Circ-PAX2 was a direct binding target of miR-186 and served as an absorber of miR-186. A. The interaction analysis of circRNA/miRNA was based on MicroRNA, miRanda and TargetScan. B. Luciferase reporter assay revealed the luciferase vitality in A549 cells when co-transfected with microRNAs mimics and circ-PAX2 (mutant and wild type). C. RT-PCR results reveal miR-186 expression level in lung cancer tissue compared to adjacent non-tumor tissue. D. Pearson's correlation analysis shows the correlations between circ-PAX2 and miR-186 expression in 86 cases of LSCC tissue (r=-0.521, P≤0.05). E, F. Xenograft mice assay in vivo shows the tumor volume and weight in nude mice injected A549 cells transfected with circ-PAX2 and negative control. Data are expressed as mean ± SD. *P<0.05, represents significant difference.

Now that circ-PAX2 was validated to have up-regulated expression in lung cancer tissues and lung cancer cells, the further functional experiments were necessary to verify other biological effects. In vitro assay, including proliferation assay and invasion assay, revealed knockdown of circ-PAX2 suppressed cell proliferation and invasion in lung squamous cell cancer. Furthermore, in vivo circ-PAX2 knockdown suppressed the tumor growth in volume and weight. Thus, circ-PAX2 was found to have an oncogenic role of lung cancer carcinogenesis, providing a valuable gene-marker for therapy of lung cancer.

The regulation pattern of circRNAs involved mainly transcriptional control and post-transcriptional control [13]. For example, circRNA-7 bound miR-7 and regulated many tumors' progression, including stomach cancer and liver carcinoma [14, 15]. Overexpression of cir-GLI2 in osteosarcoma tissues and cells exerted an oncogene role and negatively targeted miR-125b [16]. An article reported hsa_circ_0013-

958 was identified as a miR-134 absorber; it promoted cell proliferation and invasion and was used as a potential non-invasive biomarker in lung adenocarcinoma [17].

From the above study, the regulatory role of circRNAs is to bind microRNAs and target functional gene mRNA severing as molecular absorber of microRNA [18]. The signal pathways of circRNA/microRNAs/mRNA have been made many records in human malignant tumor [19-21]. In our study, nude mice study and invasion assay revealed that silencing of circ-PAX2 reduced the volume of tumor borne, lightened the weight of tumor and decreased the cell numbers of invasion compared to control group, suggesting circ-PAX2 could regulate the lung cancer progression. Three associated microRNAs were predicted by bioinformatics theories; only miR-186 was verified ultimately as the effective microRNA. miR-186 was proven to have the reverse function of circ-PAX2 on lung cancer tumorigenesis. It suggested the circ-PAX2 was served as an absorber of miR-186, regulating expression of PAX2 mRNA and translation.

In conclusion, the circRNAs expression profiles showed up-regulated circ-PAX2 in lung cancer tissue, and circ-PAX2 confirmed the pro-tumor roles by validation experiments in lung carcinoma tissues and lung cancer cells. Circ-PAX2 served as an oncogene by absorbing miR-186, stopping apoptosis, and promoting cell proliferation and migration in lung carcinoma. These results suggest that circ-PAX2 might be a novel regulator of lung cancer cell behavior and might represent a novel target for gene-targeted lung cancer therapies.

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Disclosure of conflict of interest

None.

Abbreviations

Circ-RNAs, circular RNAs; lncRNAs, long noncoding RNA; mi-RNA, micro-RNA; LC, Lung carcinoma.

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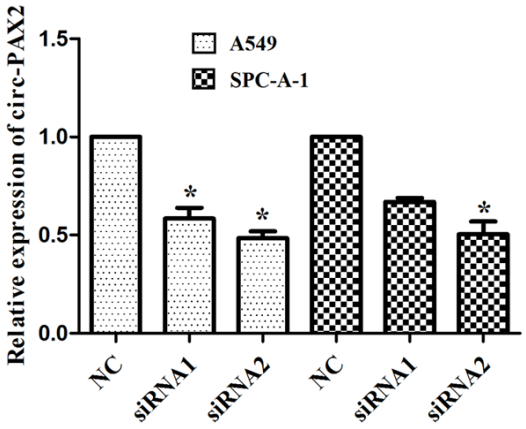

Figure S1. Circ-PAX2 expression via siRNA in lung cancer cell lines.