European Review for Medical and Pharmacological Sciences 2020; 24: 10557-10565

Circular RNA circ-PRKCI promotes lung cancer progression by binding to microRNA-1324 to regulate MECP2 expression

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Abstract. – OBJECTIVE: We aimed to investigate the expression and specific molecular mechanism of circ-PRKCI in lung cancer (LCa).

PATIENTS AND METHODS: The relationship between the expression level of circ-PRKCI and the prognosis of patients was analyzed. The impacts of circ-PRKCI on the invasiveness of LCa cells were examined by Cell Counting Kit-8 (CCK-8) experiments, clone formation experiments, and transwell invasion experiments. Subcellular localization of circ-PRKCI was determined through nuclear separation experiments. Downstream target genes that can bind to circ-PRKCI was predicted through bioinformatics analysis, and was then verified by Dual-Luciferase experiments, quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) experiments, and Western blot experiments.

RESULTS: Circ-PRKCI level was remarkably elevated in LCa tumor tissues and cell lines. At the same time, highly expressed circ-PRKCI was correlated with the poor prognosis of LCa patients. In vitro cell experiments revealed that inhibition of circ-PRKCI in LCa cell lines remarkably inhibited cell invasiveness and proliferation. In addition, circ-PRKCI can compete with MECP2 to bind microRNA-1324 and thus affect the progression of LCa.

CONCLUSIONS: Our study shows for the first time that circ-PRKCI modulates the progression of LCa through microRNA-150-5p/MECP2 axis.

Key Words:
LCa, Circ-PRKCI, CeRNA, Cell proliferation, Cell invasion.

Introduction

Lung cancer (LCa) is one of the malignant tumors with the highest morbidity and mortality worldwide[1,2]. In recent years, lung adenocarcinoma has become the most common pathological type of LCa. According to epidemiological studies, the occurence of LCa accounts for about 40% of all LCa cases[3]. It is also the most common type of LCa in nonsmokers, with a particularly significant incidence in women and young patients[4,5]. With the advancement of medical level, great improvements have been made in the diagnosis and treatment of LCa. However, due to the high recurrence rate and easy metastasis of LCa, the prognosis of most patients is extremely poor, with the 5-year survival rate less than 20%[6,7]. Therefore, it is urgent to find new therapeutic targets to develop more effective treatment strategies for LCa.

Circular RNAs (circRNAs) are a class of newly discovered non-coding RNAs that lack a 5'-3 'end and have a covalent closed structure. Generally, circular RNA molecules are stable in structure, and some circular RNAs have effective regulatory capacity by adsorption of genes[8]. CircRNA regulate multiple biological processes through a variety of mechanisms[8-10], such as the adsorption of microRNAs to regulate the expression of related genes and protein synthesis. Jin et al[11] has shown that dysregulation of circular RNA is involved in the development of many cancers. In fact, has-circ-0032462, has-circ-0028173 or has-circ-0005909 promote the progression of osteosarcoma by regulating CADM1 expression[12] and circ-VANGL1 promotes the proliferation, migration and invasion of bladder cancer cells by adsorbing microRNA605-3p/VANGL1[13]. Besides, has-circ-0007534 inhibits breast cancer growth by regulating the microRNA593/MUC19 pathway[14].

MicroRNAs (miRNAs) are a series of endogenous non-coding small RNAs with a length of about 18-22 nucleotides[15] that regulate gene ex-

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pression by combining with the messenger RNA (mRNA) of the target gene, leading to mRNA degradation or translation inhibition[16]. Currently, more than 50% of protein-coding genes have been found to be regulated by miRNAs, so changes in their expression may lead to the development of malignant tumors[17,18]. MiRNA-15b upregulation in the tumor environment negatively regulates anti-tumor immune function by inhibiting the function of CD8 + T cells[19], inhibiting miRNA-29c and its downstream pathways can regulate the tumor progression of triple-negative breast cancer[20], and microRNA1 and microRNA145 can suppress tumor generation and metastasis of gallbladder cancer. However, the specific role of miRNAs in LCa still remains elusive.

Circ-PRKCI plays a significant part in many diseases, such as esophageal squamous cell carcinoma[21], gastric cancer[22], and liver cancer[23]. It can serve as an oncogene by prompting the malignant growth and metastasis of tumor cells, but its expression and biological function in lung adenocarcinoma have not been reported. Therefore, this study preliminary explored the level of circ-PRKCI and its possible molecular mechanism in LCa.

Patients and Methods

Sample Collection

60 pairs of tumor tissue and adjacent non-tumor tissue confirmed pathologically were obtained from patients with LCa undergoing surgical resection. All patients did not receive any treatment, such as radiotherapy and chemotherapy before the surgery and all collected tumor samples were confirmed as lung cancer by postoperative pathology. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). This study complies with the Declaration of Helsinki and it was approved by the Ethics Committee of Shandong Shanxian Central Hospital. Signed written informed consents were obtained from all participants before the study.

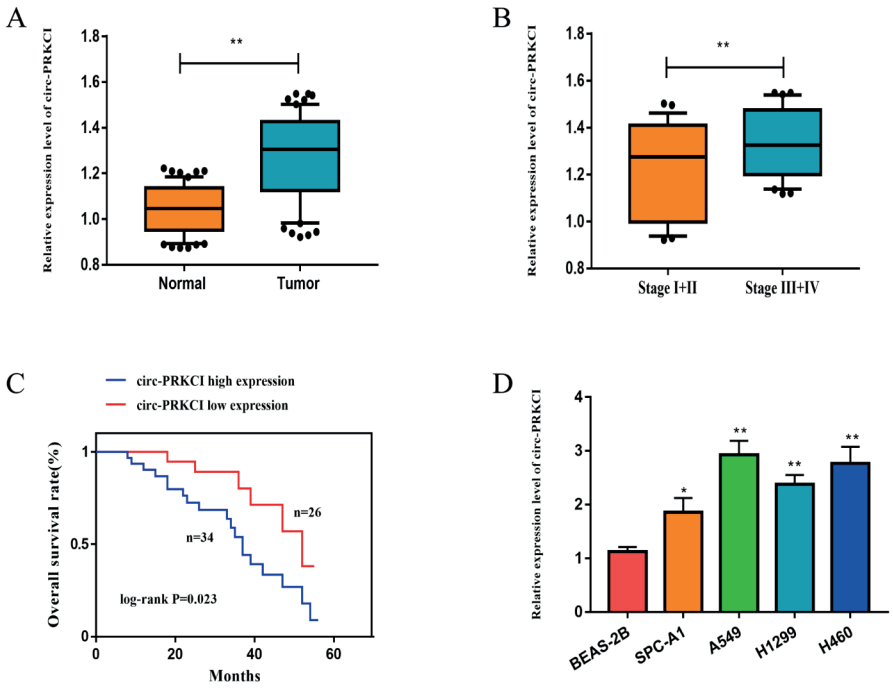

Figure 1. Circ-PRKCI expression was significantly increased in lung adenocarcinoma. **A,** qRT-PCR was used to detect the expression of circ-PRKCI in lung adenocarcinoma tissues and normal control tissues. **B,** The expression level of circ-PRKCI in patients with different stages of lung adenocarcinoma was analyzed by qRT-PCR. **C,** Kaplan-Meier was used to depict survival curves for lung adenocarcinoma patients in the circ-PRKCI high expression group and the circ-PRKCI low expression group. **D,** The expression of circ-PRKCI in normal control cells BEAS-2B and lung adenocarcinoma cell lines (SPC-A1, H1299, A549 and H460) was detected by qRT-PCR. * $p<0.05$; ** $p<0.01$

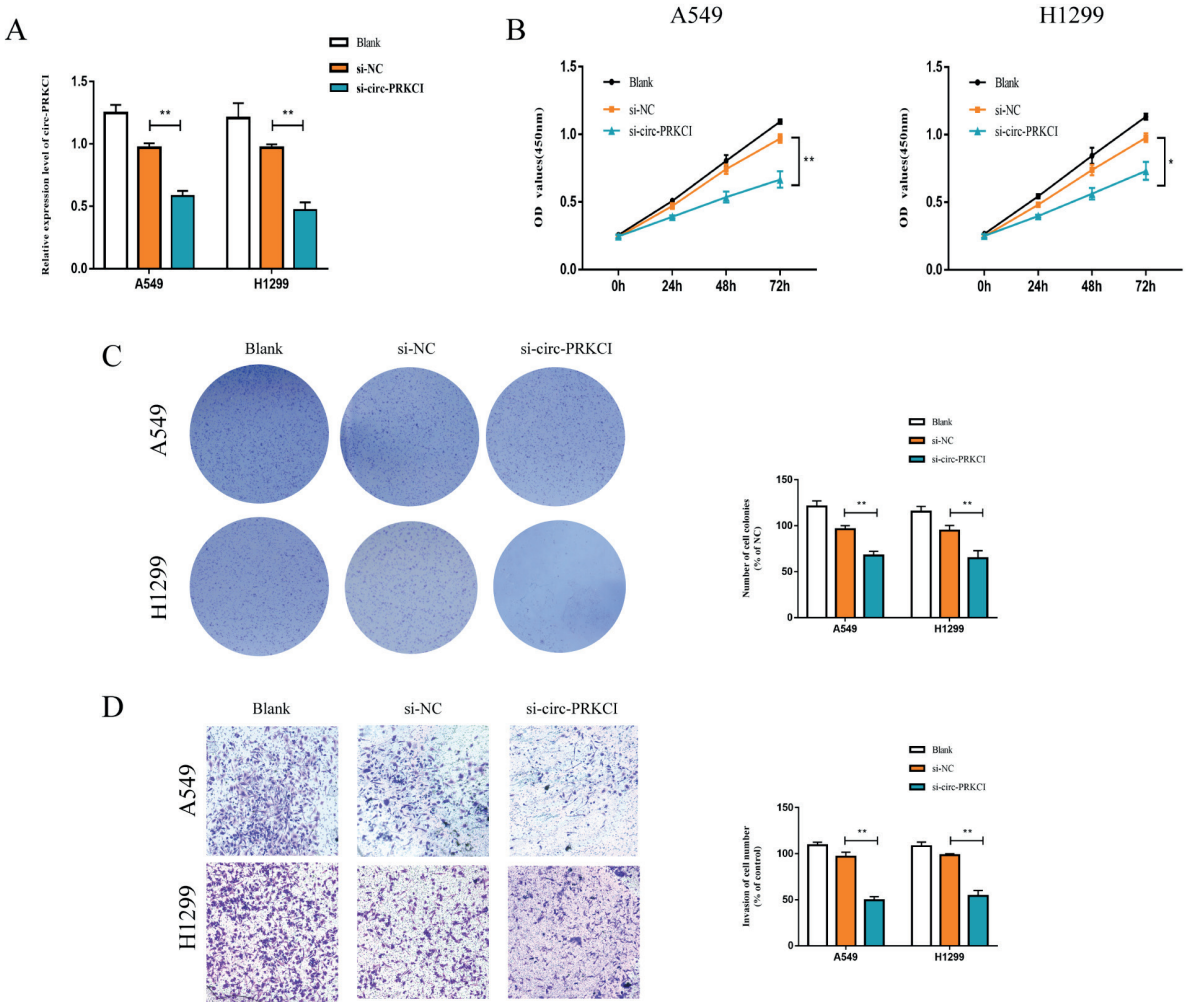

Figure 2. Inhibiting circ-PRKCI can inhibit the proliferation and invasion of lung adenocarcinoma cells. **A,** Circ-PRKCI siRNA transfection efficiency was detected by qRT-PCR. **B,** The effect of circ-PRKCI on the proliferation of A549 and H1299 cells was detected by the CCK8 experiment. **C,** Clone formation experiment detected the effect of circ-PRKCI on colony formation of A549 and H1299 cells (magnification: 40×). **D,** The effect of circ-PRKCI on the invasion ability of A549 and H1299 cells was tested by transwell invasion experiment (magnification: 40×). * $p<0.05$; ** $p<0.01$.

Cell Culture

Human normal bronchial cells BEAS-2B and LCa cell lines SPC-A1, A549, H1299, and H460 were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin/streptomycin in a cell incubator at 37°C with 5% CO_2.

Cell Transfection

For transient transfection, Lipofectamine 2000 reagent was mixed with siRNAs (GenePharma, Shanghai, China) or microRNA-1324 mimics (RiboBio, Guangzhou, China), and then, added into cells when cell density reached to more than 60%.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cultured cells using TRIzol reagent (TaKaRa, Otsu, Shiga, Japan). Quantitative PCR was carried out using the SYBR Green Real Time PCR kit (TaKaRa, Otsu, Shiga, Japan), with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 used as internal references. The primer sequences are shown as follows: circ-PRKCI Forward: 5′-TAGCAGTTC-CCCAATCCTTG-3′, Reverse: 5′-CACAAATTC-

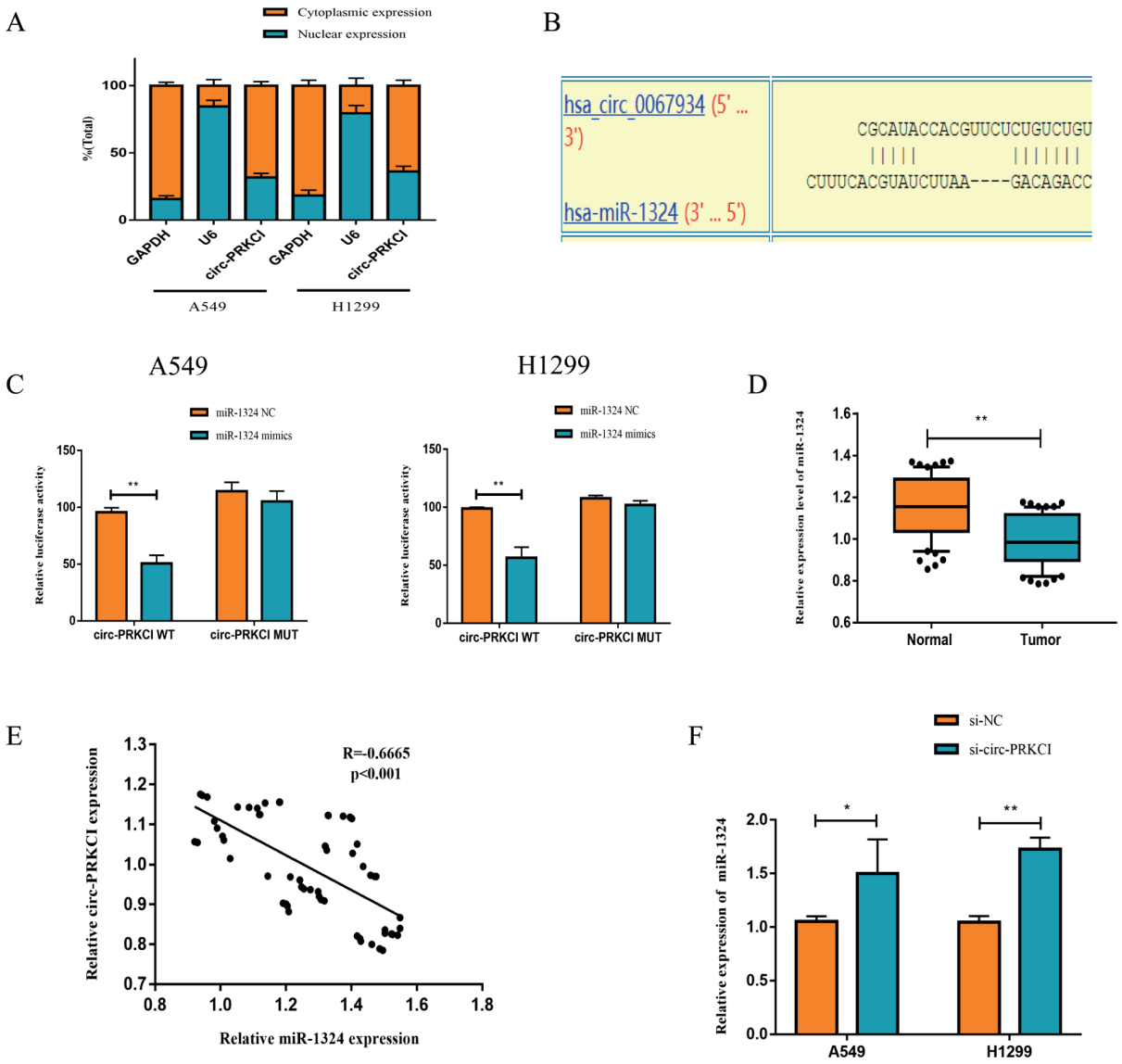

Figure 3. Circ-PRKCI can bind to miR-1324. **A,** Circ-PRKCI distribution in cytoplasm and nucleus of A549 and H1299 was detected by nuclear-plasma separation experiment. **B,** The binding sites of circ-PRKCI and miR-1324 were predicted and circ-PRKCI wild-type and mutant plasmids were constructed (circ-PRKCI-WT and circ-PRKCI-MUT). **C,** The binding relationship between circ-PRKCI and miR-1324 was detected by a dual luciferase reporter gene experiment. **D,** The expression of miR-1324 in lung adenocarcinoma tissues and normal control tissues was detected by qRT-PCR. **E,** Analysis of the correlation between circ-PRKCI and miR-1324 expression in lung adenocarcinoma tissues by Spearman's correlation. **F,** The expression of miR-1324 in A549 and H1299 cells was detected by qRT-PCR after the expression of circ-PRKCI was inhibited. * $p<0.05$; ** $p<0.01$

CCATCATTCCC-3′; microRNA-1324 Forward: 5′-ACACTCCAGCTGGGCCAGACAGAATTC-TATGC-3′, Reverse: 5′-CTCAACTGGTGTC-GTGGAGTCGGCAATTGCAGGA-3′; MECP2 Forward: 5′-TGACCGGGGACCCATGTAT-3′, Reverse: 5′-CTCCACTTTAGAGCGAAAGGC-3′; GAPDH Forward: 5′-CGGAGTCAACGGATTTG-GTCGTAT-3′, Reverse: 5′-AGCCTTCTC-CATGGTGGTGAAGAC-3′; U6 Forward: 5′-GCTGAAAAACTG-3′, Reverse: 5′-GCCTC-CCAGTTTCATGGACA-3′.

Cell Counting Kit-8 (CCK-8) Assay

Cells were plated in 96-well plates ($3*10^3$ cells/well) in 100 uL culture medium. CCK-8 assay (Dojindo Molecular Technology, Kuma-

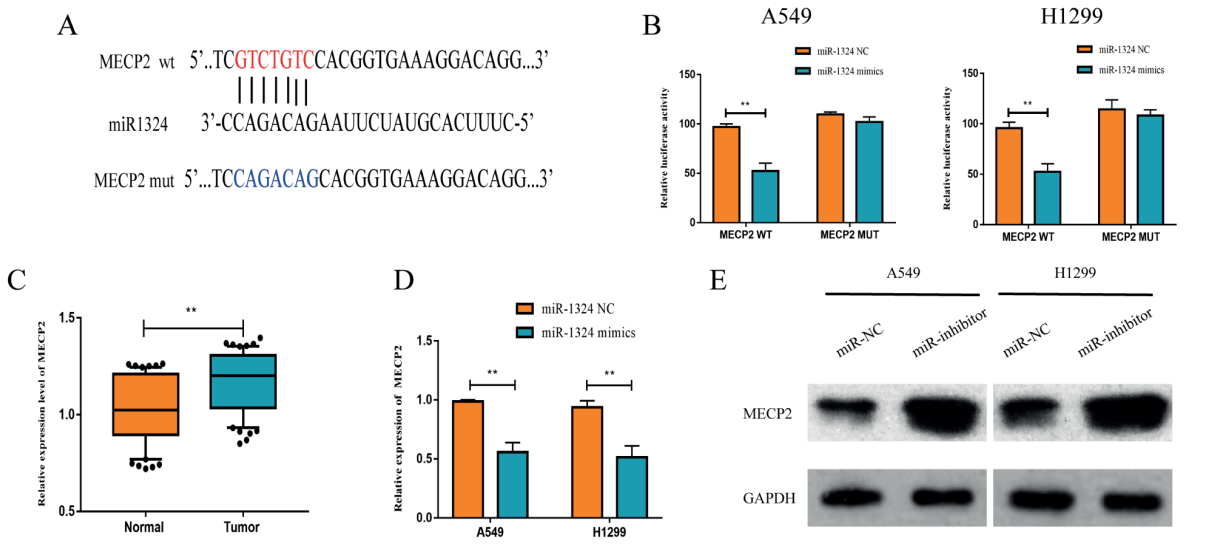

Figure 4. MiR-1324 can negatively regulate MECP2 expression. **A,** The binding sites of MECP2 and miR-1324 were predicted and MECP2 wild-type and mutant plasmids were constructed (MECP2-WT and MECP2-MUT). **B,** The binding relationship between MECP2 and miR-1324 was detected by Dual-Luciferase reporter gene experiments. **C,** MECP2 expression was detected by qRT-PCR in lung adenocarcinoma tissues and normal control tissues. **D,** MECP2 mRNA expression was detected by qRT-PCR after miR-1324 NC and miR-1324 mimics were transfected in A549 and H1299 cells. **E,** MECP2 protein expression was detected by Western blot after transfecting A549 and H1299 cells with miR-1324 NC and miR-1324 inhibitor. ** $p<0.01$.

moto, Japan) was performed according to the manufacturer's protocol.

Clone Formation Assay

Transfected LCa cells A549 and H1299 were cultured in 6-well plates at a density of 400 cells/well and cultured in the incubator at 37°C and 5% CO_2 for 2 weeks, and the medium was changed every 7 days. Cells were fixed with methanol and stained with crystal violet.

Transwell Assay

24 h after transfection, A549 and H1299 cell lines were prepared into cell suspensions and seeded in the upper chamber (10,000 cells/well) in serum-free medium, and then, 10% FBS medium was added to the lower compartment of the chamber. The migrated cells were counted after washing with crystal violet after washing.

Nuclear Separation Assay

Nuclear lysates were configured with 1% TritonX-100 (Solarbio, Beijing, China), 50 mM Tris pH 7.8, 140 mM NaCl, 10% glycerol, 1 mM EDTA (ethylenediaminetetraacetic acid), and protease inhibitor (without EDTA). Nuclei and cytoplasm were separated according to the product instructions using the PARIS kit (Thermo Fisher Scientific, Waltham, MA, USA).

Luciferase Assay

The specific binding sites of miR-1324 and circ-PRKCI or MECP2 were predicted. The wild-type plasmid or the mutant plasmid was cloned to the pGL3 vector (Promega, Madison, WI, USA), respectively. According to the product instructions, Luciferase reporter miR-1324 and circ-PRKCI or MECP2 were transfected in A549 and H1299 using Lipofectamine 2000 for 24 hours. The Luciferase detection kit (GeneCopoeia, Rockville, MD, USA) was used to detect Luciferase activity.

Western Blot

Proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer and quantified by bicinchoninic acid (BCA) assay (Beyotime, Shanghai, China). Relative protein markers were immunoprecipitated from 50 ug of total protein extracts and subjected to electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel.

Statistical Analysis

All experimental data statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 20.0 (IBM Corp., Armonk, NY, USA). Student's *t*-test was used to compare the differences between the samples analyzed.

Results

Circ-PRKCI Level was Remarkably Increased in LCa

In comparison to the normal control group, circ-PRKCI showed abnormally high expression in LCa tumor tissue samples (Figure 1A). By analyzing clinical data, we found that the relative expression of circ-PRKCI in tumor tissue of patients in advanced stage was higher than those in the low-stage group (Figure 1B). At the same time, Figure 1C indicated that the increased expression of circ-PRKCI can predict a poor prognosis of LCa patients (p=0.023) (Figure 1C). In addition, through further testing, we also found that circ-PRKCI level in LCa cell lines was also remarkably higher than that of the normal control cell line BEAS-2B (Figure 1D). These results suggested that circ-PRKC played a vital role in LCa.

Inhibiting Circ-PRKCI Level can Inhibit the Proliferation of LCa Cells

To determine the biological effects of circ-PRKCI in LCa, we selected A549 and H1299 cells for *in vitro* cell experiments. First, we silenced circ-PRKCI level in A549 and H1299 cells by small interfering RNA (siRNA) (Figure 2A). Then, we explored the effect of circ-PRKCI on the proliferation ability of A549 and H1299 cells through CCK-8 and cell clone formation assays. The results showed that circ-PRKCI knockdown can remarkably inhibit the proliferation ability of LCa cells (Figure 2B). Figure 2C showed that low expression of circ-PRKCI inhibited the colony forming ability of LCa cells (Figure 2C). Then, we explored the effect of circ-PRKCI on cell invasion ability through transwell invasion assay. As shown in Figure 2D, the invasion ability of A549 and H1299 cells transfected with circ-PRKCI siRNA was remarkably reduced as compared with the si-NC group. These results showed that circ-PRKCI knockdown can inhibit the proliferation of LCa cells.

Circ-PRKCI Can Adsorb MicroRNA-1324

We first performed subcellular localization of circ-PRKCI by nuclear separation assay. The results showed that circ-PRKCI was mostly distributed in the cytoplasm of A549 and H1299 cells (Figure 3A), which indicated that circ-PRKCI may have the ability to act as ceRNA. Then, we predicted through the bioinformatics website (https://circinteractome.nia.nih.gov/) and found that microRNA-1324 and circ-PRKCI may have possible binding sites with a high binding score (Figure 3B). Luciferase results showed that microRNA-1324 mimics could effectively decrease the luciferase activity, indicating that microRNA-1324 can bind to circ-PRKCI (Figure 3C). Through qRT-PCR detection, we found that microRNA-1324 was remarkably reduced in LCa tissues (Figure 3D), which showed that microRNA-1324 may serve as a cancer-inhibiting gene in LCa. The Spearman rank correlation analysis indicated that the relative expression of circ-PRKCI in LCa tissues was negatively correlated with microRNA-1324 expression ($R = -0.6665$, $p<0.001$) (Figure 3E). To verify the regulatory effect of circ-PRKCI on microRNA-1324, we inhibited circ-PRKCI level in A549 and H1299 cells and detected microRNA-1324 expression by qRT-PCR. The results showed that microRNA-1324 expression was remarkably increased in LCa cells after suppressing circ-PRKCI level (Figure 3F). In summary, all experimental results indicated that circ-PRKCI was able to bind microRNA-1324 and regulate its expression.

MicroRNA-1324 Can Target MECP2

To explore the molecular mechanism of circ-PRKCI in LCa, we predicted the possible target genes that could bind with microRNA-1324 through the bioinformatics website. After screening and expression detection, we found that the binding score of MECP2 and microRNA-1324 was relatively high, and the pre-experimental results showed that MECP2 expression was abnormally increased in LCa tissues, so we chose it for subsequent research. We then constructed the MECP2 wild-type overexpression plasmid (MECP2-WT) and the MECP2 mutant over-expression plasmid (MECP2-MUT) (Figure 4A). Luciferase reporter assay verified that microRNA-1324 was able to target MECP2 (Figure 4B). Figure 4C indicated that MECP2 expression in LCa tissues was remarkably increased (Figure 4C), which was consistent with our preliminary experimental results. In addition, we found that overexpression of microRNA-1324 remarkably inhibited the expression of MECP2, while the opposite result was observed after knockdown of microRNA-1324 (Figure 4D, 4E). The above observations indicate that circ-PRKCI

may bind to microRNA-1324 to promote MECP2 expression and thus participate in LCa progression.

Discussion

In recent years, LCa has become one of the most common malignant tumors in the world[24]. About 85% of LCas are non-small cell LCa (NSCLC), of which about 50% are lung adenocarcinoma. However, due to late diagnosis and limited treatment, the five-year survival of LCa patients is only 10%-15%[25,26]. Therefore, the identification of new biomarkers and therapeutic targets for LCa will supply a basis for improving diagnosis and treatment of LCa.

CircRNAs, discovered in RNA viruses in 1976, are by-products of abnormal RNA splicing. With the advancement of high-throughput sequencing technology, especially RNA sequencing, as many as 30,000 circRNAs have been discovered[27]. In recent years, there have been more and more studies on circRNAs in LCa, playing the role of tumor suppressor genes or oncogenes in the process of tumor onset. In fact, circCRIM1 inhibits the invasion and metastasis of LCa through microRNA-182/microRNA-93 leukemic inhibitory factor receptor pathway[28]. Circ-0006427 inhibited the progression of LCa by regulating microRNA6783-3p/dkk1-axis inactivation of Wnt/beta-catenin signaling pathway[29]. In addition, the highly expressed circ-0012673 adsorbs microRNA22 to promote the proliferation of LCa[30]. A previous study showed that high expression of the circular RNA circPRKCI drives lung adenocarcinoma tumorigenesis, however, the potential molecular mechanism remains unclear[31]. In this study, we indicated the abnormally high expression of circ-PRKCI in LCa by qRT-PCR and revealed that the increased expression of circ-PRKCI predicted the poor prognosis of LCa patients. Subsequently, through *in vitro* experiments, we found that inhibition of circ-PRKCI level in LCa cells could remarkably inhibit cell proliferation.

MiRNAs can be involved in tumorigenesis, cell migration, and invasion through a variety of signaling pathways, and may become a new therapeutic target for tumors[31-33]. MiRNAs have previously been shown to regulate a variety of biological processes, including cell development, cell proliferation, cell differentiation, and cell death[34,35]. Therefore, studies[36,37] on miRNA in lung adenocarcinoma have become increasingly popular in recent years. So, members of the microRNA143/microRNA145 family act as tumor suppressor genes in LCa. MicroRNA944 inhibits the growth of LCa cells by targeting STAT1 regulation[38].

Through bioinformatics analysis, we speculated that circ-PRKCI might bind microRNA-1324 and act as ceRNA to promote the expression of MECP2, and we verified this hypothesis by *in vitro* cell experiments. MECP2 is an epigenetic regulator with important functions in the brain and neurons[39,40]. MECP2 is associated with many molecular functions, such as transcriptional regulation, protein translation, RNA splicing, etc.[41,42]. Recently, MECP2 has been observed[43,44] to play a role in the progression of prostate cancer, breast cancer, and gastric cancer. In the present study, we showed that MECP2 was involved in the tumor proliferation in LCa.

However, there are still many shortcomings in this study. First of all, the nucleus-plasma separation experiment may not be so convincing as the fluorescence *in situ* hybridization results. At the same time, we still need to explore and study the biological functions of microRNA-1324 and MECP2 in future studies. Besides, the cancer-promoting properties of circ-PRKCI have not been further investigated in animals in this study, which needs to be further studied.

Conclusions

This study preliminarily identified the abnormally high expression of circ-PRKCI in lung adenocarcinoma. Circ-PRKCI may enhance invasiveness and proliferative capacity of lung adenocarcinoma cells *via* combining microRNA-1324 to promote the expression of MECP2, which may be a potential therapeutic target for this cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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