The role of Circ_PRKCI/miR-24-3p in the metastasis of prostate cancer

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Summary

Purpose: The purpose of this study was to explore the role of circ_PRKCI in regulating prostate cancer (PCa) development and the underlying molecular mechanism.

Methods: Circ_PRKCI levels in 40 PCa tissues and adjacent normal ones were detected. The relationship between circ_PRKCI level and pathological indicators in PCa patients was explored. After transfection of sh-circ_PRKCI in DU-145 and PC-3 cells, changes in viability, numbers of migratory and invasive cells, and wound closure were examined. Finally, the downstream target of circ_PRKCI was confirmed by dual-luciferase reporter assay and their involvement in PCa development was finally illustrated by rescue experiments.

Results: Circ_PRKCI was upregulated in PCa tissues than adjacent normal ones. PCa patients expressing a high level of circ_PRKCI had high risks of lymphatic metastasis and distant metastasis. Knockdown of circ_PRKCI weakened proliferative and metastatic abilities of PCa cells. As the downstream target of circ_PRKCI, miR-24-3p was negatively regulated by it. Moreover, circ_PRKCI/miR-24-3p axis was responsible for triggering proliferative and metastatic potentials in PCa.

Conclusions: Circ_PRKCI is upregulated in PCa tissues, and its level is linked to metastasis rate in PCa patients. It triggers proliferative and metastatic potentials in PCa by downregulating miR-24-3p

Key words: Circ_PRKCI, MiR-24-3p, Prostate cancer (PCa), metastasis.

Introduction

Prostate cancer (PCa) is a common malignancy in the male urogenital system. Its incidence ranks first in male malignancy [1,2]. It is reported that in 2016, there were 180,890 new cases and 26,120 deaths of PCa in the United States. The incidence of PCa varies with races and regions, which is much lower in our country than that in Western countries [1,3]. With the improvement in life quality and popularization of tumor screening, the incidence and mortality of PCa are on the rise [3,4]. Although radical prostatectomy, radiation therapy, endocrine therapy and other anti-cancer strategies have been progressed, we experience high rates of metastasis and recurrence [5,6]. Metastasis is a complex progression involving detachment of cancer cells from primary cancerous foci, tissue infiltration by blood or lymph diffusion, distant proliferation of cancer cells and finally formation of metastases [7,8]. Some dysfunctional genes in cancer microenvironment lead to abnormal expressions of metastasis-associated genes, thus inducing cancer cell metastasis [9,10]. Therefore, it is urgent to analyze molecular mechanisms underlying PCa metastasis [10].
CircRNAs are featured by the covalently closed loop structure, high conservatism, stability and specificity [11,12]. They are widely expressed in cells and exert functions by miRNA sponge effect, interaction with RNA-binding proteins, and gene regulations [13,14]. Owing to the special structure, circRNAs are more resistant to RNase than traditional linear RNAs. A growing body of evidence has proven that circRNAs have giant potentials in cancer diagnosis and treatment [15,16]. Previous studies have identified that circ_PRKCI is closely linked to tumor development [17-19]. Here, we collected 40 PCa tissues and adjacent normal ones to detect differential expression of circ_PRKCI in PCa samples, and its potential regulatory effects on PCa cell phenotypes.

**Methods**

**PCa samples**

A total of 40 pairs of PCa and adjacent normal tissues that were pathologically confirmed by hematoxylin and eosin (H&E) were collected. All samples were stored at -80°C for RNA extraction. PCa patients treated by chemotherapy and/or radiotherapy before surgery were excluded. This study got approval by Ethics Committee of The First Affiliated Hospital of Jiangxi Medical College and it was conducted after informed consent was obtained from each subject.

**Cell lines and reagents**

PCa cell lines (PC-3, DU-145, 22RV1 and Lncap) and the prostate stromal immortalized cell line (WPMY-1) were provided by American Type Culture Collection (ATCC) (Manassas, VA, USA). Cells were cultured in F-
12k or Roswell Park Memorial Institute 1640 (RPMI 1640) (HyClone, South Logan, UT, USA) with 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA) in a 5% CO₂ incubator at 37°C.

**Transfection**

Transfection plasmids were constructed by Gene Pharma (Shanghai, China). Cells were cultured to 30-60% confluence in 6-well plates and transfected with plasmids using Lipofectamine 2000. 48 hours later, cells were collected for testing transfection efficacy and functional experiments.

**Cell proliferation assay**

Cells were inoculated in a 96-well plate with 2×10⁵ cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the cell counting kit-8 (CCK-8) kit (RIBOBIO, Guangzhou, China) for plotting the viability curves.

**Transwell assay**

Transwell chambers (Millipore, Billerica, MA, USA) were inserted in each well of a 24-well plate. 200 μL of suspension (5×10⁵ cells/mL) was applied in the upper layer of the chamber with 700 μL of medium containing 20% FBS in the bottom. After 48-h incubation, migratory cells in the bottom were reacted with methanol for 15 min, crystal violet for 20 min and captured using a microscope. Migratory cells were counted in 10 random selected fields per sample. Invasion assay was similarly conducted where transwell chambers were pre-coated with Matrigel and incubated overnight.

**Wound healing assay**

Cells were inoculated in 6-well plates and grown to 90% confluence. After creation of an artificial wound in cell monolayer, medium with 1% FBS was replaced. 24 hours later, wound closure was captured for calculating the percentage of wound healing.

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**Figure 2.** Circ_PRKCI triggered proliferative and metastatic potentials of PCa cell lines. **A:** Viability in DU-145 and PC-3 cells transfected with sh-circ_PRKCI or sh-NC. **B:** Migration and invasion in DU-145 and PC-3 cells transfected with sh-circ_PRKCI or sh-NC. **C:** Wound healing percentage in DU-145 and PC-3 cells transfected with sh-circ_PRKCI or sh-NC. Data were expressed as mean±SD. *p<0.05, **p<0.01.
Quantitative real-time polymerase chain reaction (qRT-PCR)

Extracted RNAs by TRizol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using Primescript RT Reagent (TaKaRa, Otsu, Japan). The obtained cDNAs underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Japan). Each sample was performed in triplicate. Relative level was calculated by $2^{-ΔΔCt}$ and normalized to that of glyceraldheyde 3-phosphate dehydrogenase (GAPDH) or U6. circ_PRKCI: forward: 5'-ATTCAGGAGACCCCGTTCTT-3', reverse: 5'-CTCTTCAGAACACTTCGGAGTTCTT-3'; U6: forward: 5'-CTCGTTTGGCGACGACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'; miR-24-3p: forward: 5'-ACCTGAGATGAGACAGGAGT-3', reverse: 5'-GTAGAATCCTTTGCGGTCACAA-3'; GAPDH: forward: 5'-CGCTCTCTGCTCCTCCTGTTC-3', reverse: 5'-ATCCGTTGACCTGGACCTTCAC-3'.

Dual-luciferase reporter assay

HEK293T cells inoculated in a 24-well plate were co-transfected with NC mimic/miR-24-3p mimic and circ_PRKCI-WT/circ_PRKCI-MUT. They were lysed at 48h and subjected to measurement of luciferase activity (Promega, Madison, WI, USA).

Statistics

SPSS software 22.0 (IBM, Armonk, NY, USA). Differences between groups were analyzed by the t-test. Chi-square analysis was conducted for analyzing the relationship between circ_PRKCI level and pathological indicators in PCa patients. Pearson correlation test was applied for evaluating the relationship between relative expressions of two genes. Kaplan-Meier curves were depicted for survival analysis in PCa patients. p<0.05 was considered as statistically significant.

Results

Circ_PRKCI was highly expressed in PCa tissues and cell lines

We collected 40 pairs of PCa and adjacent normal tissues. As qRT-PCR data revealed, circ_PRKCI was highly expressed in PCa tissues than adjacent ones (Figure 1A). According to the median level of circ_PRKCI in the 40 PCa tissues, corresponding patients were classified into high and low expression groups. Case number in each group was recorded, and the relationship between circ_PRKCI level and pathological indicators in PCa patients was analyzed. It was shown that a high level of circ_PRKCI indicated high susceptibilities to lymphatic metastasis and distant metastasis in PCa patients. Nevertheless, it was unrelated to age, sex and tumor staging. As expected, higher abundance of circ_PRKCI was seen in PCa patients accompanied by lymphatic metastasis or distant metastasis than those without metastases (Figure 1B).

In vitro expression of circ_PRKCI was identically upregulated in PCa cell lines (Figure 1C). In the four tested PCa cell lines, DU-145 and PC-3...
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Cells expressed the highest level of circ_PRKCI, so they were used for establishing circ_PRKCI knockdown model by transfection of sh-circ_PRKCI (Figure 1D).

**Circ_PRKCI triggered proliferative and metastatic potentials in PCa cell lines**

CCK-8 assay showed the decreased viability of DU-145 and PC-3 cells transfected with sh-circ_PRKCI than that of controls, indicating the weakened proliferative potential (Figure 2A). The numbers of migratory and invasive PCa cells with circ_PRKCI knockdown were smaller than those of controls (Figure 2B). Similarly, wound closure percentage was reduced after knockdown of circ_PRKCI in PCa cells, further supporting the role of circ_PRKCI in triggering metastasis in PCa (Figure 2C).

![Figure 4. Circ_PRKCI/miR-24-3p axis regulated PCa cell phenotypes.](image-url)

A: MiR-24-3p level in DU-145 and PC-3 cells co-transfected with sh-NC+NC inhibitor, sh-circ_PRKCI+NC inhibitor and sh-circ_PRKCI+miR-24-3p inhibitor. B: Viability in DU-145 and PC-3 cells co-transfected with sh-NC+NC inhibitor, sh-circ_PRKCI+NC inhibitor and sh-circ_PRKCI+miR-24-3p inhibitor. C: Migration in DU-145 and PC-3 cells co-transfected with sh-NC+NC inhibitor, sh-circ_PRKCI+NC inhibitor and sh-circ_PRKCI+miR-24-3p inhibitor. Data were expressed as mean±SD. *p<0.05, **p<0.01.
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Interestingly, miR-24-3p was upregulated in DU-145 and PC-3 cells transfected with sh-circ_PRKCI (Figure 3A). In PCa tissues, miR-24-3p was lowly expressed and displayed a negative correlation with circ_PRKCI level (Figure 5B). Similarly, it was downregulated in PCa cell lines (Figure 5C). Based on the predicted binding sites in the 3’UTR of circ_PRKCI and miR-24-3p, we carried out dual-luciferase reporter assay. Overexpression of miR-24-3p was able to decrease luciferase activity in wild-type circ_PRKCI vector rather than the mutant-type one, confirming that miR-24-3p was the target binding circ_PRKCI (Figure 3D).

Circ_PRKCI/miR-24-3p axis regulated PCa cell phenotypes

We found that the upregulated miR-24-3p in DU-145 and PC-3 cells with circ_PRKCI knockdown was reversed by co-silence of circ_PRKCI and miR-24-3p (Figure 4A). Notably, the inhibited viability and migratory potential in PCa cells induced by circ_PRKCI knockdown were abolished by downregulated miR-24-3p (Figure 4B, 4C).

Discussion

The incidence of PCa increases with age. Globally, the incidence and mortality of PCa ranks 3rd and 6th in male malignancies, which is 1st and 2nd in the United States, respectively [1,2]. At present, the extensively applied detection of prostate specific antigen (PSA) level contributes to rapid increase in the incidence of PCa. Moreover, the proportion of PCa patients in the middle and advanced stage is relatively high in newly diagnosed cases. As a result, they are poorly responded to anti-cancer treatment and suffer a low survival mainly because of metastases [3,4]. In addition, the occurrence and development of PCa are complicated and prone to pathological changes such as bone metastasis and castration resistance [5-8].

CircRNAs are formed by joining the 3’ and 5’ end in a covalently closed continuous loop, which are responsible for gene regulation [11-13]. As early as 1976, it was discovered that viroids are composed of circRNAs. However, they have been regarded as post-transcriptional splicing intermediates, by-products or accidental splicing errors, and have not been taken seriously [20]. With the development of next-generation high-throughput sequencing technology and the improvement of bioinformatics tools, the structure and function of circRNAs have been gradually identified. Many studies have demonstrated the involvement of circRNAs in tumor diseases [11,14-16]. A large number of differentially expressed circRNAs have been found between tumor tissues and paracancerous ones, and they are also detected in plasma of tumor patients and healthy subjects [16-19]. CircRNAs may be utilized as tumor hallmarks for specific detection and prognosis judgement. In this paper, we found that circ_PRKCI was upregulated in PCa tissues and cell lines. A high level of circ_PRKCI indicated high susceptibilities to lymphatic metastasis and distant metastasis in PCa patients. Hence, we believed that circ_PRKCI could be a tumor promoter involved in PCa progression. Experimental results proved that circ_PRKCI was able to promote proliferative and metastatic potentials in PCa cells. The specific molecular mechanisms, however, remained unclear.

It has been widely studied that circRNAs exert sponge effects by sponging miRNAs [12,13]. Through predicting binding sites in the 3’UTR of circ_PRKCI and miR-24-3p, luciferase vectors of wild-type and mutant-type circ_PRKCI were constructed. Dual-luciferase reporter assay confirmed the binding relationship between circ_PRKCI and miR-24-3p. Subsequently, biological functions of miR-24-3p in regulating PCa cell behaviors were explored. MiR-24-3p was detected to be downregulated in PCa samples, displaying a negative correlation to circ_PRKCI level. Higher viability and metastasis were seen in PCa cells with co-silence of circ_PRKCI and miR-24-3p compared with those with solely circ_PRKCI knockdown. It is indicated that miR-24-3p is involved in the malignant progression of PCa regulated by circ_PRKCI. Our findings provide potential hallmarks for the detection, treatment and monitoring of PCa.

Conclusions

Circ_PRKCI is upregulated in PCa tissues, and its level is linked to the metastasis rate in PCa patients. It triggers proliferative and metastatic potentials in PCa by downregulating miR-24-3p.

Conflict of interests

The authors declare no conflict of interests.
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References