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Original Research Article

ARPC4 gene silencing inhibits T24 cell invasion and metastasis via a mechanism involving Arp2/3/cofilin-1 signaling pathway

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Abstract

Purpose: *To study the influence of ARPC4 gene silencing on human urinary bladder cancer (T24) cell proliferation, invasiveness and migration, and the mechanism(s) involved.*
Methods: *Short interfering RNA (siRNA) ARPC4 silencing fragment was transfected into T24 cells. Transfection efficiency was measured with qRT-PCR. Cell proliferation, invasiveness and migratory potential were determined with CCK-8, Transwell invasion assay, and immunofluorescence assay, respectively. Protein expressions of ARPC4 and cofilin-1 were assayed using Western blotting.*
Results: *Short interfering RNA (siRNA) silencing of ARPC4 gene led to the downregulation of mRNA and protein expressions of ARPC4 ($t = 14.898$, $p < 0.05$; $t = 7.686$, $p < 0.05$). It also significantly downregulated cofilin-1 protein, while inhibiting proliferative capacity, invasiveness and pseudopodia-formation capacity of T24 cells ($t = 8.042$, $p < 0.05$).*
Conclusion: *The results obtained suggest that ARPC4 gene silencing inhibits T24 cell invasion and metastasis via a mechanism involving regulation of the Arp2/3/cofilin-1 signaling route. This provides new leads for gene therapy.*

Keywords: *ARPC4, Bladder carcinoma, Gene silencing, Invasiveness, Cell proliferation*

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INTRODUCTION

Bladder carcinoma refers to different kinds of cancer that affect urinary bladder tissues. It refers to malignant tumor of the mucous membrane of the bladder, and it is highly prevalent among the Chinese. Bladder cancer typically affects older adults aged 50 to 70 years [1]. About 90 % of patients with bladder cancer initially show signs of hematuria (blood in the urine). As the disease progresses, bladder irritation, dysuria and upper urinary tract obstruction may occur, which seriously affect the quality of life of sufferers [2]. The condition is treatable if diagnosed early.

Since bladder cancer is likely to recur, follow-up tests are usually recommended. The treatments used for bladder cancer are cystectomy, biological therapy, radiotherapy and chemo-

therapy. The pathogenesis of bladder cancer is complex and yet to be fully understood. It is thought to be caused by genetic and environmental factors. A highly invasive tumor, bladder cancer is characterized by rapid metastasis and recurrence, and the 5-year survival and overall prognosis are unsatisfactory [3]. At present, there is a dearth of biomarkers that can be used to effectively assess metastasis and prognosis of this disease. This has necessitated the search for novel molecules/proteins of high diagnostic and prognostic values for the disease.

Actin-related protein 2/3 complex subunit 4 (ARPC4) is a subunit of Arp2/3 complex. It functions as actin-binding portion of Arp2/3 complex which controls actin polymerization, and acting in concert with NPF, regulates the generation of branched actin networks [4].

Actin-related proteins-2/3 (Arp2/3) complex is the main nucleating agent of actin assembly which is involved in the maintenance of cell morphology, cytoskeleton formation and movement [4]. It controls cell morphogenesis via the modulation of cell polarity. Studies have shown that ARPC4 expression is significantly upregulated in carcinoma of the pancreas, and that the degree of migration and invasion of the cells are significantly reduced after ARPC4 silencing [5]. The expression of ARPC4 influences the biological behavior of tumor cells [6]. The aim of this research was to study the effect of ARPC4 gene silencing on T24 cell proliferation, invasiveness and migration, and the mechanism(s) involved.

EXPERIMENTAL

Materials

Phosphate-buffered saline (PBS) was a product of Wuhan Yipu Biotechnology Co. Ltd; RPMI1640 culture medium was obtained from Shanghai Jingke Chemical Technology Co. Ltd, while BCA protein assay kits were purchased from Beijing Bio-Lab Technology Co. Ltd. Sealing liquid was obtained from Beijing YiAobo Science & Trade Co. Ltd., while ARPC4 and cofilin-1 primary antibodies were products of Shanghai Wuhao Biotechnology Co. Ltd. Ponceau protein dye was purchased from Shanghai Yiyan Biotechnology Co. Ltd.

Micro sampler was a product of Shanghai Boguang Biotechnology Co. Ltd. Electrothermal thermostatic bath was obtained from Shanghai Xinyu Biotechnology Co. Ltd. Cryogenic (-80 ^{o}C) refrigerator was purchased from Shanghai Fuze Trading Co. Ltd, while PVDF membrane was obtained from Bio-Easy Technology Co. Ltd. (Beijing). Decolorization shaker was a product of Nanjing Beideng Medical Co. Ltd. Electrophoresis tank was purchased from Shanghai Tuseng Visual Technology Co. Ltd. Low-temperature centrifuge was obtained from Sichuan Shuke Instrument Co. Ltd. Vortex oscillator was a product of Guangzhou Haohan Instrument Co. Ltd. Optical microscope was purchased from Shanghai Yiji Industrial Co. Ltd., while digital gel imaging system was obtained from Shanghai Qinxiang Scientific Instruments Co. Ltd.

Cell line and culture

Human urinary bladder cancer cell line (T24) was obtained from Shanghai Xinyu Biotech. Company Limited, and was maintained for 24 h at 37 °C in RPMI-1640 containing 10 % FBS and streptomycin/penicillin (1%) in a 5 % incubator until they attained 80 % confluency. The medium was replaced with fresh one every two days. After 1 week of incubation, the adherent confluent cells were trypsinized with 0.25 % trypsin-EDTA (2 mL), cultured again, and passaged for later use. Cells in logarithmic growth phase were selected and used in this study.

Cell transfection

The T24 cells were seeded in 6-well plates at a density of 2 x 10^4 cells/well until they attained 80 % fusion. Then, the cells were maintained in serum-free medium with equal volume of si-ARPC4 (ARPC4 silenced group) or siRNA (negative control group), each at a concentration of 10 µmol/L. Incubation was carried out at room temperature for 6 h. Lipofectamine 2000 was dissolved in serum-free medium and incubated for proper mixing for 10 min at laboratory temperature. The mixture was added to cells in each group and cultured at 37 ^{o}C in a humidified atmosphere of 5 % CO_2 and 95 % air for 48 h. Normal cell culture without siARPC4 or siRNA served as normal control group. The transfection efficiency was assessed using qRT-PCR.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

After 48 h of cell transfection, total RNA was extracted from cells of each group using Trizol reagent. The RNA was reverse-transcribed to cDNA using appropriate kits. Then, Light Cycler 1536 RT-PCR detector was employed for determination of expression levels of ARPC4. Differences in cDNA levels were subjected to

normalization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The qRT-PCR reaction conditions were: pre-denaturation at 95 ºC for 5 min, PCR reaction at 95 ºC for 5 sec and 60 ºC for 30 sec, and a total of 40 cycles in a 20-µL reaction solution comprising dH_2O, primer (10 µM), cDNA and Premix Ex Taq™ II (10 µL). Determination of relative transcription levels was done with $2^{-\Delta\Delta Ct}$ method.

Cell proliferation assay

Cell proliferation was measured with CCK-8 assay kit. The cells were plated in 96-well plates [4×10^3 cells/well (100 µL/well)] and maintained for 24 h at 37 °C in a 5 % CO_2 incubator. Then, following addition of 10 µL of CCK-8, the mixture was subjected to culturing for 72 h. The optical density of the sample was measured at 450 nm. The assay was done thrice.

Assay of cell invasiveness

The degree of invasiveness of T24 cells was estimated with Transwell invasion procedure. The cells were placed in Transwell chamber coated at a concentration of 5×10^5 cells/mL, with substrate, and were cultured in serum-free medium. The lower chamber contained medium with 10 % FBS. After 48 h, cells that passed through the matrix gel membrane were stained with crystal violet after fixation, photographed and counted using an inverted microscope.

Immunofluorescence assay

Immunofluorescence assay was used to determine morphological changes in T24 cells. The cells were subjected to fixation in 4 % paraformaldehyde on gelatin-coated coverslips and rinsed twice with PBS, each rinse for 5 min. Following treatment with 5 % BSA, overnight incubation with 1º antibodies was done. After washing 3 times with PBS, FITC/TRITC-labeled secondary antibody was added, followed by incubation for 60 min under laboratory conditions. Thereafter, the cells were stained with DAPI for 15 min and washed for 5 min. After the addition of 50 % glycerol, the cells were subjected to fluorescence microscopic examination and captured in camera.

Western blotting

The cells were subjected to lysis with ice-cold RIPA buffer laced with protease inhibitor. Following centrifugation, the protein level of the supernatant was measured with BCA method in line with the kit protocol. Then, 40-µg protein fractions were subjected to SDS-PAGE, followed by electroblotting onto PVDF membrane for 2 h. Thereafter, the membrane was blocked by incubation with 3 % non-fat milk solution, after which incubation with 1º antibodies for ARPC4, cofilin-1 and β-actin (all diluted 1: 800) was done for 12 h at 4 °C. Following rinsing with TBS-T, the membrane was subjected to incubation with HRP-linked goat anti-rabbit IgG 2º antibody at laboratory temperature for 90 min. Blot development was done with X-ray film, while ImageJ Launcher software was used for Grayscale analysis. β-Actin served as standard.

Statistics

The results are presented as mean ± SEM, and they were statistically analyzed with SPSS (version 21.0). Comparison of groups was done with Student's *t*-test. Values of $p < 0.05$ indicated significant differences.

RESULTS

Influence of ARPC4 gene silencing on ARPC4 mRNA expression in T24 cells

As indicated in Figure 1, ARPC4 mRNA was significantly lower in ARPC4-silenced cells, relative to negative control. In contrast, ARPC4 mRNA expressions in negative and normal control groups were comparable.

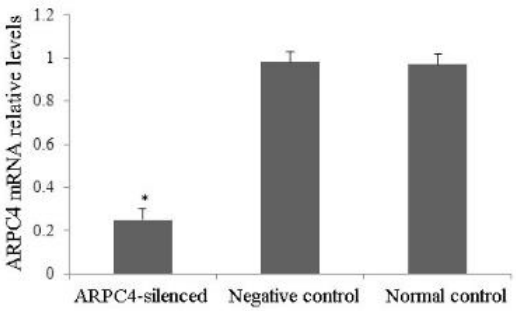

Figure 1: Influence of ARPC4 silencing on ARPC4 mRNA expression in T24 cells. *$p < 0.05$, vs negative control

Influence of ARPC4 gene silencing on T24 cell proliferation

As shown in Table 1, ARPC4 gene silencing markedly suppressed the proliferative potential of T24 cells.

Effect of ARPC4 silencing on T24 cell invasiveness

Human urinary bladder cancer (T24) cells in ARPC4-silenced group were significantly less

invasive than cells in the negative control group ($p < 0.05$; Table 2).

Table 1: Effect of ARPC4 gene silencing on T24 cell proliferation (mean ± SD, n = 6)

Group	Time (h)		
	48	***72***	***96***
ARPC4-silenced	0.61 ± 0.05*	0.80 ± 0.09*	0.91 ± 0.08*
Negative control	0.81 ± 0.04	1.13 ± 0.07	1.52 ± 0.08
Normal control	0.80 ± 0.06	1.20 ± 0.07	1.61 ± 0.07

*$P < 0.05$, vs -ve control at same period

Table 2: Effect of ARPC4 silencing on T24 cell invasiveness (mean ± SD, n = 6)

Group	No. of transmembrane cells
ARPC4-silenced	32.40 ± 2.48*
Negative control	59.05 ± 1.86
Normal control	62.02 ± 1.77

*$P < 0.05$, vs -ve control at same period

Effect of ARPC4 gene silencing on T24 cell morphology

The results of immunofluorescence assay showed that ARPC4 gene silencing significantly inhibited pseudopodia formation in T24 cells ($p < 0.05$; Figure 2).

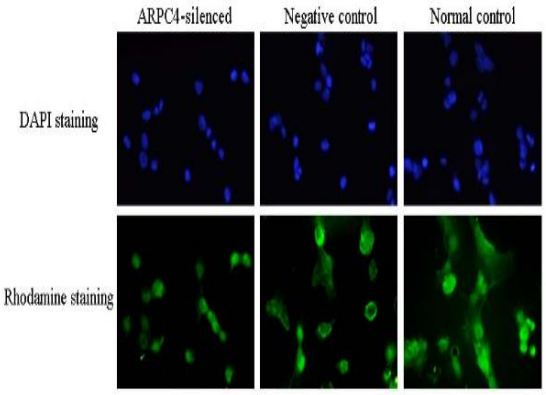

Figure 2: Influence of ARPC4 gene silencing on T24 cell morphology

Effect of ARPC4 silencing on protein expressions of ARPC4 and cofilin-1

The silencing of ARPC4 gene led to the downregulation of ARPC4 and cofilin-1 protein expressions ($p < 0.05$; Table 3, Figure 3).

Table 3: Effect of ARPC4 silencing on ARPC4 and cofilin-1 protein expressions

Group	ARPC4	Cofilin-1
ARPC4-silenced	0.25 ± 0.04*	0.37 ± 0.08*
Negative control	0.92 ± 0.18	0.90 ± 0.11
Normal control	0.89 ± 0.20	0.87 ± 0.13

*$P < 0.05$, vs negative control

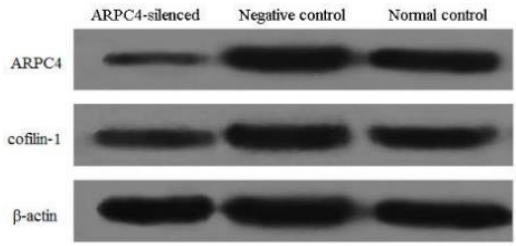

Figure 3: Effect of ARPC4 silencing on ARPC4 and cofilin-1 protein expressions

DISCUSSION

Bladder cancer, a frequently-occurring malignancy in the urinary tissues, is pathologically classified into two types: muscle invasive and non-muscle invasive bladder cancer. Muscle-invasive bladder cancer is highly metastatic, with a 5-year survival of about 50 % [7]. Studies on the mechanism of bladder cancer cell infiltration and metastasis are centered on novel therapeutic targets that can effectively improve prognosis and survival of patients.

The pathogenesis of this disease is complex and multifactorial [8]. Abnormal protein expressions are thought to contribute to the disease onset. Gene mutations affect the use of nutrients and increase the risk of cancer. Increased expression of oncogenes and downregulation or inactivation of tumor suppressor genes are key factors in tumor formation. An important characteristic of tumor cells is their ability to proliferate without restriction, thereby eventually invading other cells or tissues.

The ARPC4, a subunit of Arp2/3 complex, participates in the aggregation and depolymerization of actin. It influences cell motility, and also promotes the synthesis of microfilaments, formation of cytoskeleton and invasive pseudopodia in cancer cells [10]. The cytoskeleton is a complex and dynamic network of interlinking cytoplasmic protein filaments. It plays important roles in the maintenance of cell morphology, motility and other biological behaviors of tumor cells, such as invasion and

metastasis. Therefore, structural changes in the cytoskeleton contribute significantly to the initiation and progression of tumors [11,12].

Studies have shown that ARPC4 protein expression is significantly upregulated in gastric cancer cells, and it is linked to tumor volume, degree of infiltration, invasion and metastasis [13,14]. Similarly, significantly high expression of ARPC4 is found in the serum of colorectal cancer (CRC) patients, and is correlated with lymph node and distant metastasis. Results from animal studies have demonstrated that the downregulation of ARPC4 protein significantly inhibited the invasion and metastasis potential of CRC cells [15-18].

In the present study, siRNA silencing of ARPC4 gene led to the downregulation of mRNA and protein expressions of ARPC4. It also markedly suppressed the proliferative capacity and invasiveness of T24 cells. Results from immunofluorescence assay revealed that the formation of pseudopodia in T24 cells was significantly affected by ARPC4 silencing. These results indicate that ARPC4 silencing may inhibit T24 cell proliferation and invasion, and block the formation of pseudopodia via the regulation of Arp2/3-cofilin-1 signaling pathway.

CONCLUSION

The results obtained in this study suggest that ARPC4 gene silencing inhibits T24 cell invasion and metastasis via a mechanism involving the Arp2/3-cofilin-1 signaling pathway. These findings can provide new leads for gene therapy.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

This study was done by the authors named in this article, and the authors accept all liabilities resulting from claims which relate to this article and its contents. The study was conceived and designed by Guoqiang Chen. Shunyi Pang, Zeqin Yao, Chao Wang and Guoqiang Chen collected and analyzed the data, while Shunyi Pang and Zeqin Yao wrote the manuscript. All authors read and approved the manuscript for publication.

Shunyi Pang and Zeqin Yao contributed equally to this work and should be considered as co-first authors.

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