Overexpression of $\beta$-arrestin2 induces G1-phase cell cycle arrest and suppresses tumorigenicity in renal cell carcinoma


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Abstract. – OBJECTIVE: The objective of this study was to investigate the role of $\beta$-arrestin2 in the proliferation, migration, apoptosis, cell cycle and clone formation of renal cell carcinoma (RCC) cell lines and to explore the possible mechanism of $\beta$-arrestin2 in RCC invasion and metastasis to find a new therapeutic target.

MATERIALS AND METHODS: Cell proliferation, migration, apoptosis, cell cycle and clone formation were analyzed after RCC cell lines (786-0 and CaKi) and transfected with $\beta$-arrestin2 overexpression plasmid. Using small interfering RNA (siRNA) interference technology abrogates $\beta$-arrestin2 overexpression, and changes in cell proliferation, migration, apoptosis, cell cycle and clone formation were analyzed. The expression levels of total IkBa, IkBa phosphorylation (P-IkBa) and NFkB P65 in 786-0 cells were examined after transfection with $\beta$-arrestin2 overexpression plasmid to explore the mechanism of $\beta$-arrestin2.

RESULTS: After transfection with $\beta$-arrestin2 overexpression plasmid, the abilities of proliferation, migration, and cloning formation in 786-0 and CaKi cells decreased significantly, the apoptosis rate increased significantly, and the cell cycles were blocked in the G1 phase. After siRNA reduced the expression of $\beta$-arrestin2, the abilities to proceed through cell proliferation, migration, apoptosis, the cell cycle and clone formation were enhanced. The P-IkBa level in 786-0 cells decreased significantly after transfection, while the expression of P-IkBa in the control group remained high. The expression of NFkB P65 was high in the control group and low in the transfection group.

CONCLUSIONS: The overexpression of $\beta$-arrestin2 can inhibit the growth of RCC cells in vitro, and $\beta$-arrestin2 acts as a tumor suppressor gene in RCC. The main mechanism may directly suppress the phosphorylation of IkBa and indirectly suppress NFkB activation. Thus, $\beta$-arrestin2 is expected to be an important marker of RCC prognosis and a new therapeutic target.

Key Words
$\beta$-arrestin2, Renal cell carcinoma, Suppressor genes.

Introduction
Renal cell carcinoma (RCC) is a malignant tumor originating in the renal parenchyma urinary tubular epithelial system. RCC is also called renal adenocarcinoma and includes the various subtypes originating in different urinary tubular areas. Early RCC is confined to the kidney, so surgical resection can produce a good therapeutic effect. However, surgery is often less effective for RCC with local invasion or distant metastasis. In recent years, molecular targeted therapy has undergone considerable development, but the treatment effect is not satisfactory, as the 5-year survival rate of advanced RCC patients is still less than 10%. With deep research on the mechanisms of RCC, multiple signaling pathways were confirmed to participate in the development process. One or more of these signaling pathways and molecular changes can induce tumorigenesis and regulate the proliferation, invasion and metastasis of tumor cells. Beta-inhibition protein ($\beta$-arrestin) is an important soluble protein with mediated receptor desensitization, including $\beta$-arrestin1 and $\beta$-arrestin2. $\beta$-arrestin is a well-known negative regulator of G-protein-coupled receptor (GPCR) and plays an essential role in signal transduction, desensitization, internalization, sensitive reaction, cell proliferation and gene transcription. $\beta$-arrestin2 can inhibit cell migration, proliferation and differentiation. This study aims to investigate the role of $\beta$-arrestin2 in cell proliferation, migration, apoptosis, cell cycle
changes and clone formation by controlling the expression of β-arrestin2 in RCC cell lines and then to explore its molecular mechanisms, which might provide a useful foundation for developing a molecular targeted drug.

Materials and Methods

Experiment Materials and Instruments

RCC cell lines (786-0 and CaKi-1) were purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Songjiang, Shanghai, China). Other materials included LB culture mediums (Sigma-Aldrich, St. Louis, MO, USA), RPMI-1640 cultures (ScienCell Research Laboratories, San Diego, CA, USA), opti-MEM (Invitrogen, Carlsbad, CA, USA), PCR kits (TaKaRa Bio Inc., Otsu, Shiga, Japan), dsDNA oligos (Audiocodes Biotechnology Company, Beijing, China), T4 DNA ligase kits (New England Biolabs, Beijing Branch, Beijing, China), CCK8 kits (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), MITT kits (Biotium, Hayward, CA, USA), pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA), Lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA), Transwell chambers (Corning Inc., Corning, NY, USA), ApoScreen™ Annexin V Apoptosis Kits (SouthernBiotech, Birmingham, AL, USA). Equipment included a high speed centrifuge (Sigma-Aldrich, St. Louis, MO, USA), a horizontal electrophoresis tank (Bio-Rad, Hercules, CA, USA), a UV/visible light gel imaging system (UVP Company, Upland, CA, USA), a PE9600 PCR device (PE Applied Biosystems, Foster City, CA, USA), a constant temperature incubator (Jianqiao Testing Equipment Co., Ltd, Shenzhen, China), and an Accuri™ C6 Flow Cytometer (BD Pharmingen Inc., San Diego, CA, USA).

Cell Culture

786-0 and CaKi-1 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin streptomycin and 100 mg/L streptomycin. All cell lines were cultured at 37°C in a humidified incubator (5% CO₂).

Cell Transfection

Human β-arrestin2 gene (NM_001257328.1) was cloned into a pcDNA3.1(+) vector. β-arrestin2 was amplified by polymerase chain reaction (PCR); then, the PCR products were double-digested by restriction endonucleases of Pmel and NotI (Takara Bio, Inc., Otsu, Shiga, Japan). Subsequently, the digested products were sub-cloned into the Pmel and NotI sites of the psiCHECK-2 luciferase vector. An empty vector served as a negative control (NC). The small interfering RNA (siRNA) of β-arrestin2 and negative control siRNA (si-NC) was obtained from GenePharma (Zhangjiang, Shanghai, China). Cells were seeded in 96-or 6-well plates 24 h before the experiment. These oligonucleotides were transiently transfected into RCC cells using lipofectamine™2000 according to the manufacturer’s instructions.

CCK 8 Method for Determining Cell Proliferation

Both 786-0 and CaKi-1 cells were inoculated into 96-well cell culture plates at a density of 2×10³ per well. When cell confluence reached 70%-80%, the cell culture medium was changed to SFM and incubated 12 h for synchronization. It was then added to the different treated groups every 24 h as a testing point. Each testing point included 3 wells. Following the introduction of the CCK-8 Kit, the absorbance at 450 nm was measured by ELISA. The values of the control group and positive group were calculated about the value of the blank. Cell vitality in the control group was 100% and was obtained according to the following formula: cell viability (%) = (A value/control A value). Each assay was performed in triplicate.

Apoptosis

Both 786-0 and CaKi-1 cells were washed with phosphate buffered saline (PBS) 2 times. After digestion, 100 µl binding buffer and 10 µl fluorescein isothiocyanate-labeled annexin V (Annexin V-FITC) (20 mg/l) were added to the renal carcinoma cells, and they were kept for 30 min at room temperature away from light. Then, 5 µl propidium iodide (PI, 50 mg/l) was added to the cells. After 5 min, 400 µl binding buffer was added to the cells, and then they were immediately (no more than 1 h) analyzed using FACScan flow cytometry. The cells without Annexin V-FITC and PI cells formed the negative control group. Each test was repeated in triplicate.

Transwell Assay for Determining Invasive Ability

Each transwell chamber was coated with 10 µl fibronectin (0.5 mg/ml) and then dried on an extremely clean bench, with the fibronectin...
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Verify the Effect of β-arrestin2 on IκBα phosphorylation

The experimental transfection group consisted of 786-0 cells transfected with β-arrestin2 overexpression plasmid, while non-transfected 786-0 cells served as the control group. At time points of 0 h, 0.5 h, 2 h, 12 h and 24 h, nuclear protein and cytoplasm protein were extracted separately. The expression levels of P-IκBα and total IκBα were determined by Western blot. At the same time, the NFκB P65 expression level was determined to confirm the activation and nuclear translocation of NFκB.

Statistical Analysis

Experimental data are presented as the mean ± standard deviation and were analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA), with Student’s t-test or one-way analysis of variance and the Student-Newman-Keuls method. A value of \( p < 0.05 \) was considered statistically significant.

Results

Comparison of Cell Activity among Groups

After transfection with β-arrestin2 overexpression plasmid, the cell activities of the experimental groups decreased significantly. The activity levels of transfected 786-0 and CaKi cells were (0.85±0.10) and (0.78±0.06), respectively, whereas the results were (1.40±0.14) and (1.18±0.08) in the control group. These differences were statistically significant (\( t = 13.23, p < 0.01 \), \( t = 21.09, p < 0.01 \)) (Figure 1A, Figure 1B). After down-regulation of β-arrestin2 expression by siRNA, the 786-0 and CaKi cells showed enhanced activity: the 786-0 cell activity was (1.02±0.05), and CaKi cell activity was (1.06±0.03), both of which were higher than the control group results. The difference was statistically significant \( (p < 0.01) \) (Figure 1C, Figure 1D).

Comparison of Apoptosis Rates among Groups

After transfection with β-arrestin2 overexpression plasmid, the apoptosis rate of the experimental groups increased significantly. The apoptosis rates of transfected 786-0 and CaKi cells were 4.5% and 19.1%, respectively (Figure 2A), while the results were 3.5% and 19.5% in the control groups (Figure 2B). These differences were statistically significant \( (p < 0.05) \).
Figure 1. The activity of 786-0 and CaKi-1 cells after transfection or siRNA interference. Notes: A, Activity of 786-0 cell transfected with β-arrestin2 overexpression plasmid; B, Activity of CaKi-1 cell transfected with β-arrestin2 overexpression plasmid; C, Activity of 786-0 cells after siRNA interference; D, Activity of CaKi-1 cells after siRNA interference.

Figure 2. Comparison of apoptosis rates. Notes: A, Apoptosis of 786-0 cells transfected with β-arrestin2 overexpression plasmid; B, Apoptosis of CaKi-1 cells transfected with β-arrestin2 overexpression plasmid.
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Comparison of Invasion Ability among Groups

In the 786-0 cells, the numbers of cells permeating the septum (NCS) of β-arrestin2 overexpression plasmid transfected groups and the control groups were 10.3±4.0 and 59.0±3.6, respectively (t=11.21, p<0.01) (Figure 3A). In the CaKi-1 cells, the NCS values of the transfection group and the control group were 8.7±1.2 and 36.2±4.2, respectively (t=12.80, p<0.01) (Figure 3B). After down-regulation of β-arrestin2 expression by siRNA, in the 786-0 cells, the NCS values of the transfection groups and the control groups were 32.1±1.2 and 10.1±2.2, respectively (t=9.1, p<0.01) (Figure 3C); in the CaKi-1 cells, the NCS values of the transfection groups and the control groups were 21.6±2.4 and 9.0±1.6 (t=8.21, p<0.01) (Figure 3D).

Comparison of Cell Cycle among Groups

In both 786-0 and CaKi-1 cells, transfection with β-arrestin2 overexpression plasmid induced G1-phase cell cycle arrest and produced a significant increase in the G1-G0 phase population compared with the control groups (Figure 4A, Figure 4B). After down-regulation of β-arrestin2 expression by siRNA, the experimental groups showed a significant decrease in the G1-G0 phase population, while the G2-S phase population increased (Figure 4C, Figure 4D).

Comparison of Clone Forming Ability among Groups

After transfection with β-arrestin2 overexpression plasmid, the cell proliferation ability of the experimental groups decreased significantly. The cell proliferation levels of 786-0

Figure 3. The invasion abilities of transfected cells determined using a Transwell assay. Notes: A, Invasion ability of 786-0 cells decreased after β-arrestin2 overexpression plasmid transfection. B, Invasion ability of CaKi-1 cells decreased after β-arrestin2 overexpression plasmid transfection. C, Using siRNA increased the invasion ability of 786-0 cells. D, Using siRNA increased the invasion ability of CaKi-1 cells.
cells in the transfection groups and the control groups were 13.5±4.1 and 89.2±11.6, respectively (t=14.21, p<0.01) (Figure 5A), while the cell proliferation levels of CaKi-1 cells in the transfection groups and the control groups were 24.0±4.8 and 85.1±10.2 (t=13.06, p<0.01) (Figure 5B). After down-regulation of β-arrestin2 expression by siRNA, the cell proliferation ability was enhanced. The cell proliferation levels of 786-0 cells in the transfection groups and the control groups were 68.8±6.9 and 36.4±12.2, respectively (t=11.32, p<0.01) (Figure 5C), while the cell proliferation levels of CaKi-1 cells in the transfection groups and the control groups were 87.6±4.8 and 23.5±5.0 (t=16.76, p<0.01) (Figure 5D).

β-arrestin2 Can Regulate the Phosphorylation of IkBa

After transfection with β-arrestin2 overexpression plasmid, the expression level of P-IkBa decreased significantly in 786-0 cells (Figure 6A), NFkB P65 expression was also low (Figure 6B), while the expression levels of P-IkBa and NFkB P65 in the control group were high (Figure 6A, Figure 6B).

Discussion

RCC is the most common malignant tumor in the urinary system, accounting for 2% to 3% of the adult malignancy incidence. This incidence is different in countries and regions; developed countries show a higher incidence than developing countries. In recent years, the incidence has increased year by year in China. Approximately 30% of patients have metastasis at diagnosis, eliminating the opportunity for surgery. The 5-year survival rate for stage I of RCC is more than 90%, whereas the survival rate of stage IV is less than 20%-30%. Early diagnosis is the key to improving the prognosis of RCC. Several studies have shown that the occurrence and development
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Figure 5. Cell proliferation ability was determined by the clone formation assay. Notes: A, After transfection with β-arrestin2 overexpression plasmid, 786-0 cell proliferation ability decreased significantly. B, After transfection with β-arrestin2 overexpression plasmid, CaKi-1 cell proliferation ability decreased significantly. C, After down-regulation of β-arrestin2 expression using siRNA, 786-0 cell proliferation ability increased. D, After down-regulation of β-arrestin2 expression using siRNA, CaKi-1 cell proliferation ability increased.

of RCC are related to the abnormal activation and expression of various oncogenes and tumor suppressor genes. β-arrestin is a class of intracellular proteins that mediate the desensitization of seven transmembrane-coupled receptors, which play a role in tumor cell invasion and metastasis. β-arrestin regulates signal pathways by regulating the phosphorylation, ubiquitination or intracellular localization of signaling molecules, thereby regulating the life course of tumor cells. Recently, attention has been drawn to determining the biological effects of β-arrestin on tumors and the application of β-arrestin targeted drugs in cancer therapy. β-arrestin2 is an important mediated receptor-desensitization soluble protein and an important negative regulator of GPCR signaling pathways. It has been found to have a certain significance in the prognosis prediction of breast cancer and prostate cancer. Raghuwanshi et al. reported that in rat lung cancer models, β-arrestin2 has the effect of inhibiting tumor growth and metastasis. At present, there are few studies on renal carcinoma and β-arrestin2. In this study, we examined the effects of β-arrestin2 on the proliferation, migration, apoptosis, cell cycle and cloning of renal carcinoma cell lines. It was found that after overexpression plasmid transfection, the RCC migration and cloning ability decreased significantly, the proportion of apoptosis of RCC increased significantly, and the cell cycle was blocked in the G1 phase. When we down-regulated the expression of β-arrestin2 using siRNA, cell proliferation and migration ability clearly increased, and the ability of cell cloning was enhanced, which was similar to the results of Alemayehu et al. Therefore, we speculate that β-arrestin2 may be a tumor suppressor gene in RCC. NFkB is a nuclear transcription factor that occurs widely in many types of cells. It is involved in the development and metastasis of cancer.
NFkB is activated in many types of tumors. IkBa is a key regulator of NFkB. When NFkB is not activated, it forms a complex with IkBa. IkBa is phosphorylated at Ser32 and Ser36 when stimulated by inflammatory factors, growth factors, or chemokines. In normal cells, β-arrestin2 binds to the NFkB inhibitor to prevent the phosphorylation and degradation of NFkB, leading to the inactivation of NFkB in these cells, thus inhibiting tumorigenesis. The results of animal experiment about myocardial infarction showed that the up-regulated expression may inhibit the expression and nuclear translocation of NFkB. The results of our experiments in vitro showed that the level of P-IkBα in the 786-0-cell line after transfection was significantly lower than in the control group, while the expression of P-IkBα in the transfected group was higher than in the control group. The expression of nuclear protein NFkB P65 in the transfected group was significantly higher than in the control group. We speculated that β-arrestin2 may be one of the tumor suppressor genes of RCC and expect it to become a new therapeutic target.

Conclusions

We found that β-arrestin2 plays an important role in RCC and could become a therapeutic target for RCC, suggesting a new train of thought. The results showed that the overexpression of β-arrestin2 could inhibit the proliferation and migration of 786-0 and CaKi-1 RCC lines, induce G1-phase cell cycle arrest, and suppress tumorigenicity in vitro. Through the regulation of IkBa phosphorylation, β-arrestin2 indirectly affects NFkB activation to achieve tumor inhibition. We speculate that β-arrestin2 may be one of the tumor suppressor genes of RCC and expect it to become a new therapeutic target.

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Conflict of interest
The authors declare no conflicts of interest.

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