SERUM AND GLUCOCORTICOID-INDUCED KINASE 1 INHIBITS APOPTOSIS IN PROSTATE CANCER BY PHOSPHORYLATING FOXO3A

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ABSTRACT

Objective: *To observe and analyse the role of serum and glucocorticoid-induced kinase 1 (SGK1) in inhibiting the apoptosis of prostate cancer cells by regulating the phosphorylation of Foxo3a.*

Methods: *PC3 cells were transfected with SGK1 interfering slow virus plasmids, SGK1 overexpressing plasmids and corresponding empty plasmids, labelled as SGK1 interfering group, SGK1 overexpressing group and control group, respectively. The apoptosis rate was detected by flow cytometry and the expression levels of SGK1, Foxo3a and corresponding phosphorylated proteins were detected by western blotting. The SGK1 overexpression group was further transfected with Foxo3a small interference plasmid and control plasmid, labelled as SGK1 overexpression-Foxo3a interference group and SGK1 overexpression-control group, and SGK1 overexpression-blank group, respectively, to detect the apoptosis rate, SGK1, Foxo3a and corresponding phosphorylated protein expression levels in each group.*

Results: *Phosphorylation of Foxo3a (Thr-32) decreased compared with control group and the blank group, phosphorylation SGK1 interference group, SGK1 (Ser-78) and phosphorylated Foxo3a (Ser-253). the apoptosis rate, SGK1 in express group phosphorylation SGK1 (Ser-78) and phosphorylated Foxo3a (Ser-253), and phosphorylation Foxo3a (Thr-32) increased, the decrease in the total cell apoptosis rate, the difference had statistical significance (P<0.05). Foxo3a in the SGK1 interference group was concentrated in the nucleus, while a large amount of Foxo3a was transferred from the nucleus to the cytoplasm in the SGK1 overexpression group, the control group and the blank group. The levels of Foxo3a, phosphorylated Foxo3a (Ser-253) and phosphorylated Foxo3a (Thr-32) decreased and the total apoptosis rate increased in the SGK1 overexpression control group and SGK1 overexpression blank group, respectively (P<0.05).*

Conclusion: *SGK1-induced Foxo3a phosphorylation may be one of the mechanisms responsible for the abnormally decreased apoptosis rate of prostate cancer cells.*

Keywords: *Prostate cancer, serum and glucocorticoid-induced kinase 1, forkhead protein O3a, cell apoptosis.*

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Introduction

Prostate cancer is one of the major diseases endangering male health(1). It is imperative to explore the underlying mechanism of its occurrence and development and seek more effective treatment strategies. Serum- and glucocorticoid-inducible kinase 1 (SGK1) corresponds to the occurrence and development of tumours, and SGK1 levels are highly expressed in a variety of tumours(2-4). It has been reported(5) to promote the growth of prostate cancer with and without androgen receptors, and down-regulation of SGK1 can inhibit the growth of prostate cancer cells with and without androgen receptors. But the specific mechanism of SGK1 in prostate cancer is not clear. Activation of SGK1 causes an increase in concentration of many enzymes, including protein phosphorylation fork O3a (forkhead transcription-factor O3a, Foxo3a), glycogen synthase kinase 3. Phosphorylation ubiquitin ligase as NEDD4-1 appears 2(6), such as the Foxo3a by inducing cell cycle arrest and apoptosis, in turn, promote apoptosis. Phosphorylation of Foxo3a after the loss of activity can lead to the decrease in the rate of apoptosis and

increase in proliferation activity[7]. However, it is not clear whether SGK1 can inhibit apoptosis by phosphorylating Foxo3a in prostate cancer.

In this study, prostate cancer PC3 cells were used to investigate the role of Foxo3a in prostate cancer apoptosis.

Materials and methods

Materials and reagents

PC3 cells and SGK1 interfering lentiviral vector (LV2-shSGK1), SGK1 overexpressed lentiviral vector LV6-SGK1, corresponding blank vector and the Foxo3a interfering sequence were purchased from Shanghai Jima Pharmaceutical Technology (Shanghai). Lipofectamine 2000 in vitro DNA transfection reagent, SGK1 primary antibody, phosphorylated SGK1, Foxo3a and phosphorylated Foxo3a primary antibody were purchased from Abcam, USA. Hrp-labelled goat anti-mouse secondary antibody and AV/7-add apoptosis kit were purchased from BD, USA.

Methods

Experimental Design

• Developed SGK1 interference PC3 cells in model group (SGK1 interference) and SGK1 overexpression of PC3 cells model (SGK1 express group), the control group (with empty plasmid transfection) and blank group (not to carry the plasmid transfection). Detect inhibition of SGK1 expression, phosphorylation of SGK1, Foxo3a and phosphorylation Foxo3a expression level, observe Foxo3a positioning within the cell, and determine apoptosis in each cell.

• Used SGK1 express group and small interference RNA to investigate suppression of Foxo3a, detect Foxo3a expression (SGK1 expression-Foxo3a inhibiting group), SGK1 expression-the control group (with nonspecific small interference RNA transfection), and SGK1 expression-blank group (not are compared, and small interfering RNA transfection). It was also used to detect apoptosis and SGK1 phosphorylation, constituting the SGK1, Foxo3a and phosphorylation Foxo3a expression levels.

Establishment of SGK1-Interfering and Overexpressed PC3 Cell Model

PC3 cells were inoculated on 6-well plates and transfected with SGK1-interfering or overexpressed lentivirus when they reached 30-50 % cell density. After 12~24 h, they were replaced with fresh complete medium. After 72-96 h, the stable cells were screened by puromycin 2-4 g/mL. The SGK1 protein expression was detected by western blotting to determine the transfection effect.

Foxo3a SiRNA Transfection

Non-specific control siRNA sequence: UUCUCCGAACG UGUCACGUdTdT, Foxo3a siRNA sequence: GCUGUCUCUCCAUGGACAAUATT. Lipofectamine 2000 was used for transfection of small interfering RNA. After 36-48 h transfection, fluorescence intensity was observed and total protein and RNA were extracted to detect interference efficiency.

Cell Apoptosis Rate Detected by Flow Cytometry

PC3 cells were transfected with lentivirus or small interfering RNA. After 5×105 cells were cultured in 6-well plates, they were digested with trypsin without EDTA, washed twice with PBS, centrifuged at 2000 RPM for 3-5 min, and 1-5×105 cells were collected. 500 μL 1×binding buffer was added to prepare the cell suspension then 5 μL Annexin V-FITC and 10 μL propidium iodide were added and mixed. The cell apoptosis rate was detected by upflow cytometry after the light avoidance reaction at room temperature for 10 min.

The Expression Levels of SGK1, Foxo3a and Corresponding Phosphorylated Proteins in the Cells Were Analysed by Western Blotting

The total protein of the cells was extracted. The total protein concentration was determined by the BCA protein concentration assay kit, and protein denaturation was carried out according to the measured concentration. The PVDF membrane was obtained after loading, electrophoresis and membrane transformation. A TBST solution of 5 % skim milk powder was applied to a PVDF membrane, sealed for 1-2 h, then diluted with TBST solution of 5 % BSA and incubated overnight at 4 °C.

The primary antibody was recovered, and the membrane was washed with TBST solution 3x for 10 min each. Then the TBST solution was absorbed, the diluted secondary antibody was added, and incubated in a room temperature shaker for 1-2 h.

The membrane was washed with TBST 3x for 10 min each. A chemiluminescence reaction was performed with ECL luminescence kit, and semi-quantitative analysis was conducted after scanning the film. GAPDH bands were used as internal parameters to calculate the relative expression of each protein (= grey value of protein bands/grey value of GAPDH bands).

Detection of Foxo3a Protein Localization by Cellular Immunoluminescence

An appropriate number of cells was added to the 6-well plate to achieve a cell density of 40–50 %. Medium was absorbed and washed with PBS 2–3 x to remove residual liquid.

After fixture with 3 % paraformaldehyde at room temperature for 30 min, PBS was used 3 x to remove residual liquid. One mL 0.1 % tritonx-100 was used to break the film, then washed with PBS 3 x to remove the residual liquid, then 5 % goat serum was added, the plate sealed at 30 °C for 2 h. The liquid was discarded, the primary antibody added and the plate incubated overnight at 4 °C. It was washed with PBS 3 x to remove the residual liquid. Fluorescent secondary antibodies were added and plates incubated at 37 °C for 2 h in the dark. They were washed with PBS 3 x to remove residual liquid and 1 mL DAPI (5 g/mL) dye was added to the core at room temperature for 15 min and washed with PBS 3 x. The expression and localisation of related proteins was observed by inverted fluorescence microscopy.

Statistical method

SPSS 25.0 was used to process the data, and the data were expressed as ($\bar{x}\pm s$). The single-factor analysis of variance and the test of SNK-q were used among the groups, and P<0.05 was considered statistically significant.

Results

Effect of SGK1 expression on Foxo3a phosphorylation

After lentiviral transfection, PC3 cells with stable low or high SGK1 protein expression were obtained, namely, the SGK1 interference group and SGK1 overexpression group.

There was no significant difference in SGK1 expression between the control group transfected with empty plasmid and the blank group without plasmid transfection (P>0.05). Western blotting results showed that there was no statistically significant difference in Foxo3a expression between the SGK1 interference group, the SGK1 overexpression group, the blank group and the control group (P>0.05), but the SGK1 (Ser-78), Foxo3a (Ser-253), and Foxo3a (Thr-32) were significantly decreased in the SGK1 interference group (P<0.05). SGK1 (S78), Foxo3a (Ser-253) and Foxo3a (thr-32) were significantly increased in the SGK1 overexpression group (P<0.05). Expression results are in Table 1.

Group	SGK1	p-SGK1 (Ser-78)	Foxo3a	p-Foxo3a (Ser-253)	p-Foxo3a (Thr-32)
SGK1^{+}	1.704±0.103$^{*\#\Delta}$	0.827±0.035$^{*\#\Delta}$	1.820±0.114	0.582±0.047$^{*\#\Delta}$	0.531±0.032$^{*\#\Delta}$
SGK1*	0.527±0.107$^{*\#}$	0.206±0.038$^{*\#}$	1.781±0.108	0.175±0.041$^{*\#}$	0.105±0.038$^{*\#}$
Control	0.924±0.095	0.671±0.035	1.882±0.115	0.301±0.044	0.317±0.042
Blank	0.938±0.103	0.685±0.031	1.827±0.120	0.310±0.041	0.302±0.040
F	69.731	180.433	0.397	46.993	62.361
P	0.000	0.000	0.759	0.000	0.000

Table 1: Comparison of relative expressions of SGK1, Foxo3a and corresponding phosphorylated proteins in the four groups after regulating SGK1 expression.

*Note: *Compared with SGK1 overexpression-control group, P<0.05; Compared with SGK1 overexpression-blank group, P<0.05. SGK1+: SGK1 overexpression; SGK1*: SGK1 interference.*

Effect of SGK1 expression regulation on apoptosis rate of PC3 cells

There was no significant difference in the early apoptosis rate, late apoptosis rate and total apoptosis rate between the control and the blank groups (P>0.05). The early apoptosis rate, late apoptosis rate and total apoptosis rate in the SGK1 interference group were all higher than those in the control group and the blank group, with statistically significant differences (P<0.05).

The total apoptosis rate of SGK1 overexpression group was lower than that of control and blank groups, and the difference was statistically significant (P<0.05). Apoptosis rates are in Table 2.

Group	Early apoptosis (Q4)	Late apoptosis (Q2)	Total rate of apoptosis (Q2+Q4)
SGK1^{+}	1.83±0.31$^{\Delta}$	8.31±0.71$^{\Delta}$	9.14±0.73$^{*\#\Delta}$
SGK1*	4.35±0.38$^{*\#}$	14.26±0.87$^{*\#}$	18.61±0.79$^{*\#}$
Control	1.70±0.35	9.56±0.70	11.26±0.81
Blank	1.81±0.30	9.81±0.68	11.62±0.69
F	43.828	36.672	88.825
P	0.000	0.000	0.000

Table 2: Comparison of apoptosis rates among the four groups after regulating SGK1 expression (%).

*Note: *Compared with SGK1 overexpression-control group, P<0.05; Compared with SGK1 overexpression-blank group, P<0.05. SGK1+: SGK1 overexpression; SGK1*: SGK1 interference.*

SGK1, Foxo3a and corresponding phosphorylated proteins in the SGK1, Foxo3a overexpression group were regulated

Foxo3a in the SGK1 interference group gathered in the nucleus, while the SGK1 overexpression group, the control group and the blank group showed a large amount of Foxo3a transferred from the nucleus to the cytoplasm. There was no signifi-

cant difference in SGK1, phosphorylated SGK1 (Ser-78), Foxo3a, phosphorylated Foxo3a (Ser-253) and phosphorylated Foxo3a (Thr-32) between the control group and the control group (P>0.05).

The levels of Foxo3a, phosphorylated Foxo3a (Ser-253) and phosphorylated Foxo3a (Thr-32) decreased significantly in the SGK1 overexpression control group and SGK1 overexpression blank group (P<0.05), but the levels of SGK1 and phosphorylated SGK1 (Ser-78) did not change significantly (P>0.05). See phosphorylation results in Table 3.

Group	SGK1	p-SGK1 (Ser-78)	Foxo3a	p-Foxo3a (Ser-253)	p-Foxo3a (Thr-32)
SGK1^{+}-Foxo3^{-}	1.703±0.085	0.715±0.038	1.203±0.114$^{*\#}$	0.202±0.035$^{*\#}$	0.358±0.048$^{*\#}$
SGK1^{+}-control	1.682±0.093	0.782±0.031	1.910±0.104	0.517±0.030	0.583±0.042
SGK1^{+}-blank	1.631±0.102	0.758±0.038	1.885±0.105	0.571±0.038	0.564±0.038
F	0.460	2.694	41.576	100.155	25.423
P	0.652	0.146	0.000	0.000	0.001

Table 3: SGK1, Foxo3a and corresponding phosphorylated proteins in the SGK1, Foxo3a overexpression group after regulation of Foxo3a expression.

*Note: *Compared with SGK1 overexpression-control group, P<0.05; Compared with SGK1 overexpression-blank group, P<0.05. SGK1^{+}-Foxo3^{-}: SGK1 overexpression-Foxo3a inhibition group; SGK1^{+}-control: SGK1 overexpression-control group; SGK1^{+}-blank: SGK1 overexpression-blank group.*

Apoptosis of SGK1 cells after Foxo3a expression was regulated

The late apoptosis rate and total apoptosis rate of SGK1 overexpression-Foxo3a inhibited group was significantly high than that of the SGK1 overexpression-control group and SGK1 overexpression-blank group (P<0.05). Regulated Foxo3a apoptosis is in Table 4.

Group	Early apoptosis (Q4)	Late apoptosis (Q2)	Total apoptosis rate (Q2+Q4)
SGK1^{+}-Foxo3^{-}	2.11±0.48	10.37±0.82$^{*\#}$	12.48±0.75$^{*\#}$
SGK1^{+}-control	2.25±0.53	6.81±0.71	9.06±0.77
SGK1^{+}-blank	2.21±0.52	6.61±0.77	8.82±0.71
F	0.060	22.763	22.732
P	0.942	0.002	0.002

Table 4: Apoptosis of SGK1 cells after Foxo3a expression was regulated (%).

*Note: *Compared to SGK1 overexpression-control group, P<0.05; Compared with SGK1 overexpression-blank group, P<0.05. SGK1^{+}-Foxo3^{-}: SGK1 overexpression-Foxo3a inhibition group; SGK1^{+}-control: SGK1 overexpression-control group; SGK1^{+}-blank: SGK1 overexpression-blank group.*

Discussion

Prostate cancer has become common in Chinese men[8]. Exploration of the molecular mechanisms of its occurrence and development is needed to carry out targeted therapy and improve the prognosis of patients[9]. SGK1 is a serine/threonine protein kinase, which plays an important role in the tumour-signalling pathway and is involved in cell transformation, tumour progression, radiotherapy and chemotherapy resistance[10-12]. This study found that inhibition-mediated SGK1 slow virus infection can induce PC3 cells apoptosis, enhance the level of SGK1 expression and decrease the rate of PC3 cell apoptosis, suggesting SGK1 is involved in the occurrence and development of prostate cancer. Inhibition of SGK1 may be promising for prostate cancer treatment, results consistent with Sherk[13].

However, the molecular mechanism of SGK1 inhibiting apoptosis in prostate cancer cells had not previously been fully explored. The results of this study suggest that Foxo3a, a downstream factor of SGK1, may play an important role in this process.

The targeted phosphorylation sequences of SGK1 are R-X-R-X-X-(S/T) -phi and R-R-X-S /T (X for any amino acid, R for arginine, S for serine, T for threonine and phi for hydrophobic amino acid) [14], which can phosphorylate the ser-253 and thr-32 sites of Foxo3a[15], leading to the inactivation of the latter protein. Foxo3a that had not been inactivated has been found mainly in the nucleus and transferred to the cytoplasm after inactivation[16].

This study found that SGK1 PC3 cells expression level does not directly affect Foxo3a expression, but the slow virus SGK1 can inhibit phosphorylation of Foxo3a silence, causing Foxo3a to gather within the nucleus. SGK1 expression levels can trigger Foxo3a phosphorylation, and from the nucleus to the cytoplasm, prompt PC3 cells are widespread SGK1 phosphorylation Foxo3a phenomenon.

Foxo3a acts as a transcription factor in the nucleus, regulating the expression of various genes, such as Bim and p27[17-18]. Bim and p27 are both pro-apoptotic proteins, and Bim can bind to bcl-2 and inhibit the latter's anti-apoptotic effect, leading to apoptosis[19-20]. P27 is a cell cycle regulator with high expression leading to cell cycle stagnation and low or no expression leading to apoptosis[21-22].

Thus, the phosphorylation of Foxo3a leads to a decrease in apoptosis rate. In this study, it was found that after inhibiting SGK1 expression, the apoptosis rate of PC3 cells increased, while promoting SGK1

expression could further inhibit apoptosis. Foxo3a may play a key role in the above changes.

For further analysis of the apoptosis inhibition effect, this study utilised the small RNA interference suppression SGK1 group to express Foxo3a expression level and found Foxo3a silenced suppression of Foxo3a phosphorylation and improved PC3 cell apoptosis. This suggests that blocking the SGK1 phosphorylation Foxo3 process, to a certain extent, will correct the PC3 cells abnormal apoptosis resistance.

This is confirmed by SGK1 phosphorylation of Foxo3a, causing inactivation of the latter as one reason for the low PC3 cell apoptosis rate. It has been reported(23) that promoting Foxo3a activity can induce apoptosis and inhibit T24 cell apoptosis in bladder cancer. Other studies(24) have shown that inhibition of Foxo3a phosphorylation can promote autophagy and induce apoptosis.

The above conclusions suggest that inhibiting the phosphorylation of Foxo3a by SGK1 may be one of the therapeutic targets for prostate cancer, which is consistent with the report of Fitzwalter et al.(25), but the effectiveness remains to be confirmed by subsequent studies.

In conclusion, this study found that SGK1-induced Foxo3a phosphorylation may be one of the reasons for the abnormal decline in the apoptosis rate of prostate cancer cells, which has a specific guiding role in the targeted treatment of prostate cancer.

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