


A Vector-Based Short Hairpin RNA Targeting Aurora B Suppresses Human Prostatic Carcinoma Growth

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Mei Cao, PhD^{1,2}, Panpan Qi, MS¹, Chong Chen, MS¹, Liju Song, MS¹, Xuege Wang, MS¹, Ningzhe Li, MS¹, Daoyan Wu, MS¹, Guoku Hu, PhD^{2,3}, and Jian Zhao, PhD¹

Abstract

Aurora kinase B, playing a vital, important role in mitosis, is frequently detected to be overexpressed in many cancer cell lines and various tumor tissues, including prostatic carcinoma. Given the essential function of Aurora kinase B in mitosis and its association with tumorigenesis, it might be a drug target for prostatic carcinoma treatment. In our study, short hairpin RNA targeting Aurora kinase B was cloned into a pGPU6 plasmid vector and then transfected into human prostatic carcinoma cells. The expression level of Aurora kinase B was verified by reverse transcription-polymerase chain reaction and Western blot. At the same time, cell apoptosis was detected by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, fluorescent staining, and flow cytometric analysis. Furthermore, prostate carcinoma cells were injected into mice to establish a tumor xenograft model. Previous studies have shown the effect of pGPU6-shAURKB plasmid on tumor growth in a prostate carcinoma xenogenic implantation model. From the study, we knew that the Aurora kinase B was significantly downregulated in prostate carcinoma cells, and cell apoptosis was also detected higher in treated groups than that in control groups. Moreover, in the prostate carcinoma xenogenic implantation model, compared with the control groups, the tumor growth was inhibited about 78.7% in the pGPU6-shAURKB plasmid-treated group, and cell apoptosis in the experimental group was notably higher than that in control groups. The average duration of tumor-bearing mice was prolonged to about 35 days. The results of experiment indicated that specific knockdown of Aurora kinase B led to prostate carcinoma cells apoptosis and inhibited tumor growth. Our data clearly confirmed that specific knockdown of Aurora kinase B expression by vector-based short hairpin RNA/liposome may be a potential new approach to treat human prostatic carcinoma.

Keywords

Aurora B, RNA interference, prostatic carcinoma, gene therapy, cell apoptosis

Abbreviations

Aurora A, B and C, Aurora kinase A, B, and C; CPC, chromosomal passenger protein; dUTP, deoxyuridine triphosphate; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IV, intravenous; mRNA, messenger RNA; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; PBS, phosphate-buffered saline; PC, prostate carcinoma;

¹ Key Laboratory of Biological Resource and Ecological Environment of Chinese Education Ministry, College of Life Sciences, Sichuan University, Chengdu, People's Republic of China

² Core Laboratory, School of Medicine, Sichuan Provincial People's Hospital Affiliated to University of Electronic Science and Technology of China, Chengdu, People's Republic of China

³ Department of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, NE, USA

Corresponding Author:

Jian Zhao, PhD, Key Laboratory of Biological Resource and Ecological Environment of Chinese Education Ministry, College of Life Sciences, Sichuan University, Chengdu, 610064, People's Republic of China.
Email: zj804@163.com



PEG, polyethylene glycol; PI, propidium iodide; RNAi, RNA interference; RPMI, Roswell Park Memorial Institute; shRNA, short hairpin RNA; SqRT-PCR, semiquantitative reverse transcription-polymerase chain reaction; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

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Introduction

Prostatic carcinoma is the most common neoplasia in men and the second leading cause of death after cardiovascular diseases.¹ The faithful chromosome segregation is crucial for the maintenance of genomic balance and normal growth in mitosis, implicating that chromosomal instability leads to the generation of numerical and structural changes in the preneoplastic and neoplastic stages of prostate carcinoma (PC) cells. It is currently acknowledged that the unstable chromosome number in cancer cells acts as a driving force during the malignant progression, and aneuploidy is the most prevalent genomic alteration identified in solid tumor.² Loss or gain of the mitotic regulation is a probable cause of aneuploidy in human epithelial malignancy, and this has been presumed to create an abnormal nuclear morphology in cancer cells.

Aurora kinases represent a novel group of serine/threonine kinases, which conserved across yeast, nematodes, and mammalian cells. In humans, there are 3 known family members, the Aurora kinase A, B, and C. These isoforms differ in localization, expression levels, and timing of activity.^{3,4} Aurora kinases play a regulatory role from G2 to cytokinesis, encompassing the key cell cycle events such as monitoring the mitotic checkpoint, creation of bipolar mitotic spindle, and alignment of centrosomes on it, also modulating centrosome separation, bio-orientation of chromosomes, and cytokinesis. Any errors in mitotic signaling pathways can result in uncontrolled proliferation, which is regarded as one of the cardinal characteristics of tumors.⁵

Aurora kinase B is a chromosome passenger protein, mapping to chromosome 17q13, localized on the centromeres from the prophase through the metaphase–anaphase transition.⁶ Nuclear expression of Aurora B in prostatic intraepithelial neoplasia lesions correlated with clinical staging of tumor, whereas cytoplasmic expression in tumors correlated with seminal vesicle invasion.⁷ Aurora kinase B has been identified as an oncogene due to its overexpression in many human tumors, and this has been shown to cause centrosome amplification, chromosome instability, and cell tumorigenic transformation both in vitro and in vivo. Due to its crucial role in chromosome dynamics and common feature of aneuploidy in PCs, Aurora B has been recognized as the marker in neoplastic stages of PC. Additionally, Aurora B has also been considered as one of the negative prognostic factors and targets of gene therapy in patients with diverse cancers.

Exploiting the RNA interference (RNAi) pathway is known as a potential therapeutic strategy against several cancers.^{8–10} Eukaryotic RNAi pathways are activated by double-stranded RNA. Short hairpin RNAs (shRNAs) driven by polymerase III

promoters have been demonstrated as an alternative tactics to suppress gene expression more stably in mammalian cell lines. However, its application is limited due to immune responses and half-life period in animal bodies. However, these drawbacks could be improved by liposome combination. Polyethylene glycol (PEG)-coated (containing PEG-derivatized phospholipid) liposome, a long circulating liposome, is promising for aclacinomycin A stable delivery and circulation in vivo and deducing its toxicity.¹¹ Polyethylene glycol-coated long circulating liposome encapsulating prednisolone could be more effective in the treatment of experimental autoimmune encephalomyelitis.¹² The purpose of this study is to investigate the inhibitory effect of pGPU6-shAURKB/liposome on the expression of Aurora B and on the proliferation of PC3 cells. This may lead to the exploration of the potential application of RNAi technique and liposome delivery system by targeting Aurora B gene for the treatment of malignant tumor.

Materials and Methods

Cell Lines

Human prostatic carcinoma cell lines, PC3, were grown in RPMI 1640 (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) and 5% penicillin/streptomycin. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂. The PC3 cells were obtained from the State Key Laboratory of Biotherapy, West China Medical School, Sichuan University.

Plasmid Constructions

Fragments caccGGTGATGGAGAATAGCAGTtcaagagACTGCTATTCTCCATCACCtttttg and gatccaaaaaGGTG ATG GAGAATAGCAGTctcttgACTGCTATTCTCCATCACC were annealed and inserted into Bbs I/BamH I sites of plasmid pGPU6. The pGPU6-shAURKB was constructed for expressing shRNAs, specific for interfering with expressions of Aurora B, whereas pGPU6-shNC was prepared as irrespective sequence control. All the constructed plasmids were confirmed by DNA sequencing. Large-scale plasmid DNA was purified using an EndoFree Plasmid Giga kit (Qiagen, Hilden, Germany). After endotoxin detection, DNA was eventually dissolved in sterile endotoxin-free water and stored at –20°C before use.

Transfection

Twenty-four hours before transfection, cells were trypsinized and seeded in plates. The DNA (pGPU6-shAURKB or pGPU6-shNC)/liposome complexes were prepared and maintained

in RPMI 1640 with free serum at room temperature for 30 minutes. The PC3 cells were incubated with the above agents for 4 hours, rinsed 3 times with phosphate-buffered saline (PBS), and then RPMI 1640 supplemented with 10% FBS was added, with a continued incubation for an additional 48 hours for further studies.

RNA Extraction and SqRT-PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer. Semiquantitative reverse transcription-polymerase chain reaction (SqRT-PCR) was used for the analysis of Aurora B messenger RNA (mRNA) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The PCR primers were as follows: Aurora B, forward (5-ATGGAGAATAGCAGTGG GACA-3) and reverse (5-CAGATTGAAGGGCAGAGGG-3); GAPDH, forward (5-TCATCTCTGCCCTCTG-3) and reverse (5-CCTGCTTCACCACCTTCTTG-3).

Western Blot

Whole cell lysates were obtained with lysis buffer plus protease inhibitors (50 mM NaH₂PO₄, 10 mM Tris-HCl, 250 mM NaCl, 100 µg/mL leupeptin, 1 mM phenylmethanesulfonyl fluoride, pH 8.0). Antibodies to Aurora B (Sigma Aldrich, St. Louis, MO, USA) and GAPDH (Sigma Aldrich, St. Louis, MO, USA) were used. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and electronically transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking, the membranes were incubated with recommended dilution primary antibodies against Aurora B or GAPDH, followed by incubation with peroxidase-conjugated secondary antibodies. Quantitative analysis of bands was performed with Quantity One software v4.6.9. Densitometric level of Aurora B was quantified and expressed as its ratio to GAPDH.

3-(4,5-Dimethyl-2-thiazolyl)-2,5-Diphenyl-2H-Tetrazolium Bromide Assay

Cells (1×10^4) were added into a 96-well plate and incubated overnight. The DNA/liposome complexes were transfected into the cells. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma Aldrich, St. Louis, MO, USA) was added after 48 hours. After 4-hour incubation, the MTT formazan precipitate was dissolved in dimethyl sulfoxide (100 µL/well) on a shaker before reading the absorbance at 570 nm.

Flow Cytometric Analysis

Cells were harvested with trypsin, washed with PBS, then pelleted and suspended in propidium iodide (PI)/RNase/PBS (100 µg/mL PI and 100 µg/mL RNase A). After gentle vortexing, the cells were incubated for 30 minutes in the dark and analyzed by flow cytometry.

Nuclear Staining and Fluorescent Microscopy

Cells were stained using Hoechst33258 (Beyotime, Shanghai, China) as recommended by the manufacturer. The morphology of the cell nuclei was observed using a fluorescence microscope after adding antifade reagent.

Tumor Xenograft Model and shRNA Treatment

All animal procedures were approved by the Institutional Animal Care and Treatment Committee of Sichuan University. Tumor xenograft model was established by injecting 1×10^7 PC3 cells into the skin of the back of female athymic nude mice (BALB/c, 6-8 weeks of age, nonfertile, and 18-20 g each). When tumors were palpable, mice were randomly distributed into the following 4 groups (N = 7) and injected intravenously (IV) through the tail vein—(i) treatment with 5% glucose solution; (ii) treatment with liposome; (iii) treatment with pGPU6/shNC; and (iv) treatment with pGPU6-shAURKB complexes. The IV route for shRNA delivery was selected in 200 µL volume with a regimen of 1 time per 3 days for 8 times. Tumor size was measured using the formula: tumor size = (length) × (width)² × (π/6).¹³ Animals were sacrificed 1 week after the last treatment, and tumor tissue was fixed in 10% neutral-buffered formalin and embedded in paraffin. To monitor drug toxicity, body weight was measured and pathological sections were analyzed including heart, liver, spleen, lungs, and kidneys.

In Situ Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling Assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed with an in situ cell death detection kit according to the manufacturer's directions (Roche, Molecular Biochemicals, Basel, Switzerland). Five equal-sized fields were randomly chosen and analyzed. Density was evaluated in each field to calculate the density of apoptotic cells.

Immunohistochemistry

After deparaffinization, antigen retrieval was performed by heating slides in an autoclave in 10 mM sodium citrate buffer at 120°C for 5 minutes (pH 6.0). Sections were incubated with Aurora B antibodies at 4°C overnight. As the second step, biotinylated goat anti-rabbit IgG was applied and detected using the Strept Avidin Biotin Complex Elite kit (Boster, Wuhan, China) with diaminobenzidine as the substrate.

Statistical Analysis

All data were presented as means ± standard deviation (SD) and were analyzed by 1-way analysis of variance and Tukey test. Differences between means as appropriate were considered significant when yielding $P < .05$.

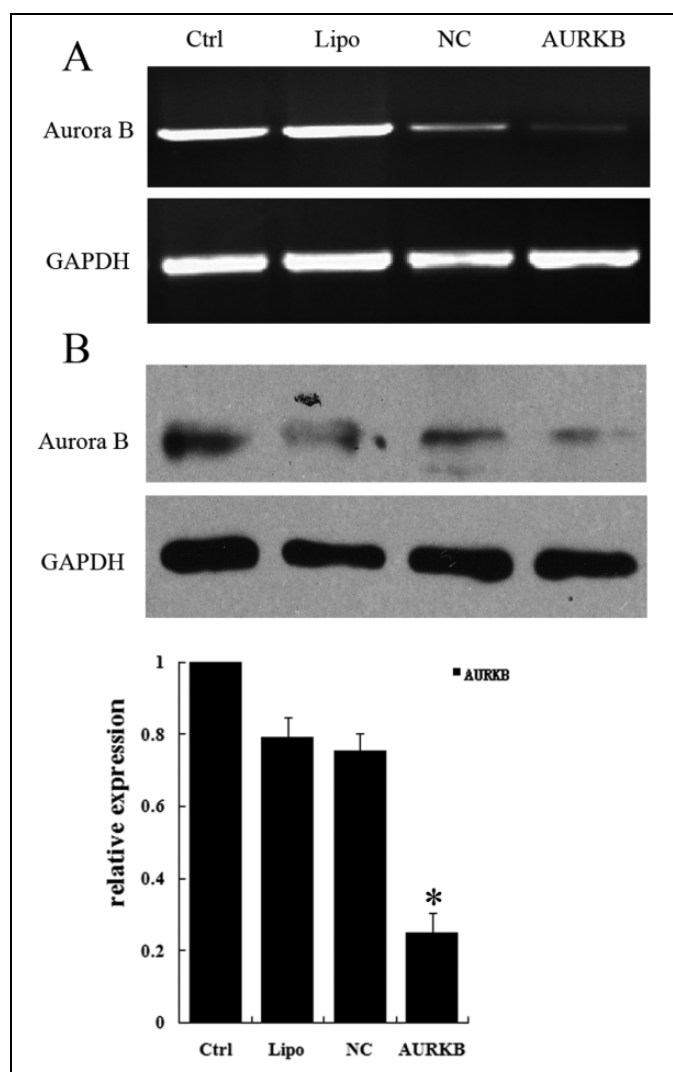


Figure 1. Expression of Aurora kinase B in PC3 cell lines treated with pGPU6-shAURKB after 48 hours was reduced. The PC3 cells were transfected with pGPU6-shAURKB (AURKB) or pGPU6-shNC (NC)/liposome complex or liposome (Lipo) or medium (Ctrl) separately as control for 48 hours. A, SqRT-PCR. Expression of Aurora B was detected by SqRT-PCR. B, Western blot. Expression of Aurora B and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were detected by Western blot, and GAPDH expression was monitored as the control. The ratio of Aurora B/GAPDH was calculated using densitometry. * $P < .05$.

Results

Specific Knockdown of Aurora B in Prostatic Carcinoma Cell Lines

After endotoxin detection ($<0.001\text{EU}/\mu\text{g DNA}$), pGPU6-shNC plasmid and pGPU6-shAURKB plasmid were transfected into PC3 cells, respectively. The cells were harvested after 48 hours, and the expression levels of Aurora B were analyzed, respectively, by SqRT-PCR and Western blot. Both mRNA and protein expression of Aurora B were dramatically suppressed in PC3 cell lines treated with pGPU6-shAURKB (Figure 1).

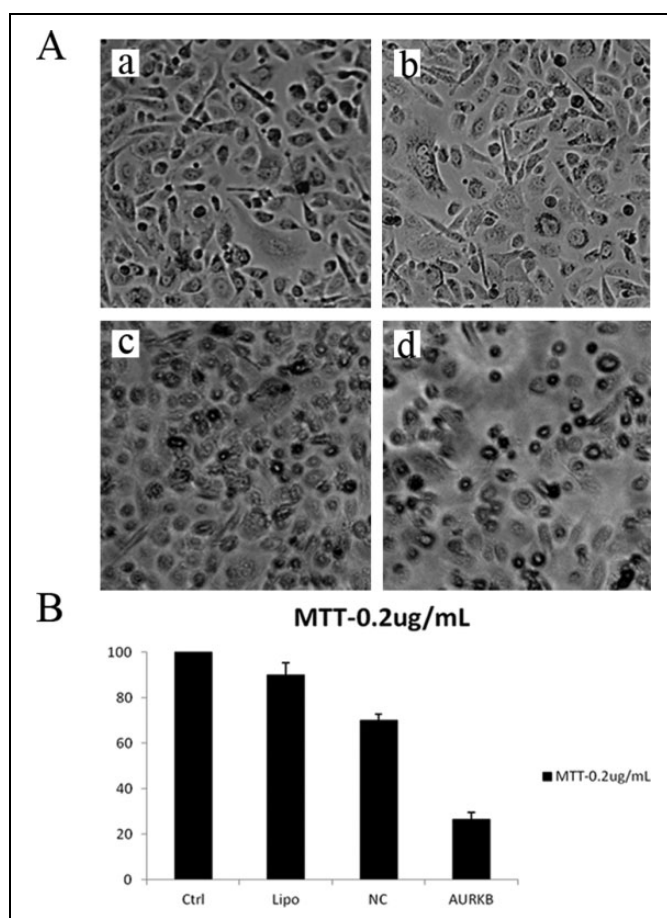


Figure 2. pGPU6-shAURKB inhibited cell proliferation in vitro. A, Morphologic changes of PC3 cells transfected with pGPU6-shAURKB. (a) Untreated PC3 cells; (b) treated with liposome; (c) transfected with pGPU6-shNC; and (d) transfected with pGPU6-shAURKB. The adherent cells became round and fell off the culture substance in the pGPU6-shAURKB-treated group. B, Effect of Aurora kinase B gene silencing on cell proliferation. AURKB indicates transfected with pGPU6-shAURKB; Ctrl, untreated cells; Lipo, treated with liposome; NC, transfected with pGPU6-shNC. * $P < .05$.

Quantification of band intensities of Aurora B in Western blot showed that the treatment with pGPU6-shAURKB plasmid reduced the expression of Aurora B by 75.1% ($P < .05$).

Knockdown of Aurora B Reduced Cell Proliferation and Induced Cell Apoptosis In Vitro

The observation of cell morphology, anchorage-dependent rate, and MTT colorimetry preliminarily was used to estimate the growth of cells. The adherent cells became round and fell off the culture substance (Figure 2A). The MTT analysis revealed that the cell proliferation of PC3 cells transfected with pGPU6-shAURKB was prominently inhibited at approximately 73.6% ($P < .05$) compared with control groups (Figure 2B). The ratio of apoptotic cells was about 41.0% after pGPU6-shAURKB treatment for 48 hours in PC3 cell lines by flow cytometric analysis, which showed significant difference with that in the pGPU6-shNC group ($P < .05$; Figure 3A).

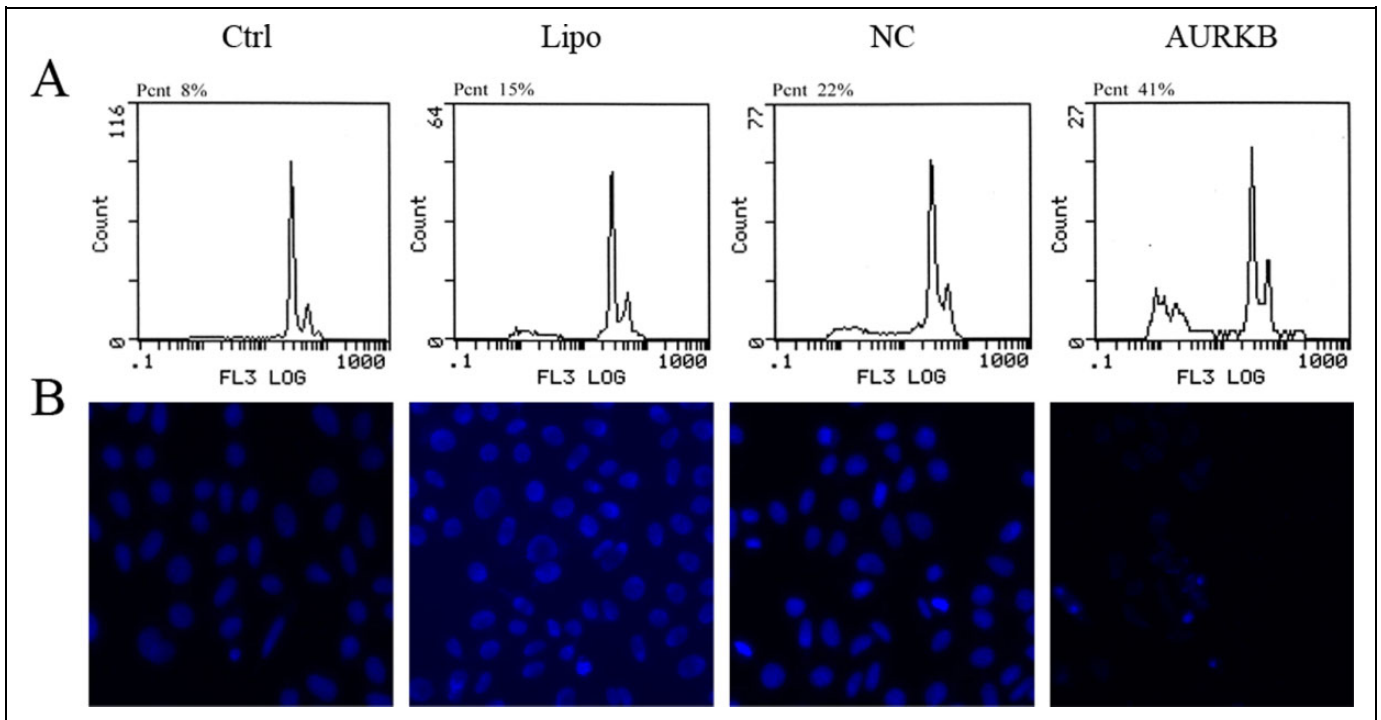


Figure 3. pGPU6-shAURKB induces apoptosis in vitro. A, Flow cytometry analysis of the apoptosis of PC3 cells after silencing the expression of Aurora kinase B in vitro. $*P < .05$. B, Fluorescence microscopy appearance of cells stained with Hoechst33258. Almost all cells underwent apoptosis in the pGPU6-shAURKB-treated group, which was significantly different from the other 3 groups. AURKB indicates transfected with pGPU6-shAURKB; Ctrl, untreated cells; Lipo, treated with liposome; NC, transfected with pGPU6-shNC.

In addition, nuclear staining with Hoechst3325 was performed to further evaluate apoptosis result from morphological changes. The morphological changes of cells at 48 hours post-transfection monitored by fluorescence microscopy were the characteristics of apoptosis (Figure 3B). These results indicated that the shRNA-mediated knockdown of Aurora B led the prostatic carcinoma cells to apoptosis.

Knockdown of Aurora B Inhibited the Growth of PC3 Cells In Vivo

The xenograft mouse model of human prostate cancer was used to evaluate the effect of RNAi-based strategy to suppress Aurora B expression in inhibiting tumor growth in vivo. The xenograft mouse model of human prostate cancer was injected with 25 μ g pGPU6-shAURKB into the caudal vein of each mouse, and the control groups were injected with 5% glucose, liposome alone, or 25 μ g pGPU6-NC.

The treatment of pGPU6-shAURKB resulted in an obvious inhibitory effect on tumor growth versus group glucose, liposome, and pGPU6-shNC. The mean tumor volume was about 826 mm³ in the pGPU6-shAURKB group, which was much smaller than the other groups ($P < .05$; Figure 4A). The survival rate of mice treated with pGPU6-shAURKB was 83.3% after 100 days from the initial time of seeding PC3 cells, which was distinctly higher than the other groups (Figure 4B). The TUNEL assays were carried out to detect apoptosis in tumor tissues. Within a similar field of view, more apoptotic cells

were observed in tumor tissues from the mice treated with pGPU6-shAURKB plasmid than glucose, liposome only, and pGPU6-NC treatment groups (Figure 5A). We further examined the expression of Aurora B in tumor tissues. Similar to the results in vitro, systematic administration of plasmid reduced the expression of Aurora B (Figure 5B).

Compared with the treatment with 5% glucose, the tumor growth was inhibited about 78.7% by pGPU6-shAURKB, and the average duration of tumor-bearing mice was prolonged to about 35 days. The survival rate was 83.3% after 100 days from the initial time of seeding PC3 cells. Infiltrative lymphocytic and zone of necrosis were observed in the tumor section after hematoxylin and eosin staining. Compared with other treatments, there were more apoptotic cells, and the expression of Aurora B was downregulated after pGPU6-shAURKB treatment.

Toxicity Assessment

In addition, the mice treated with pGPU6-shAurora B had been investigated for potential side effects. Present studies have shown that there were no significant adverse consequences in gross measures, including weight loss, ruffling of fur, life span, behavior, and feeding. Furthermore, no pathological changes in heart, liver, spleen, lungs, or kidneys were found by microscopic examination (data not shown).

Our results informed that the expression of Aurora B gene was dramatically suppressed in human prostatic carcinoma cell

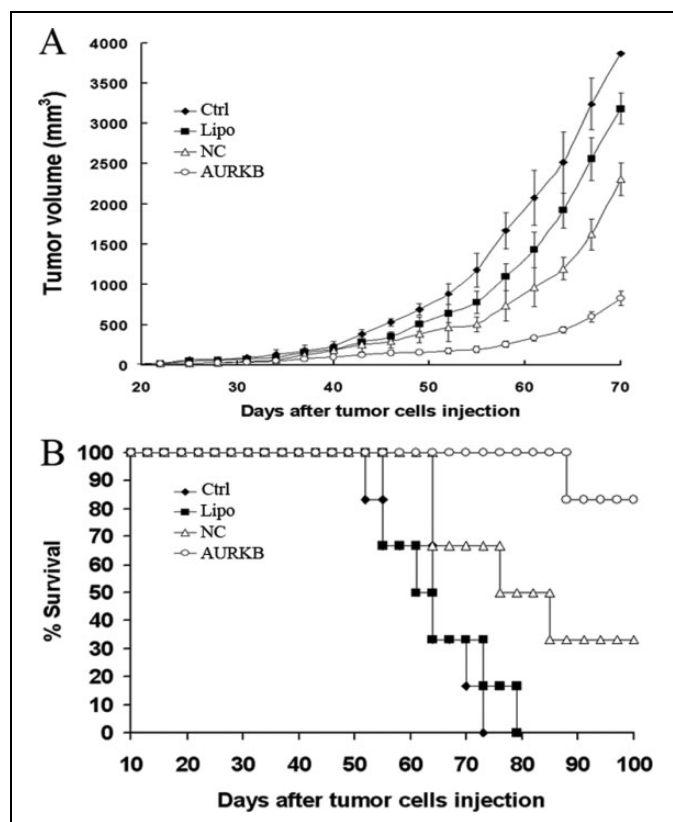


Figure 4. pGPU6-shAURKB suppressed tumor growth in vivo. A, Suppression of tumor growth in mice after the treatment with differently recombinant plasmids. Graph of systemic therapy with pGPU6-shAURKB resulted in significant tumor growth inhibition versus group glucose ($P < .05$), liposome ($P < .05$), and pGPU6-NC ($P < .05$). B, The average duration of tumor-bearing mice (day). Graph of systemic therapy with pGPU6-shAURKB resulted in significantly prolonging duration of tumor-bearing mice. Glucose indicates treated with 5% glucose; liposome, treated with liposome alone; pGPU6-NC, treated with pGPU6-NC; pGPU6-shAURKB, treated with pGPU6-shAURKB.

line PC3 after the transduction with pGPU6-shAURKB. Consequently, the proliferation of PC3 cells in vitro transfected with pGPU6-shAURKB was much lower than those with pGPU6-shNC and liposome alone. Most importantly, the vector-based Aurora B shRNA resulted in the suppression of Aurora B protein expression, the increase of apoptosis, and the inhibition of prostatic carcinoma tumor growth. In conclusion, shRNA-based gene silencing may be developed into an effective gene therapy strategy for the treatment of Aurora B over-expressed cancers, including prostatic carcinoma.

Discussion

Aurora kinase B, a multifunctional chromosomal passenger protein (CPC), is the only kinase center of CPC, and it is the important molecule that performs a complex function in the form of substrate protein phosphorylation.¹⁴ Before mitosis, Aurora B regulates the chromatin to concentrate. In the metaphase and anaphase of mitosis, Aurora B participates in the

regulation of chromosome separation. Besides, it also plays an important role in cytokinesis in the telophase of mitosis. Furthermore, Aurora B can correct the wrong combination of centromere and microtubules.

The variation of chromosome numbers (euploid) is a major cause of malignant tumor of human beings; the inaccurate process of mitosis leads to the production of aneuploid cells and plays a promoting role in malignant tumor development.^{15,16} In the last few years, many prospective studies have demonstrated a clear association between cancer and Aurora kinases. Aurora kinases have been acknowledged to play an important role in developing new drugs to cure cancer. The specific knockdown of Aurora A expression by vector-based shRNA could increase apoptosis and reduce cell proliferation both in vitro and in vivo studies.¹⁷ The expression of Aurora B in high-grade gliomas was associated with a histologic malignant degree and the clinical pathology characteristic.¹⁸ In addition, the high expression of Aurora B was also found in colorectal cancer, breast cancer, thyroid cancer, pancreatic cancer, endometrial carcinoma, and so on,^{5,19-22} including pancreatic cancer.³ An Aurora B inhibitor, S39, was a broad-spectrum and potent inhibitor of various tumor cell lines, such as Hela, HepG2, and SW620.²³ The current data indicated that the proliferation of many kinds of cancer cells was suppressed by inhibiting the expression of Aurora B, through RNAi technology and Aurora B inhibitor.

By inhibiting the activity of Aurora B, tumor cells can prevent the kinetochore distinguishing abnormal microtubule interaction, stopping the arrangement and separation of chromosome, which can lead to disastrous mitosis, inducing cell apoptosis. Since Aurora B expresses and becomes activated only in the process of mitosis, Aurora B inhibitors are valid for the proliferative cells only. In the human body, most of the normal cells have a low proliferation rate. Therefore, compared with other nonspecific cytotoxic drugs, inhibiting Aurora B will have a greater advantage. Pharmacology and gene interference studies have confirmed that the tumor cells treated with Aurora kinase inhibitors appear the phenomenon of apoptosis, which is largely due to the suppression of Aurora B. Compared with the Aurora A inhibition, Aurora B inhibition might more likely be an effective anticancer method, depending on inducing the extremely unstable genome and causing cell death quickly.²⁴

The plasmid pGPU6-shAURKB was constructed and transfected into human prostatic carcinoma cells successfully. The results revealed that reducing the expression of Aurora B caused the cell apoptosis and inhibited the growth of PC3. Animal experiments also showed that knockdown of Aurora B could suppress the tumor growth in mice and prolong the average duration of the tumor-burdened mice.

We screened the optimal sequence for RNAi to target sequences by comparing the inhibition efficiency of Aurora B in vitro. The liposome we used for transfection was made by our colleagues, which had high transfection efficiency in vitro and no significant toxic effect in vivo. The results of plasmid pGPU6-shAURKB revealed that reducing the

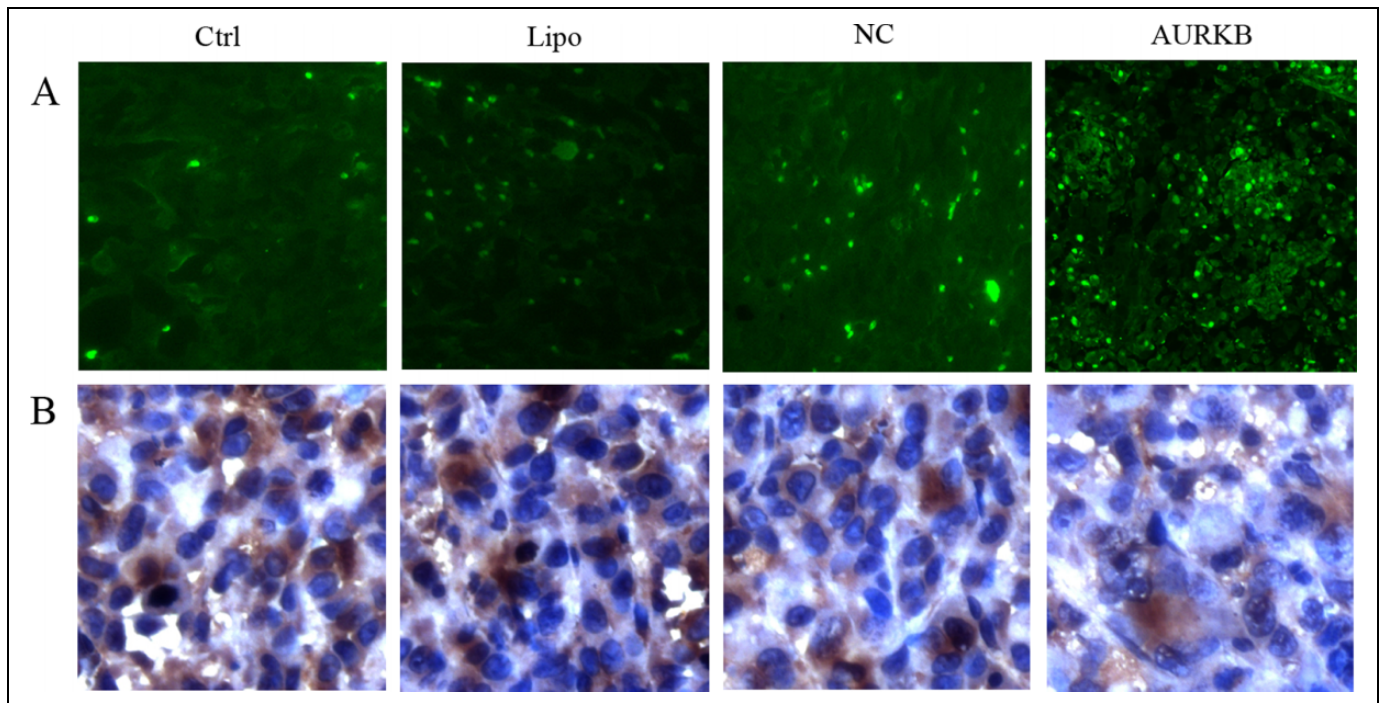


Figure 5. pGPU6-shAURKB induces apoptosis in vivo. A, Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) analysis of the apoptosis cells. Compared with the other treatments, there were more apoptotic cells in the group treated with pGPU6-shAURKB. B, Immunohistochemistry analysis of tumors. Compared with the other treatments, the expression of Aurora B was downregulated in the group treated with pGPU6-shAURKB ($\times 400$ magnification). AURKB indicates transfected with pGPU6-shAURKB; Ctrl, untreated cells; Lipo, treated with liposome; NC, transfected with pGPU6-shNC.

expression of Aurora B caused the cell apoptosis and inhibited the growth of PC3 cells. Animal experiments also showed that knockdown of Aurora B could suppress the tumor growth in vivo and prolong the average duration of the tumor-burdened mice. Meanwhile, the mice were almost not affected by the side effects, which suggested that the vector-based Aurora B-shRNA might be a potential therapy for human prostatic carcinoma. Based on the previous research, the effect of Aurora B-shRNA combined with radiation or chemotherapy might be a future direction to enhance the therapeutic effect.

Authors' Note

Mei Cao and Panpan Qi contributed equally to this work. All the authors have contributed directly to the planning, execution, or analysis of the work reported or to the writing of the paper.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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