Original Article

AURKA suppression induces DU145 apoptosis and sensitizes DU145 to docetaxel treatment

Wei He, Min-Guang Zhang, Xiao-Jing Wang, Shan Zhong, Yuan Shao, Yu Zhu, Zhou-Jun Shen

Department of Urology, Ruijin hospital, Shanghai Jiaotong University, school of medicine, Shanghai, China

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Abstract: The palliative therapy effect by docetaxel for CRPC patients makes it urgent to improve the therapy. It was suggested that PI3K and androgen receptor-directed combination therapy may be effective for prostate cancer (PCa) patients PTEN negative. However, for those patients PTEN positive, the mechanism of anti-apoptosis survival of cancer cells is not yet well defined. Amplification of AURKA has been detected in 5% of PCa. In this work, Du145, a PTEN positive PCa cell model, was employed to investigate the role of aurora kinase a (AURKA) on cell growth. Inhibition of AURKA expression by shRNA markedly reduced prostate cancer cell viability. Furthermore, we demonstrate that AURKA inhibition induced a remarkable downregulation of AKT activity and Bax induction. Moreover, specific inhibition of the activity of AURKA, but not other aurora family members, by small molecular chemical inhibitors induced significant cell killing effects. Notably, AURKA inhibition sensitized prostate cancer cells to docetaxel treatment. Our work suggests that AURKA-directed monotherapy or combination therapy with docetaxel could be a potent treatment for PCa patients in future.

Keywords: Prostate cancer, AURKA, p53, docetaxel, castration-resistant prostate cancer, aurora kinases

Introduction

Prostate cancer (PCa) is the most common male malignancy and the second leading cause of male cancer deaths worldwide, behind only lung cancer. Androgen deprivation therapy has been the standard treatment of metastatic prostate cancer for many years; however, progression to castrate resistance disease occurs in the majority of patients [1, 2]. Following the emergence of castrate-resistant prostate cancer (CRPC), docetaxel chemotherapy has been shown to be palliative with only 4 months of the median increase in survival [3]. Thus, there is an urgent need for improvements in therapy for PCa. Deletion of PTEN is present in 40% of prostate cancers. Recently, it was demonstrated that reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient PCa coordinately support PCa cells survival under androgen deprivation conditions [4, 5]. These findings provide rationale for combination therapy in PTEN negative patients. However, for those patients with positive PTEN expression, who occupied 16% or so in all PCa patients [6], the mechanism of anti-apoptosis survival of cancer cells remains unclear.

Accumulating evidences suggest that the aurora kinases often act as oncogenic drivers in many human cancers [7]. The aurora family consists of three known gene paralogs (AURKA, AURKB, and AURKC) that are key regulators of mitosis and cell cycle [8]. The genes each encode serine/threonine kinases with a significant degree of homology in the C-terminal catalytic domain. AURKA has a major role in the cell cycle and is absolutely required for the G2/M transition via phosphorylation of polo-like kinase 1 (PLK1) in concert with the cofactor Bora [9, 10]. In addition, AURKA is critical for mitotic spindle assembly and stability, as well as the regulation of centrosomal and kinetochore formation [11]. It is therefore not surprising that AURKA expression is tightly regulated throughout normal development and the cell cycle and those AURKA knockout mice are early embryonic lethal [10]. Notably, AURKA amplification/overexpression is commonly seen in a variety of human cancers, including breast,
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ovarian, colon, gastrointestinal, esophageal and prostate cancer [12]. Gene amplification of AURKA has been shown to correlate with genomic instability and clinically resistance to chemotherapy [12, 13]. Silence of AURKA by shRNAs or small molecular inhibitors induced apoptosis and attenuated cancer cells growth as well as tumor growth in various cancers [13-18]. Most interestingly, inhibition of AURKA expression can result in chemosensitizing activity to taxanes in human pancreatic cancer [13], indicating an important role of AURKA in cancer therapy. AURKA regulates several important proteins such as p53, AKT [19], p73 [12], BRCA1 [20], GSK3B [21] and N-myc [14, 22], all of which play critical roles in tumorigenesis and cancer therapy. AURKA is currently therefore a hot target for anti-cancer drug development. Several inhibitors of Aurora kinases, such as hesperidin, ZM447439, VX-680 [19] and MLN8054 [12] have been developed and are in different phases of clinical trials.

Despite that AURKA is a promising drug target in man cancers, the function of AURKA in either prostate cancer development or target therapy is little known. AURKA amplification/overexpression exists in 5% PCa and 40% neuroendocrine prostate cancer (NEPC), an aggressive subtype of prostate cancer that most commonly evolves from preexisting prostate adenocarcinoma [22], providing a rationale for oncogene addiction-based targeted therapy. Considering the emergent need to find novel targets and strategies for prostate cancer therapies, especially for those patients with function PTEN signaling, it is becoming extremely interesting to investigate whether AURKA could regulate prostate cancer cell growth. In this work, a PTEN-positive and AR-negative PCa cell model, Du145, was used to investigate the role of AURKA in cell growth. Inhibition of AURKA expression by shRNAs and small molecular inhibitors markedly induced apoptosis and resulted in chemosensitizing activity to docetaxel. Moreover, underlying signal pathway analyses revealed that p53/Bax upregulation and p-Akt downregulation coordinately contribute to the decrease in cell viability induced by AURKA inhibition. Our data shed light on the potential role of AURKA in target prostate cancer therapy.

Materials and methods

PCa cell lines, cell culture and reagents

LNCaP, Du145 and C4-2 cells (American Type Culture Collection, Rockville, Md.) were cultured in RPMI 1640 medium supplemented...
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with 10% fetal bovine serum (FBS). PC3 cells were maintained in DMEM medium with 10% FBS. The AURK inhibitors, Danusertib (PHA-739358, Selleck, S1107), Aurora A Inhibitor I (Selleck, S1451) and AMG 900 (Selleck, S2719), were used at 3 concentrations to investigate the cytotoxic effect on four PCA cell lines. For Danusertib, the concentrations are 0.3, 1.5 and 7.5 μmol/l; for Aurora A Inhibitor I, the concentrations are 0.2, 1, 5 μmol/l; for AMG 900, the concentrations are 0.05, 0.25 and 1.25 μmol/l.

**shRNA construction and lentivirus infection**

Four distinct shRNA fragments were constructed into lentivirus vector (3D-HTS company, Shanghai), and shRNA lentivirus were packaged and titered in HEK293T cells according to the manufacturer’s protocol. Du145 cells were infected by shRNA lentivirus at MOI of 10 in the presence of polybrene (8 μg/ml).

**MTS cell proliferation assay**

The tetrazolium compound (MTS) cell proliferation assay is a quantitative colorimetric assay for mammalian cell survival and proliferation. All four PCA cells (4×10³) were grown in 100 μl of culture medium containing serum per well in a 96-well plate. After 24 h, the cells were treated by shRNA lentivirus or AURK inhibitors for 72 h. Every treatment for each cell line was triplicate in the same experiment. Then 20 μl of MTS (CellTiter 96 AQueous One Solution Reagent; Promega) was added to each well for 1 to 4 h at 37°C. After incubation, the absorbance was read at a wavelength of 490 nm according to the manufacturer’s protocol.

**Quantitative PCR**

Du145 cells were infected with lentiviral shRNA under condition above mentioned. After 24 h, the culture medium was refreshed. RNA was extracted 48 h later and cDNA was synthesized using PrimeScript RT reagent kit with gDNA Eraser (Takara, RR074A) for RT-PCR with oligo-dT. Forward primer for AURKA: 5’-gca-gattttggtgtgctagct-3’; reverse primer for AURKA: 5’-tcgacctcactatta-3’. Real-time qPCR was performed on CFX-96 (Bio-lab), with endogenous control hActb. Gene expression was calculated relative to expression of hActb endogenous control and adjusted relative to expression in shControl-infected cells.

**Flowcytometry**

Cells were harvested with trypsin-EDTA, washed with PBS, and fixed with 70% ethanol at -20°C for a few days. The fixed cells were pelleted, resuspended in 100 μl of hypotonic citric buffer (192 mmol/L Na2HP04 and 4 mmol/L citric acid), and incubated for 30 minutes at room temperature. The cells were pelleted and suspended in PI/RNase/PBS (100 μg/mL propidium iodide and 10 μg/mL RNase A) overnight at 4°C. Analysis of DNA content was done on a FACSCalibur system (BD Immunocytometry Systems, San Jose, CA).

**Protein isolation and western blotting**

Cell pellets were resuspended in 1×SDS loading buffer (1 mmol-L-1 Na3VO4, 10 mmol-L-1 NaF, 1 mmol-L-1 PMSF) containing protease inhibitors. Lysates (20 μg each lane) were applied to SDS-PAGE. Immunoblotting of Abs specific for GAPDH (Abmart, 080922), p53 (ABclonal Biotechnology, A2063), Bax (ABclonal Biotechnology, A0207), AKT (Santa Cruz, sc-8312), and p-AKT (Santa Cruz, SC-7985-R) were detected using HRP-conjugated anti-mouse (Promega) or anti-rabbit (Promega) and visualized by chemiluminescence detection system (Millipore, WBKLS0500).

**Results**

Inhibition of AURKA by shRNAs attenuates Du145 cell growth

To investigate the role of AURKA in PCA cell growth, we depleted the expression of AURKA by introducing short hairpin RNA (shRNA) targeting AURKA into PCA cells as described in Materials and Methods. To avoid off-target effects of shRNA [23], we designed 4 independent shRNAs to inhibit AURKA expression. It is generally considered that a phenotype caused by two distinct shRNAs is unlikely to be the result of an off-target effect [24].

ShAURKA, the mixture of 4 independent AURKA shRNAs (see detail in Materials and Methods), shAURKA1 and shAURKA3 decreased Du145 cell viability significantly by 39% (P<0.001), 25% (P=0.006) and 17% (P=0.02), respectively (Figure 1A). AURKA silencing by shRNA was confirmed by quantitative PCR (Figure 1B). Consistently, the two AURKA-targeting shRNAs (shAURKA1 and shAURKA3) that caused signifi-
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Significant inhibition of cell growth also induced an obvious down-regulation of AURKA mRNA expression.

**Inhibition of AURKA inhibitors on prostate cancer cells**

Due to the ubiquitous overexpression and amplification of AURKA in many cancers, multipe AURKA inhibitors are currently tested in clinical trials. To further validate the inhibition effect of AURKA silencing on Du145 cell viability, we test the anti-tumor effect of AURKA inhibitors on a panel of prostate cancer cells. AURKA inhibitor I is specific to inhibit AURKA, while Danusertib (PHA-739358) and AMG 900 target to all three AURKs [25-27]. We found that both Danusertib and AURKA specific inhibitor, AURKA inhibitor I, inhibited the growth of all prostate cancers (Figure 2A and 2B). Importantly, AURKA inhibitor I had more significantly inhibitory effects on PC3 and LNCaP cells in a dose-dependent manner (Figure 2B). Considering that PC3 is a p53-null, androgen-independent cell line and LNCaP is a p53-positive and androgen-dependent cell line, our results suggest that AURKA inhibitor induces prostate cancer cell growth inhibition in a both p53- and androgen-independent way.

**AURKA inhibition induces Du145 cell apoptosis**

Previous studies have suggested that inhibition of AURKA results in cell cycle arrest and/or apoptosis in most cases [12, 13, 28, 29]. As shown in Figure 3A and 3B, we did not find cell cycle arrest after AURKA inhibition in Du145 cells. Interestingly, introduction of shAURKA1 and shAURKA3 into Du145 cells increased apoptosis from 5% to 14% and 20%, respectively (Figure 3A). Since AKT is an important regulator of cancer cell survival and previous reports showed that upregulation of AURKA expression enhance AKT phosphorylation at Ser473, we next examine whether AURKA inhibition decreases AKT activity and in turn affects Du145 survival. Western blot analysis demonstrated an obvious reduction of AKT phosphorylation (Figure 3C), indicating that impaired AKT activation might partially contribute to the inhibition effects induced by AURKA knockdown.

Given that p53 plays an important role in apoptosis and AURKA has been reported to induce p53 degradation via phosphorylation of p53 [30], we next examine whether knockdown of AURKA expression in Du145 could induce p53 activation. As shown in Figure 3D, we found an induction of p53 expression after AURKA inhibition. Bax, an apoptosis inducer, is the downstream target of p53. We also detected an increase in Bax expression upon AURKA inhibi-
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Figure 3. AURKA silencing induces Du145 cells apoptosis and inactives the AKT pathway. A. AURKA knockdown resulted in increasing of sub-G1 fraction. Cells were infected with shAURKA1, shAURKA3 or shControl. Cell cycle profiles were assessed by propidium iodide (PI) staining and fluorescence-activated cell scanning (FACS) from cell aliquots. B. Histogram represented the percentage of cells in each phase of the cell cycle. Significantly larger differences in sub-G1 phase and smaller differences in G1 phase after shAURKA1 or shAURKA3 infection were observed, indicating apoptosis induced by AURKA silencing. C. shAURKA1 or shAURKA3 infected-cells have significantly lower expression of p-AKT protein than shControl-infected cells. Du145 cells were infected with shAURKA1, shAURKA3 or shControl. Lysates were made 72 h following infection. Antibodies recognizing phosphoserine 473 and total AKT were used with GAPDH as a loading control. D. shAURKA1 or shAURKA3 infected-cells have significantly higher expression of p53 and Bax protein than shControl-infected cells. Du145 cells were infected with shAURKA1, shAURKA3 or shControl. Lysates were made 72 h following infection. Antibodies recognizing p53 and Bax were used with GAPDH as a loading control.

Collectly, these results suggest that AURKA inhibition induces apoptosis in Du145 cells via induction of Bax expression and inhibition of AKT activity.

Knockdown of AURKA significantly enhances cytotoxics of docetaxel

Docetaxel therapy is the routine treatment for CRPC patients. In addition, it was demonstrated that AURKA knockdown by shRNAs or inhibitors resulted in chemosensitizing activity to taxanes and cisplatin in pancreatic cancer and esophageal adenocarcinoma cells [13, 31, 32]. Therefore, we next investigated the synergistic effects of AURKA inhibition and docetaxel treatment in Du145 cells. Since introduction of shRNA3 induces a better knockdown of AURKA inhibition in Du145 cells, we transfected Du145 cells with lenti-shRNA3 viral vectors and treated cells with docetaxel after AURKA inhibition. Consistent with data shown in Figure 1, MTT analysis revealed that shAURKA1/3 treated cells had only 20% or so of cell death, while combination of docetaxel treatment and AURKA inhibition induced more cell death (Figure 4), indicating AURKA inhibition could sensitize cells to docetaxel treatment.

Discussion

Everlasting progression of prostate cancer eventually leads to the emergence of castrate-resistant prostate cancer (CRPC), which is diffi-
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Figure 4. Synergistic enhancement of cytotoxicity between AURKA shRNA and docetaxel. Survival cells were quantitated by MTS assay after infection of shAURKA3 or shControl only or subsequent addition of 0.9 nmol/L docetaxel. These experiments were performed for three times. Doc, docetaxel.

cult to treat and there is only palliative therapy by docetaxel. Recently, it was demonstrated that PI3K and AR combinational inhibition may be possible therapy for PTEN negative patients. However, in PCa patients, those of PTEN negative occupied 20% or so [6]. Therefore, for patients PTEN positive, it is urgent to understand the mechanism by which the cancer cells survive.

In this work, a PTEN positive PCa cell line, Du145, was employed to examine the role of AURKA in cell growth and survival. We found that AURKA knockdown induced Du145 apoptosis and sensitized cells to docetaxel treatment. Interestingly, treatment with AURKA inhibitors also reduced cell viability in p53-null PC3 cells and p53-mutated Du145 cells, indicating that AURKA inhibition induces a p53-independent cell death. However, we cannot exclude the possibility that AURKA could regulate cancer cell survival in a p53-dependent way. Du145 is a p53 mutant strain with heterozygous p53, indicating that p53 in Du145 remains partial normal function [33]. Consistent with this, WYC02-9, a novel synthetic flavonoid, has been reported to induce DNA damage and apoptosis in Du145 through activation effect of reactive oxygen species on ATM-p53-H2A.X pathway [34]. More importantly, we found that knockdown of AURKA expression induces an upregulation of p53 as well as its downstream target, Bax. Therefore, further experiments, like the knockdown of p53 expression in either Du145 or p53 wild type LNCaP cells, need to be performed to test this possibility. Interestingly, p53 has been proved to repress AURKA expression [30]. Our study, together with previous report demonstrate an inhibition of p53 by AURKA [35], suggests that there exists a reciprocal feedback regulation between p53 and AURKA. This feedback loop regulation may represent a balance between the protective mechanism for cell survival and the evolution of cancer cells for unlimited growth. Previous study has shown that cell apoptosis induced by AURKA inhibition is p73-dependent in p53 deficient cancer cells [12]. However, it was demonstrated that p73 loss is usually concomitant with p53 in androgen-independent PCA cells [36]. Therefore, it is unknown whether p73 or other signal molecular would mediate cell death induced by AURKA inhibition in p53-deficient prostate cancer cells.

Treatment with AURKA inhibitors showed different cytotoxic effects on tested PCA cell lines in our study (Figure 2), i.e., LNCaP and PC3 are more sensitive to these inhibitors than Du145 and C4-2. Among which, LNCaP, PC3 and C4-2 are PTEN negative strains, while Du145 is a PTEN positive strain. Moreover, PC3, Du145 and C4-2 are androgen-independent and LNCaP is androgen-dependent. Therefore, it seems likely that cell killing effects by AURKA inhibition is neither PTEN nor androgen signaling-dependent. Although it has been suggested that PTEN deletion and AURKA amplification occur concomitantly in colorectal cancer [37], there is no report on the correlation of PTEN loss and AURKA amplification in PCA so far. It is therefore extremely interesting to investigate the relationship between the amplification status of AURKA and other drug targets in prostate cancer.

The cytotoxic effect of AURKA inhibitors on cancer cells have been proved by previous studies and our work. However, our data demonstrate that the specific inhibitor, AURKA inhibitor I, showed most significant inhibition on prostate cancer viability, indicating that AURKA has strongest cell killing activity among three aurora family members. However, AURKA knockdown caused no or only minor growth delay in leukemic cell lines [38]. It has been shown that AURKB was overexpressed in many cancers, including high-grade prostate cancer [39]. AURKB knockdown resulted in proliferation arrest and apoptosis. AZD1152, an AURKB
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inhibitor, enhances radiosensitivity of androgen-resistant prostate cancer cell lines, PC3 and Du145 [40]. Furthermore, AURKB and EGFR siRNA in combination have shown enhanced therapeutic effect by inhibiting PC3 cell proliferation and inducing apoptosis in vitro, whereas androgen-dependent cancer cells LNCaP remain unaffected [41]. Additionally, over-expression of AURKC has also been detected in many cancers and several cancer cell lines, although it is unknown whether AURKC inhibition is associated with cancer cell death. Collectively, these data together with our data suggest that different aurora family members exhibit a specific activity in different cancers.

Recently, an AURK inhibitor, Danusertib (PHA-739358) was in phase II clinical trial for treatment of patients with metastatic castration-resistant prostate cancer after docetaxel failure [42]. The results showed that Danusertib monotherapy shows minimal efficacy in patients with castration-resistant prostate cancer. One possible reason for the failure of this trial is that patients are not selected by AURKA overexpression or other specific biomarkers. Further retrospective analysis need to be performed to identify patients on whom Aurora kinase inhibition has more significant effects. Another reason is that Danusertib might not be as effective as Aurora A Inhibitor I in prostate cancer patients, as shown in Figure 2A and 2B. It is anticipated that specific inhibition of AURKA would have significantly inhibitory effects in clinic.

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Address correspondence to: Dr. Zhou-Jun Shen, Department of Urology, Ruijin Hospital, Shanghai Jiaotong University, School of Medicine, Shanghai, China. Phone: 86-13917138608; E-mail: shenzhou-jun6@163.com

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