Antitumor effect of sunitinib in human prostate cancer cells functions via autophagy

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Abstract. The aim of the present study was to explore sunitinib-induced autophagic effects and the specific molecular mechanisms involved, *in vitro,* using PC-3 and LNCaP human prostate cancer cell lines. Cells were exposed to escalating doses of sunitinib treatment and subsequent cell viability and cell cycle analyses were performed to evaluate the inhibitory effect of sunitinib *in vitro*. Immunofluorescence staining of microtubule associated protein 1A/1B-light chain 3 (LC3) puncta was employed to assess autophagy levels after sunitinib treatment. Western blot analysis was performed to evaluate variations in the levels of LC3, sequestosome-1, extracellular signal regulated kinase 1/2 (ERK1/2), mammalian target of rapamycin (mTOR), p70 ribosomal protein S6 kinase (p70S6K) and cleaved caspase-3 proteins. The present study revealed that sunitinib treatment inhibited cell growth and triggered autophagy in a dose-dependent manner in both cell lines. In addition, sunitinib activated ERK1/2 and inhibited mTOR/p70S6K signaling. Sunitinib-induced autophagy was notably reversed by ERK1/2 kinase inhibitor, U0126. Furthermore, inhibition of sunitinib-induced autophagy by 3-methyladenine enhanced apoptosis and exhibited improved cell viability, which indicated that sunitinib induces not only apoptosis but also autophagic cell death in prostate cancer cell lines. These results may lead to an improved understanding of the mechanism of sunitinib's cytotoxic action and may provide evidence that combined sunitinib autophagy-regulating treatment may be of benefit to anti-prostate cancer therapy.

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Introduction

Prostate cancer (PCa) is the most common type of malignant tumor among men and the annual rate of PCa-related mortality is increasing rapidly worldwide (1). Treatments for prostate cancer include active surveillance, surgery, radiation therapy, chemotherapy, hormonal therapy, high-intensity focused ultrasound, or various combinations of these strategies. However, therapies for treating advanced PCa are considered to be largely ineffective (2,3).

Autophagy is an important homeostatic cellular recycling mechanism responsible for degrading unnecessary or dysfunctional cellular organelles and proteins in all living cells (4). Autophagy promotes a cell survival response and may be triggered by distinct cellular stress, including nutrient starvation, pathogen-associated molecular patterns and virus infection (5,6). Cells that undergo excessive autophagy may undergo cell death in a non-apoptotic manner (7). Additionally, the mammalian target of rapamycin (mTOR) signaling pathways have been reported to be involved in autophagy regulation in mammalian cells (8,9). Activation of extracellular signal-regulated kinases 1/2 (ERK1/2) has also been reported to be associated with autophagic regulation (10).

Sunitinib, which is a multi-targeted receptor tyrosine kinase (RTK) inhibitor, is approved for the treatment of advanced kidney cancer, imatinib-resistant gastrointestinal stromal cancer, pancreas adenocarcinoma and other types of solid-organ cancer (11-15). This anti-cancer agent has been described as an efficient therapeutic tool due to its desirable features in targeting apoptosis and oxidative stress (16,17). In PCa, preclinical and clinical studies have shown that sunitinib is able to inhibit PCa angiogenesis and proliferation, inducing prostate carcinoma cell apoptosis (18,19). However, while it may be possible that sunitinib may induce autophagy in PCa, the underlying effect and mechanism of autophagy in PCa remains unclear.

In the present study, the cytotoxicity and autophagy induced by sunitinib were explored using PC-3 and LNCaP human PCa cell lines. The effects of sunitinib on cell proliferation, cell cycle, autophagy and apoptotic cell death were investigated in PC-3 and LNCaP cell lines. Furthermore, the mechanisms

of sunitinib-induced cell death were examined, specifically ERK1/2 phosphorylation and mTOR signaling.

Materials and methods

Cell culture and materials. PC-3 and LNCaP human PCa cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 nutrient mixture (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) and RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (both HyClone; GE Healthcare Life Sciences), respectively. Sunitinib malate powder was supplied by Pfizer, Inc., (New York, NY, USA); 3-methyladenine (3-MA) and U1260 ERK inhibitor were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). All reagents were diluted in dimethyl sulfoxide (DMSO) and stored at -20°C.

Cell viability assay. Cell viability assays following suni-tinib treatment were performed using a cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). PC-3 and LNCaP cells were seeded in 96-well plates ($1x10^4$ cells/well) with culture medium supplemented with 10% FBS and were incubated at 37°C in incubator with an atmosphere of 5% CO_2 for 12 h to allow adherence. Cells were treated with 10 μl culture medium containing 0, 5, 10 or 20 μmol/l of sunitinib for 24 h. A total of 10 μl CCK-8 was added to the cells, following sunitinib treatment, and the cells were incubated for a further 2 h at 37°C. A microplate reader was used to measure the absorbance of each well at 450 nm, and inhibition rates were calculated as follows: Viability rate (%)={[A_{450}(sample)-A_{450}(blank)]/ [A_{450}(control)-A_{450}(blank)]}x100.

Cell cycle analysis. Cell cycle distribution was conducted by staining DNA with propidium iodide (PI; Sigma-Aldrich, Merck Millipore). Cells were trypsinized, fixed overnight in 70% ice-cold ethanol, washed twice with PBS, then centrifuged at 223.6 x *g*. Cells were incubated with RNase (50 μl at 100 μg/ml) for 10 min, stained with propidium iodide (200 μl at 50 μg/ml; cat. no. 81845; Sigma-Aldrich) for 1 h at room temperature. The percentage of cells in the different phases of the cell cycle were then analyzed with the FACSAria II flow cytometer using CellQuest7.6.2 software (both BD Biosciences, San Jose, CA, USA). ModFit v. 3.3.11 (Verity Software House, Topsham ME, USA) was used for data analysis.

Apoptosis assays. Apoptosis was determined by Annexin V-PE vs. 7-amino-actinomycin D (7-AAD) staining, using a PE Annexin V Apoptosis Detection kit I (BD Biosciences), according to the manufacturer's instructions. Cells were analyzed using a FACSCalibur flow cytometer and CELLQuest software (BD Biosciences).

Immunofluorescence staining. Cells were seeded on sterile cover slips in 6-well tissue culture plates at a concentration of $1x10^5$ cell/ml in a volume of 0.6 ml. Following treatment with either DMSO (control) or sunitinib (5, 10 or 20 μmol/l) for

24 h, cells were fixed onto slides with 4 % (v/v) paraformalde-hyde for 20 min, blocked with 5% bovine serum albumin for 30 min at room temperature and incubated with microtubule associated protein 1A/1B-light chain 3 (LC3) antibody (cat. no. sc292354; Santa Cruz Biotechnology, Inc., Dallas, TX, USA; dilution 1:200) overnight at 4°C. Subsequently, the treated cells were washed three times with PBS and incu-bated at room temperature with AlexaFluor 488-conjugated anti-rabbit secondary antibody (cat. no. A-11008; Thermo Fisher Scientific, Inc., Waltham, MA, USA; dilution 1:1,000) for 1 h. Fluorescence was measured using a confocal micro-scope (Leica Microsystems, Inc., Buffalo Grove, IL, USA; x630 magnification). Quantitation of the LC3 puncta was performed by counting 10 cells manually for each sample.

Western blot analysis. Cells were washed twice with ice-cold PBS and lysed in lysis buffer (1% NP-40, 5 mM NaPPi, 150 mM NaCl, 20 mM Tris HCL (pH 7.5), 5 mM Na_3VO4, 1 mM PMSF and 10 μg/ml leupeptin). Samples were vortexed briefly, incubated in lysis buffer for 30 min on ice and centrifuged at 15,000 x *g* for 15 min. Protein concentration was determined by the Bradford method. Equal amounts of samples containing total protein (20 μg) were separated using 10-15% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (cat. no. IPVH00010; Merck KGaA, Darmstadt, Germany). Membranes were blocked in 5% non-fat milk for 1 h at room temperature, incubated with the below indicated primary antibodies in nonfat milk overnight at 4°C, then washed with PBS/0.1% Tween 20 for 1 h. Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (cat. no. 111-035-003; Jackson Immunoresearch Labs, Inc., West Grove, PA, USA; dilution, 1:20,000) or rabbit goat anti-mouse IgG (cat. no. 115-035-003; Jackson Immunoresearch Labs, Inc.; dilution 1:20,000) for 1 h. Subsequently, membranes were washed with PBS/0.1% Tween-20 for 40 min and detected with enhanced chemiluminescence. Primary antibodies used in western blotting, according to the manufacturer's instruc-tions, were: Anti-LC3, anti-sequestosome-1 (SQSTM1/p62), anti-mTOR, anti-p-mTOR (Ser2481), anti-p-p70S6K (Thr389) (Cell Signaling Technology, Inc., Danvers, MA, USA), β-actin, anti-p-ERK1/2 (Thr202/Tyr204), anti-ERK1/2 (Santa Cruz Biotechnology) and anti-cleaved-caspase3 (Beyotime Institute of Biotechnology, Jiangsu, China). Bands were revealed using an enhanced chemiluminescence reagent (ECL) in an ECL Plus kit (Beyotime Institute of Biotechnology, Jiangsu, China) and recorded on X-ray films (Fujifilm Life Science, Tokyo, Japan).

MTT assay. Cell viability was measured by the 3-(4,5-dimeth-ylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (cat. no. 11465007001; Roche Diagnostics, Basel, Switzerland). Cells were plated at 5,000 cells/well in 96-well plates, in triplicate. Cells were allowed to adhere overnight, and medium containing the test drug or control media was added. After incubation for 48 h at 37°C in 5% CO_2, the drug-containing medium was removed and replaced by 100 μl fresh medium with 0.5 mg/ml MTT solution. After incubation for 4 h, the medium with MTT was removed and 100 μl solubilization solution was added to each well. The plates were then gently agitated until the color reaction
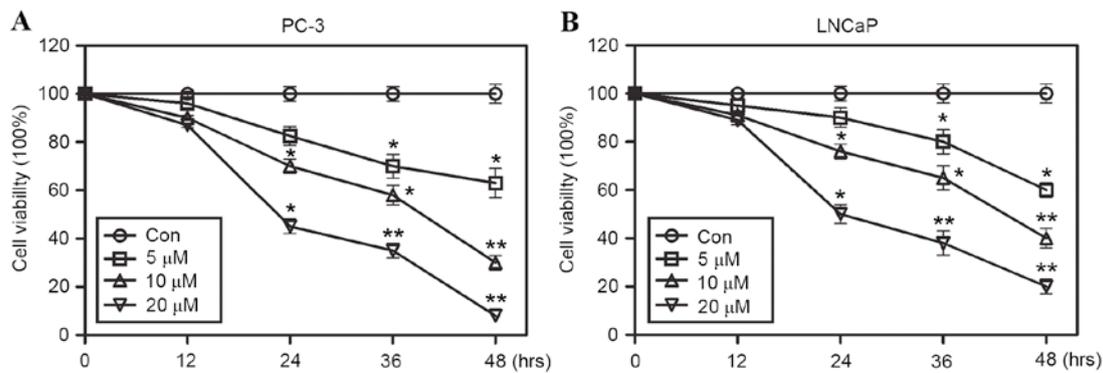
Figure 1. Effect of sunitinib on the proliferation in PC-3 and LNCaP cells. Following 5, 10 or 20 μmol/l sunitinib treatment in PC-3 and LNCaP cells for 24 h, cell viability was measured using a Cell Counting Kit-8 kit assay. Sunitinib inhibited the proliferation of (A) PC-3 and (B) LNCaP cells in a dose-dependent manner. Data are presented as mean ± standard deviation (n=4). *P<0.05 and **P<0.01 vs. Con. Con, control.

was uniform, and the OD_{570} (optical density at a wavelength of 570 nm) was determined using a microplate reader (Wellscan MK3, Labsystems Diagnostics, Vantaa, Finland). Media-only treated cells served as the indicator of 100% cell viability. Viability rate (%)={[A_{570}(sample)-A_{570}(blank)]/[A_{570}(control)-A_{570}(blank)]}x100.

Statistical analysis. All results presented were confirmed in at least three independent experiments. Data were presented as mean ± standard deviation. Statistical analysis was performed using one-way analysis of variance with Bonferroni's *post-hoc* tests for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Sunitinib inhibits the growth of PC-3 and LNCaP cells in vitro. To ascertain the inhibitory effect of sunitinib on the growth of PCa cells, cell viability assays were performed using PC-3 and LNCaP cells, either in the presence of sunitinib at different concentrations (5, 10 or 20 μmol/l) or in the absence of sunitinib and treated instead with DMSO (control) for 24 h. Significant inhibitory effects on cell growth in PC-3 and LNCaP cells were observed in a dose-dependent manner (Fig. 1A and B, respectively), which may suggest that sunitinib possesses a potent cytotoxic activity against both human PCa cell lines.

Sunitinib induces G1-phase arrest in PC-3 and LNCaP cells. Cell viability assays revealed that sunitinib treatment significantly inhibited the proliferation of PC-3 and LNCaP cells. Subsequently, whether the inhibition effect on cell proliferation was related to cell cycle progression was detected. Flow cytometric analysis indicated that 10 μM sunitinib treatment for 48 h resulted in the accumulation of cells in the G1 phase in PC-3 cells, (76.36±4.78%) compared with the control (51.82±7.02%), and in LNCaP cells (65.83±7.99%) compared with the control (53.32±6.08%) (Fig. 2). Furthermore, the populations of PC-3 and LNCaP cells in G2/M phase decreased significantly (P=0.037 and 0.032 for PC-3 and LNCaP, respectively), from 19.42±2.49% and 20.65±1.85% in the control groups to (8.58±1.03%) and (12.11±1.17%) after sunitinib treatment (Fig. 2). These results indicated that sunitinib induced G1-phase arrest in PC-3 and LNCaP cells.
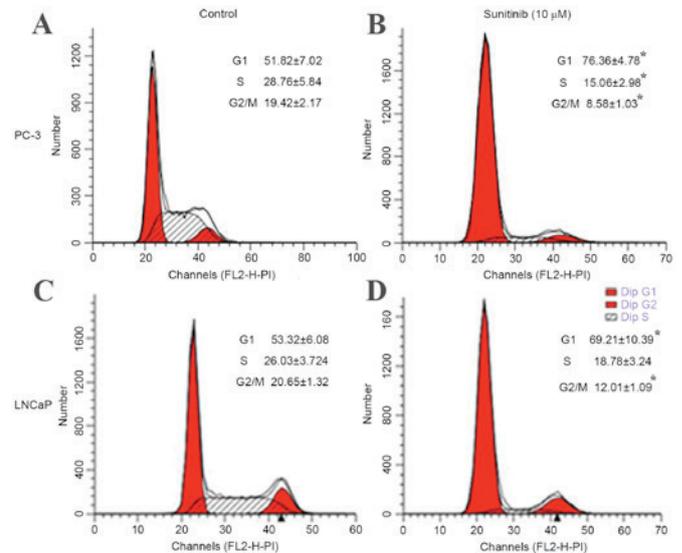

Figure 2. Cell-cycle (G0/G1, S and G2/M) analysis of PC-3 and LNCaP cells exposed to sunitinib (10 μM) or DSMO (control) for 48 h. Flow cytometric analysis was performed using PC-3 and LNCaP human PCa cells to establish the percentage of cells in G0/G1, S and G2/M phases of the cell cycle. (A) Control PC-3 cells treated with DSMO. (B) PC-3 cells treated with 10 μM sunitinib. (C) Control LNCaP cells treated with DSMO. (D) LNCaP cells treated with 10 μM sunitinib. Data are presented as mean ± standard deviation (n=4). *P<0.05 vs. control. DSMO, dimethyl sulfoxide.

Sunitinib induces autophagy in PCa cell lines. Sunitinib, which is a multi-targeted tyrosine kinase inhibitor, exhibits anti-angiogenic and anti-tumor activity and has been approved by a large body of research (20,21). It was speculated that sunitinib may also induce autophagy in PCa cells. Western blot analysis of SQSTM1/p62 and LC3 and subsequent immunofluorescence staining of LC3 puncta was performed to evaluate autophagy. PC-3 and LNCaP cells were treated for 24 h with various concentrations (5, 10 or 20 μmol/l) of sunitinib or DMSO (control), respectively. Cells were subjected to immunofluorescence staining and dose-dependent formation of LC3 fluorescence dots was observed. Limited numbers of LC3 punctas were observed in control groups (DMSO) in both cell lines. However, following sunitinib treatment, the LC3 puncta numbers increased significantly (when comparing the 5, 10 or 20 μmol/l groups with the control, P<0.001 for
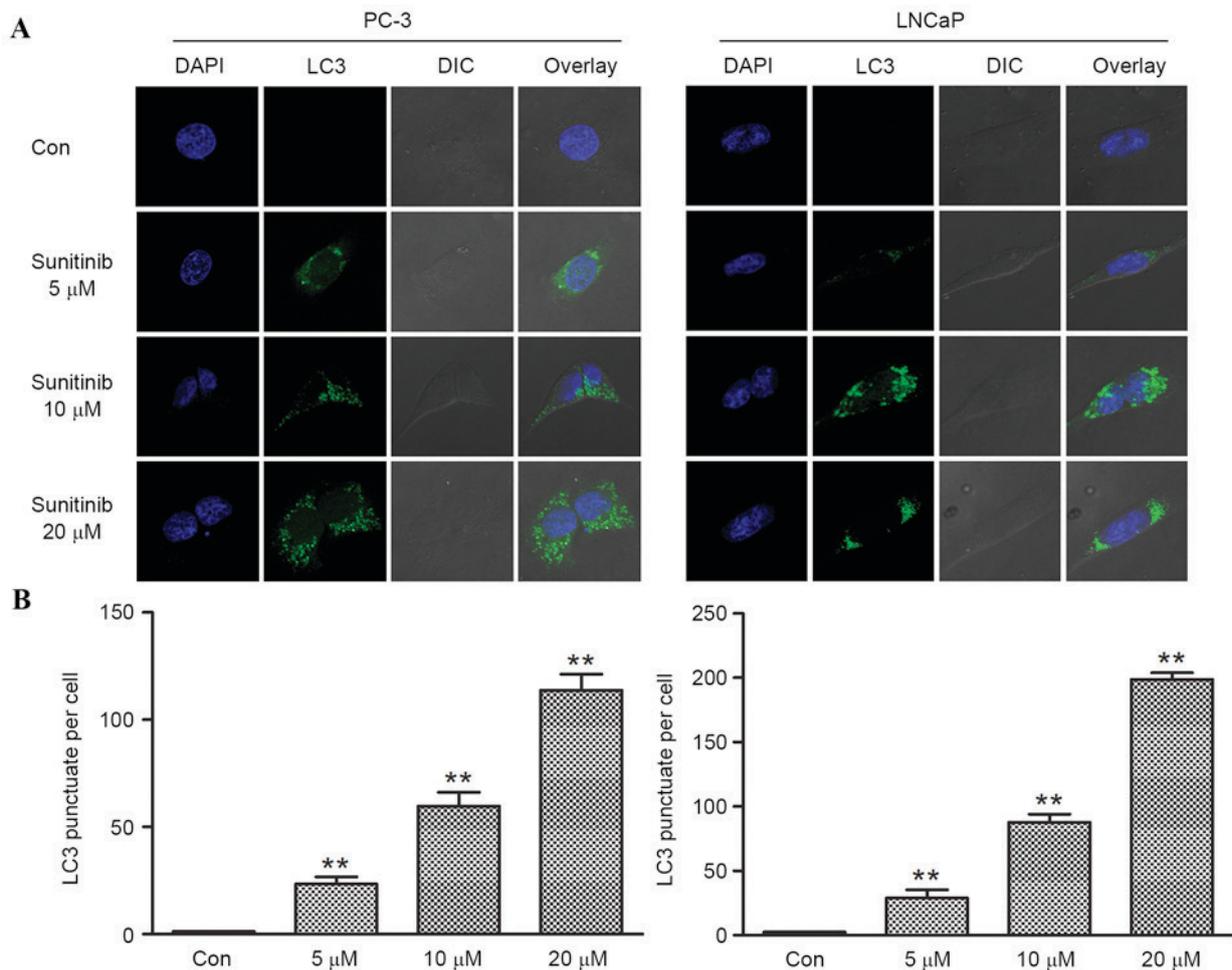
Figure 3. Effect of sunitinib on the formation of LC3 puncta in PC-3 and LNCaP cells. (A) Fluorescence images obtained from sunitinib-treated and control groups of prostate cancer cells, PC-3 and LNCaP. Cells treated with DMSO were set as control groups, the numbers of LC3 puncta per cell were counted manually. The number of LC3 puncta (green fluorescent) per cell in sunitinib-treated groups increased in a dose dependent manner (magnification, x630). (B) Limited numbers of punctas were located in control group PC-3 and LNCaP cells. The numbers of LC3 puncta per cell in PC-3 cells of 5, 10 and 20 μM sunitinib-treated groups were 23.29±3.36, 59.74±6.48 and 113.71±7.52, respectively. The numbers of LC3 puncta per cell in LNCaP cells of 5, 10 and 20 μM sunitinib-treated groups were 28.90±6.34, 87.56±6.45 and 198.56±5.33, respectively. Data are presented as means ± standard deviation (n=4). *$P<0.05$ and **$P<0.01$, vs. Con. DSMO, dimethyl sulfoxide. LC3, microtubule associated protein 1A/1B-light chain 3; Con, control; DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast.

PC-3, and $P<0.002$ for LNCaP), corresponding with increasing concentrations of sunitinib (Fig. 3A and B).

A hallmark of autophagy is the enhanced conversion of microtubule-associated protein 1 light chain 3 (LC3-I) to its faster-migrating form LC3-II, which is closely associated with the membrane of autophagosomes. Degradation of SQSTM1/p62 is used as a marker of autophagic flux (22). p62 is incorporated into autophagosomes and degraded in autolysosomes. Compared with the control groups, significantly increased expression levels of LC3-II (when comparing the 5, 10 and 20 μmol/l groups with the control, $P=0.017$, <0.001 and <0.001 for PC-3, and $P=0.028$, <0.001 and <0.001 for LNCaP, respectively), accompanied by decreased expression levels of SQSTM1/p62, were observed in dose-dependent manners in PC-3 and LNCaP cells exposed to sunitinib for 24 h (Fig. 4A-C). These findings indicated that sunitinib may have induced autophagy in the human PCa cell lines.

Regulation of phosphorylated ERK1/2 and mTOR signaling contributes to sunitinib-induced autophagy in PC-3 and

LNCaP cells. Previous studies have confirmed that activation of AKT/mTOR pathway negatively regulates autophagy (23,24). In order to assay the direct effect of sunitinib on the activation or inhibition of ERK and mTOR signaling, western blot analysis was performed to explore the role of specific components involved in autophagy flux regulation by observing the expression levels of total ERK1/2, mTOR, phosphorylated ERK1/2, mTOR and p70S6K. Treatment with sunitinib at different concentrations for 24 h induced a significant upregulation of phosphorylated ERK1/2 (when comparing the 5, 10 or 20 μmol/l groups with the control, $P=0.050$, 0.003 and <0.001 for PC-3, and $P=0.044$, 0.048 and <0.001 for LNCaP, respectively) in a dose-dependent manner for both PCa cell lines (Fig. 5). Furthermore, the expression levels of mTOR and its substrate, p70S6K, and their phosphorylated forms, were investigated to reveal whether the mTOR pathway may be responsible for sunitinib-induced autophagy. The results revealed that 10 and 20 μmol/l sunitinib treatment significantly inhibited the phosphorylation of mTOR ($P=0.008$ and 0.002 for PC-3, and $P=0.005$ and 0.001 for LNCaP,
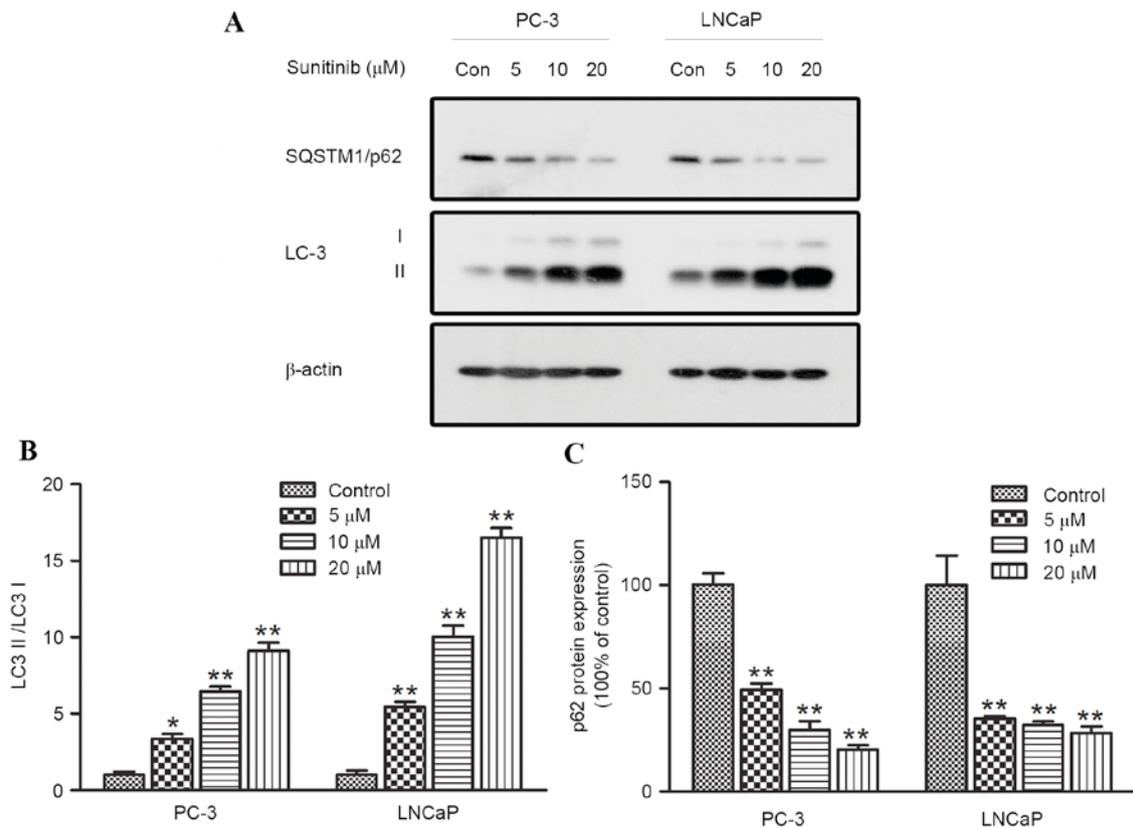
Figure 4. Western blotting analysis of sunitinib-induced autophagy in prostate cancer cells. (A) Following sunitinib treatment at different concentrations for 24 h, increased levels of LC3-II protein expression and decreased levels of SQSTM1/p62 protein expression were observed in both PC-3 and LNCaP cells. (B) Fold changes of LC3-II/ LC3-I and (C) the expression level of SQSTM1/p62 between the treatment and the control groups were determined by three independent experiments and normalized by the total protein level. Data are presented as mean ± standard error. *P<0.05 and **P<0.01, vs. Con. LC3, microtubule associated protein 1A/1B-light chain 3; Con, control; SQSTM1/p62, sequestosome-1.

respectively) in both cell lines. Simultaneously, the phosphorylation of p70S6K was also inhibited (when comparing the 5, 10 or 20 μmol/l groups with the control, P=0.003, <0.001 and <0.001 for PC-3, and P=0.010, 0.008 and <0.001 for LNCaP, respectively) (Fig. 5).

U0126 is a highly selective inhibitor of both MEK1 and MEK2, which are types of MAPK/ERK kinase (25). U0126 is able to block the ERK pathway (26); therefore, U0126 (10 μM) was used to inhibit the phosphorylation of ERK1/2 in the present study. LC3II/I ratio was significantly increased when PC-3 and LNCaP cells were treated with 10 μM sunitinib; however, a decrease in the LC3II/I ratio was observed when comparing the U1026+sunitinib group with the sunitinib group in PC-3 and LNCaP cells (P=0.008 and <0.001, respectively). In addition, quantitation analysis revealed that when phosphorylation levels of ERK were inhibited, no significant increase of LC3II/I ratio was found after sunitinib treatment when compared with the control (Fig. 6). Overall, these results suggest that ERK1/2 and mTOR activation stimulates sunitinib-induced autophagy in PC-3 and LNCaP cells.

Autophagy and apoptosis have roles in sunitinib-mediated cell death in PC-3 and LNCaP cells. To investigate whether apoptosis or autophagy is involved in the growth inhibitory effect of sunitinib, 3-MA (5 μM) was administered as a pre-treatment to inhibit sunitinib-induced autophagy and flow cytometry using Annexin V-PE and 7-AAD staining was

performed, following 10 μM sunitinib treatment for 48 h in both cell lines. Cleaved caspase-3 protein expression levels were also determined using western blot analysis.

Significant reductions in the number of viable cells (Annexin V-PE⁻/7-AAD⁻) after treatment with sunitinib only (sunitinib groups) were observed in PC-3 and LNCaP cell lines when compared with controls (P=0.007 and 0.038, respectively), accompanied by significant increases of the number of apoptotic cells (P=0.001 and 0.017, respectively) (Annexin V-PE⁺/7-AAD⁻, early apoptosis; Annexin V-PE⁺/ 7-AAD⁺, late apoptosis) (Fig. 7A and B). However, there was no significant difference observed between the 3-MA and control groups for apoptosis induction. However, when compared with sunitinib + 3-MA groups and 3-MA groups, combined sunitinib and 3-MA treatment resulted in significantly increased apoptotic cell death (P=0.003 and 0.010 for PC-3 and LNCaP cells, respectively), with a higher percentage of 7-AAD⁺ cells. These results indicated that inhibition of autophagy by 3-MA enhanced sunitinib-induced apoptosis. Western blot analysis was carried out to detect the expression of cleaved caspase-3, a hallmark of apoptotic cell death which preceded the previously observed changes in nuclear morphology (27). Sunitinib groups or sunitinib + 3-MA groups exhibited increased expression levels of cleaved caspase-3 when compared with the control or 3-MA groups. Furthermore, inhibition of autophagy by 3-MA significantly enhanced the expression levels of cleaved caspase-3 induced
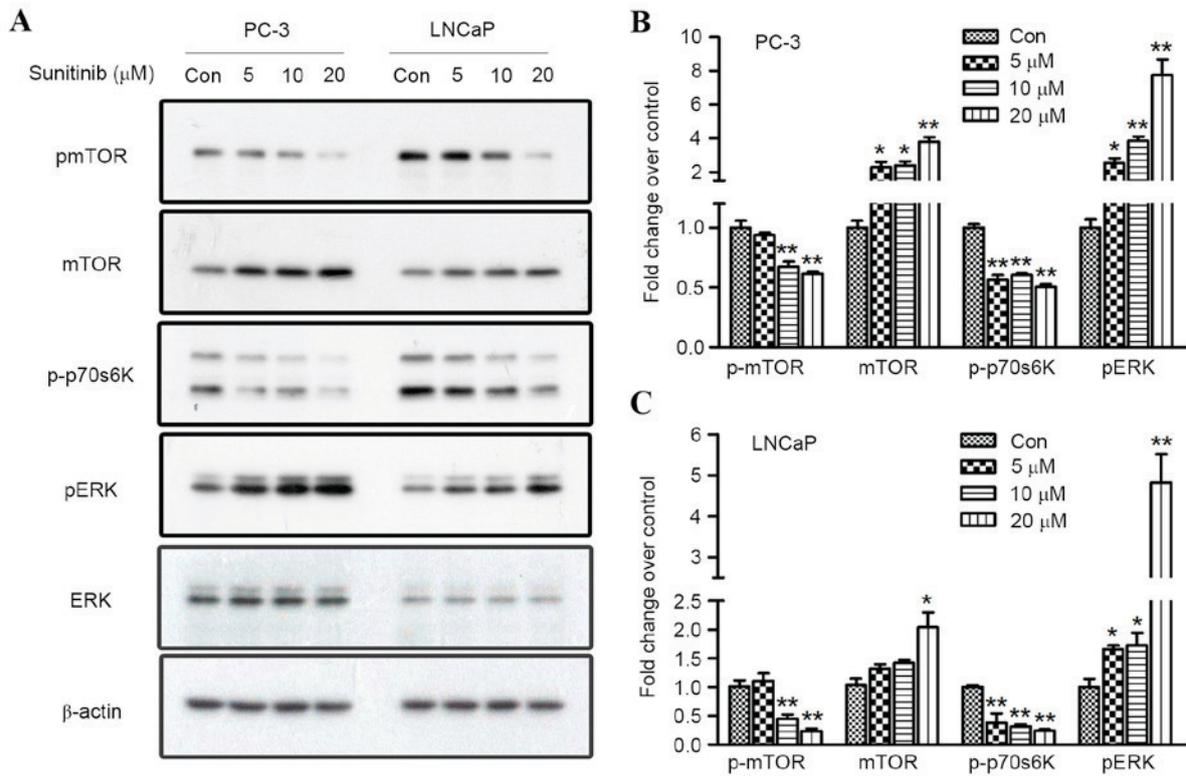
Figure 5. Effect of sunitinib on mTOR and ERK signaling in PC-3 and LNCaP cells. (A) Sunitinib treatment suppressed the phosphorylated levels of mTOR and p70S6K and activated the phosphorylated level of ERK1/2, as demonstrated by western blotting. Fold changes in p-mTOR, mTOR, p-p70S6K and p-ERK proteins in 5, 10, or 20 μM sunitinib-treated groups and control groups in (B) PC-3 cells and (C) LNCaP cells. Results were determined by three independent experiments and normalized by the total protein level. Data are presented as mean ± standard deviation. *P<0.05 and **P<0.01, vs. Con. mTOR, mechanistic target of rapamycin; p70S6K, ribosomal protein S6 kinase beta-1; ERK 1/2, extracellular signal-regulated kinases 1/2; Con, control.
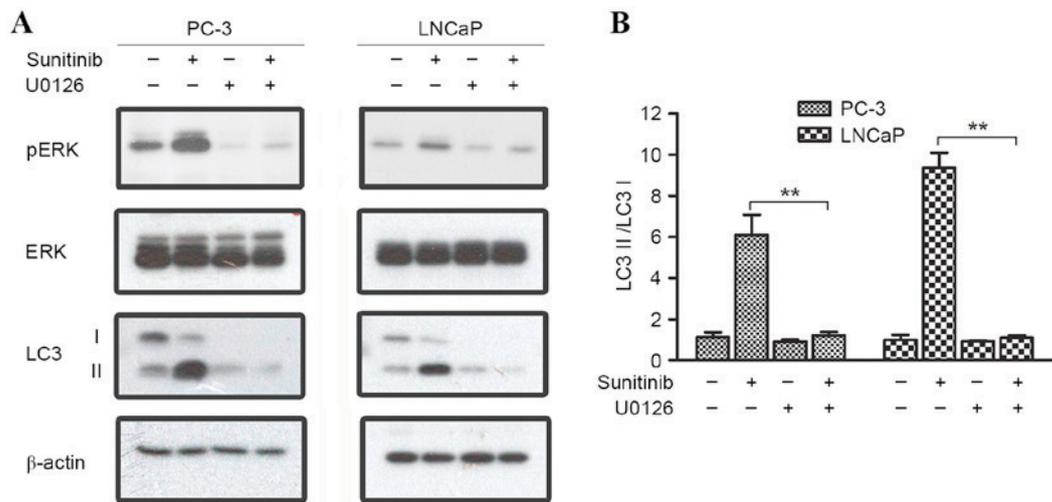

Figure 6. Inhibition of ERK1/2 by U1026 effects on the autophagy level induced by sunitinib. (A) Inhibition of the phosphorylation of ERK1/2 by U0126 (10 μM) in PC-3 and LNCaP cells suppressed the increased fold changes of LC3II/I levels triggered by sunitinib, as demonstrated by using western blotting. (B) Fold changes of LC3II/I expression levels in sunitinib-treated groups and the control groups were determined by three independent experiments and normalized by the total protein level. Data are presented as mean ± standard deviation, *P<0.05 and **P<0.01, vs. control. ERK 1/2, extracellular signal-regulated kinases 1/2; LC3, microtubule associated protein 1A/1B-light chain 3.

by sunitinib, when comparing the sunitinib groups with the sunitinib + 3-MA groups (P=0.019 and 0.038 for PC-3 and LNCaP, respectively) (Fig. 7C).

　　To further evaluate the interplay between apoptosis and autophagy and their effect on sunitinib cytotoxicity in PCa cells, a cell viability assay by MTT was performed. Following

48 h of sunitinib treatment, inhibition of autophagy by 5 μM 3-MA pre-treatment lead to a significant increase in the rate of cell viability when comparing the sunitinib group with the sunitinib + 3-MA group in PC-3 cells (P=0.40) (Fig. 8). A similar observation in the increased rate of cell viability was exhibited in LNCaP cells, although, no statistical significance
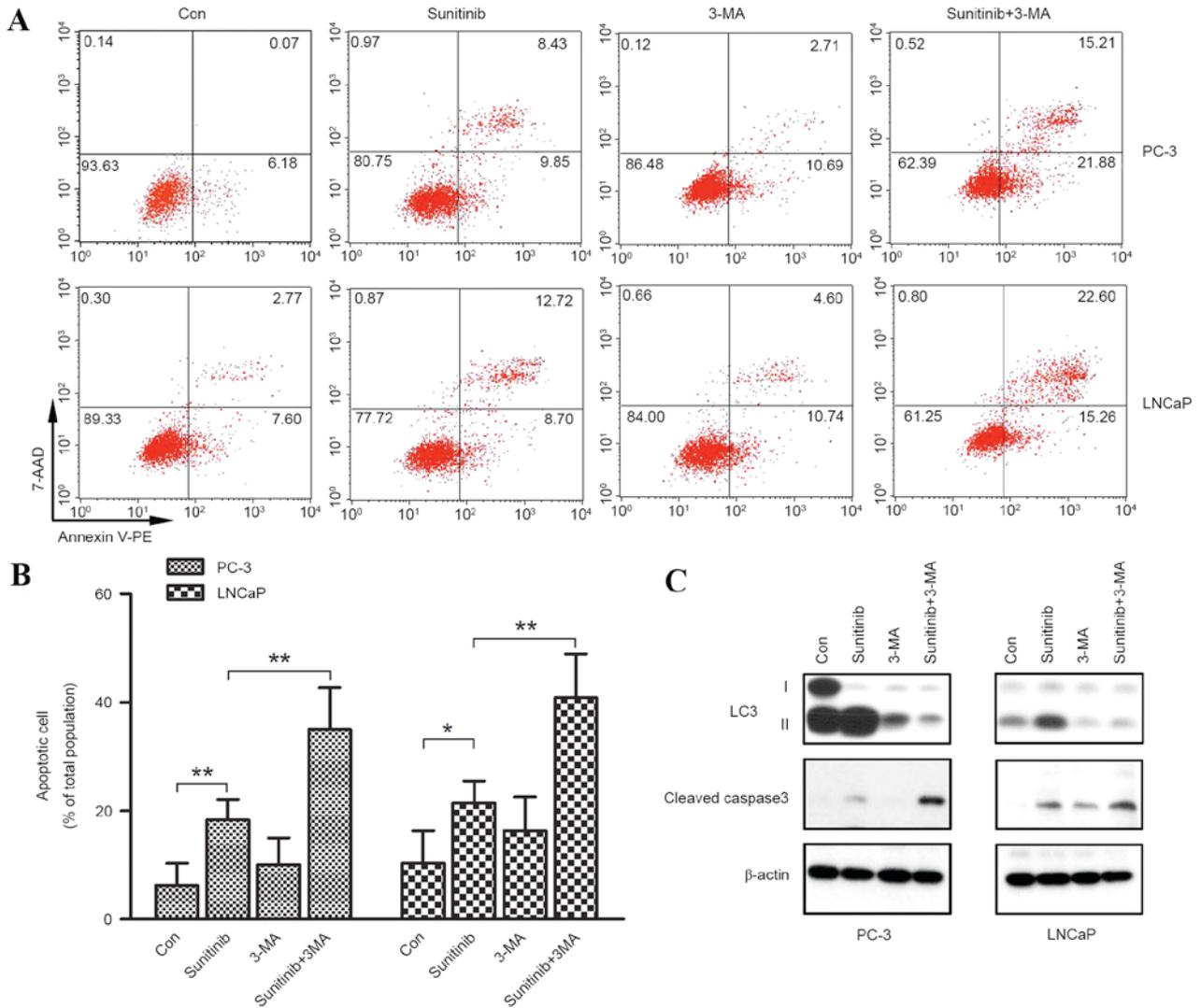
Figure 7. Inhibition of autophagy by 3-MA effects on sunitinib-induced cell apoptosis. (A) Flow cytometric analysis indicated inhibition of autophagy by 3-MA (5 μM) increased the rate of apoptosis induced by sunitinib. (B) Percentage of apoptotic cells in control, sunitinib-treated, 3-MA-treated and sunitinib + 3-MA-treated groups in PC-3 and LNCaP cells (C) Following sunitinib treatment, caspase-3 was activated and inhibition of autophagy enhanced the activation of caspase-3. Data in panel (B) represent the mean ± standard deviation of three independent experiments, *P<0.05 and **P<0.01. 3-MA, 3-methyladenine; Con, control.
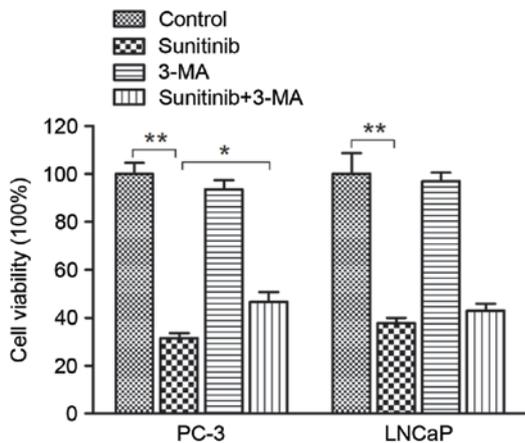

Figure 8. Inhibition of autophagy by 3-MA effects on sunitinib-induced apoptosis. Sunitinib-induced cell death was reversed by inhibition of autophagy with 3-MA (5 μM) in PC-3 cells, which was similarly observed in LNCaP cells; however, no statistical significance was detected in LNCaP cells. Data are presented as mean ± standard deviation. *P<0.05 and **P<0.01. 3-MA, 3-methyladenine.

was indicated. In consideration of the flow cytometry assay results, we conclude that sunitinib-triggered cytotoxicity was mediated by autophagy and apoptotic cell death induction in PC-3 and LNCaP cells.

Discussion

Previous studies have advanced understanding of the role of vascular endothelial growth factor (VEGF), which has led to the addition of several agents to the therapeutic landscape for various tumor types. Among these antiangiogenic agents, sunitinib is a tyrosine kinase inhibitor that targets VEGF receptors 1, 2 and 3 and has been approved for the treatment of metastatic renal carcinoma (28) and gastro-intestinal stromal tumor (29).

In accordance with our results, several studies have observed a significant inhibitory and cytotoxicity effect on PCa cell lines via the regulation of hypoxia and angiogenesis (18,30). These findings suggest a possible antitumor effect

of sunitinib in PCa. However, there is a lack of studies on the antitumor effect and mechanism of action of sunitinib in PCa.

In the present study, sunitinib was revealed to inhibit the viability of PC-3 and LNCaP cells in a dose-dependent manner. Our findings are in accordance with the results of two previous studies, concerning anti-tumor and radiosensitivity of sunitinib on PC-3 and DU145 cells *in vitro* (31) and the preventative effects in the course from non-castration to castration of LNCaP xenograft prostate (19). In addition, cell cycle analysis in the present study revealed that, following 10 μM of sunitinib treatment for 24 h, the number of PC-3 and LNCaP cells in S and G2/M phases was decreased, whereas the number of cells in G1 phase was increased, when compared with the controls. Similar results were reported when colonic stromal fibroblasts were treated with sunitinib mesylate (32). Moreover, Di Desidero *et al* (33) identified that sunitinib induced a concentration-dependent inhibition of the cyclin-D1 gene and protein expression, in HMVEC-d, 8305C and FB3 cells, respectively.

Autophagy is a highly conserved, homeostatic process by which intracellular constituents are delivered to lysosomes for degradation. It is activated in response to various environmental stresses that have been documented in human tumor cells, following treatment with chemotherapeutic drugs (34,35). Furthermore, a previous study has indicated that excessive activation of autophagy may lead to another form of programmed cell death in a non-apoptotic manner (36). LC-3 is considered to be a strong marker of autophagy. Furthermore, the conversion of LC3-I to LC3-II and formation of LC3 puncta usually demonstrates the activation of autophagy (37). In addition, p62 degraded alongside autophagosomal contents, resulting in decreased p62 levels (38). In the present study, sunitinib treatment was revealed to promote autophagy in PC-3 and LNCaP cells in dose-dependent manner, as indicated by the increases in the LC3-II/LC3-I ratio and membrane-bound lapidated form of LC3, and the degradation of p62 protein observed. Ikeda *et al* (39) reported that sunitinib induced autophagy in rat pheochromocytoma PC12 cells and that this was dependent on the suppression of mTORC1 signaling and the formation of ULK1/2-Atg13-FIP200 complexes. In addition, another study discovered that sunitinib treatment for 24 h triggers incomplete autophagy, impaired cathepsin B activation and stimulated lysosomal-dependent necrosis in bladder cancer cells (40). To the best of our knowledge, the present study is the first to propose that autophagy in PCa cell lines may be stimulated by sunitinib.

mTOR and ERK1/2 are two major pathways that regulate autophagy induced by nutrient starvation. Cadmium (41) or TNFα treatment (42) have been clearly associated with ERK activation and autophagic programmed cell death; moreover, direct ERK activation by the overexpression of active MEK may promote autophagy without any other stimulus (43). The modifications exhibited by phosphorylated ERK (p-ERK) after sunitinib treatment are conflicting, according to previous reports (44,45). Sunitinib treatment has been revealed to exhibit increased levels of p-ERK in bladder cancer cells (J82 and 5637) (40) and adrenocortical carcinoma (SW13) cells (46), whereas p-ERK levels were suppressed in human colonic stromal fibroblasts (32) and papillary thyroid cancer cells (44). The present study revealed that sunitinib significantly promoted

ERK1/2 phosphorylation and suppressed mTOR/p70S6K phosphorylation, which was consistent with the differences of LC3-II/LC3-I ratio and p62 expression observed. Furthermore, the highly selective MEK1/2 inhibitor, U0126, markedly reversed the induction of autophagy by sunitinib when compared with the sunitinib and U0126 combination group and the sunitinib group, which suggested that ERK signaling may have a role in sunitinib-induced autophagy. However, Diaz *et al* (31) reported that sunitinib promoted a decrease in the expression level of p-ERK in PC-3 cells after treatment. Considering that the maximum concentration of sunitinib used by Diaz *et al* (31) employed in that study was 5 μM, which is much lower than that of our study, both results may be reasonable and further research is required. It has been established that mTORC1 may suppress autophagy by promoting phosphorylation and inactivation of proteins involved in autophagosome formation (47,48). The role of the mTOR substrate, p70S6Kinase 1 (S6K1), in autophagy remains controversial (49). However, one study has indicated that active S6K1 may decrease the level of LC3 processing and foci formation by autophagosomal vacuoles in cells treated with sulforaphane, and diminished levels of S6K1 or a lack of S6 kinases resulted in the accumulation of autophagosomes in PC-3 cells (50). This is consistent with the variation trend of p-mTOR and p-p70S6K demonstrated in the present study, which suggested that mTOR signaling is also involved in the regulation of sunitinib-induced autophagy. Overall, the present data revealed that sunitinib may promote autophagy in PCa cells through the activation of ERK1/2 and the inhibition of mTOR.

Autophagy, which is described as a 'double-edged sword', has a key role in tumorigenesis, progression and oncotherapy. On the one hand, autophagy manifests as a survival pathway that allows tumor cells to live through various severe environments (51); whereas, autophagy has also been revealed to contribute to cell death, which was previously confirmed in cancer cells undergoing antineoplastic therapy (52). To establish the role of autophagy in sunitinib-induced cell death, 3-MA (5 μM) was used as a pre-treatment to block autophagy and subsequent flow cytometry and cell proliferation assays were employed to determine apoptosis and cell viability. These analyses revealed that sunitinib treatment induced an increase in the rate of cellular apoptosis, while inhibition of autophagy by 3-MA led to a remarkable enhancement in sunitinib-induced apoptosis. Furthermore, the expression of cleaved caspase-3 protein, a cysteine protease involved in the 'execution' phase of cellular apoptosis and a key regulator of tumor repopulation promoting generated from the dying cells (53), was detected by western blotting. Western blot analysis showed a similar changing trend in accordance with the change of apoptosis rate: Sunitinib only treatment induced significant increases in the number of apoptotic cells, while a further enhancement of the apoptotic rate was detected after the inhibition of autophagy by 3-MA when compared sunitinib + 3-MA group with sunitinib group. Coincidentally, inhibition of autophagy by 3-MA significantly enhanced the expression levels of cleaved caspase-3 induced by sunitinib, when comparing sunitinib groups with sunitinib + 3-MA groups. These findings suggest that, besides promoting autophagy, sunitinib may also generate apoptosis in a caspase-3-dependent manner in

PC-3 and LNCaP cells. Additionally, 3-MA (5 μM) was used to inhibit autophagy and cell viability following 48 h of 10 μM sunitinib treatment was determined by MTT. Unexpectedly, the cell death rate of PC-3 cells significantly decreased when treated with sunitinib and 3-MA pre-treatment; despite this, the inhibition of autophagy lead to a significant enhancement of sunitinib-induced apoptosis. Similar observations were identified in LNCaP cells; however, no statistical significance was detected. Collectively, these findings indicated that apoptosis and autophagic cell death have key roles in sunitinib-induced cytotoxicity in PCa cells. While relatively few reports focus on the induction of autophagy by sunitinib (39,40), the present results suggest that autophagy may be an important component in the cytotoxicity resulting from sunitinib treatment in PCa cells.

Phase II trials of single-agent sunitinib in metastatic castration-resistant PCa (mCRPC) have suggested that the antitumor activity, which was assessed by a >50% decline in prostate-specific antigen levels and tumor shrinkage, exhibited an acceptable safety profile (18,30). In addition, several drugs with anti-angiogenic properties have proceeded to Phase III evaluation for the treatment of patients with CRPC; however, no phase III study to date has succeeded in demonstrating a survival benefit in large randomized studies (54), which indicates that further advances are required.

In conclusion, the present study demonstrated that sunitinib manifested an antineoplastic effect on PCa cell lines *in vitro*. Sunitinib was revealed to stimulate autophagy in PCa cells via the regulation of ERK1/2 phosphorylation and mTOR signaling. Autophagic and apoptotic cell death were demonstrated to have roles in the cytotoxic effects induced by sunitinib in PCa cells. Further studies are required to fully investigate the interactions and signal pathways involved in the conversion between autophagic and apoptotic cell death. In addition, the findings of the present study suggest that implementing autophagy combined sunitinib treatment may be beneficial in devising novel anti-cancer strategies in PCa.

Acknowledgements

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