

Zoledronic Acid Suppresses Epithelial-to-Mesenchymal Transition and Invasion via Degradation of Ubiquitinated NEDD9 in PC-3 Prostate Cancer Cells

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Abstract

Objective: Zoledronic acid (ZA) is highly effective in the treatment of castration-resistant prostate cancer (CRPC) patients with bone metastases. It is one of bone modifying agents (BMAs) that has been shown to exert not only inhibiting the activation of osteoclasts but also preventing the tumor cell growth, invasion and migration in some cancers. Neural precursor cell-expressed developmentally downregulated protein 9 (NEDD9) is a key regulator of tumor aggressiveness including invasion, epithelial-to-mesenchymal transition (EMT), dedifferentiation and resistance to chemo-drugs. However, research into a biological mechanism in the inhibitory effects of ZA on prostate cancer (PCa) metastasis is still limited. In this study, we examined its effects on tumor cell invasion and EMT via the ubiquitin-proteasomal system for NEDD9 in PC-3 cells.

Methods: We assessed the expression of NEDD9 and its down-stream molecules associated with EMT in PC-3 cells exposure to ZA under the condition with/without TGF- β . By a boyden chamber assay, the suppressive effect of ZA on PC-3 cell invasion triggered by TGF- β was measured. We measured the expression levels of NEDD9 in PC-3 cells exposure to a proteasome inhibitor, MG132. In addition, we detected the effect of ZA on ubiquitinated NEDD9 using an immunoprecipitation method.

Results: ZA markedly inhibited the expression of NEDD9 and its down-stream EMT molecules. Both the invasion and expression of EMT markers of PC-3 cells triggered by TGF- β were significantly suppressed by the exposure to ZA. The exposure to MG132 inhibited the degradation of NEDD9 in PC-3 cells. The further add-on of ZA enhanced the polyubiquitination of NEDD9 in PC-3 cells.

Conclusion: The results from a current study indicate that ZA inhibited the invasion and expression of NEDD9 and its EMT markers, along with the enhanced degradation of ubiquitinated NEDD9 in PC-3 cells.

Keywords: Zoledronic acid; EMT; Invasion; NEDD9; Ubiquitination; Proteasome; degradation; PC-3 prostate cancer cells; TGF- β ; BMA

Abbreviations: PCa: Prostate Cancer; ZA: Zoledronic Acid; BMA: Bone Modifying Agent; NEDD9: Neural Precursor Cell-Expressed Developmentally Downregulated Protein 9; EMT: Epithelial-to-Mesenchymal Transition; CRPC: Castration-Resistant Prostate Cancer; Cas: Crk-Associated Substrate; MET: Mesenchymal-To-Epithelial Transition; UPS: Ubiquitination-Proteasome System; SREs: Skeletal-Related Events; RANK-L: Receptor Activator Of Nuclear Factor-kappa beta-Ligand

Introduction

Until now, some part of treatment-naïve patients diagnosed with high-risk prostate cancer (PCa) and the most of aggressive castration-resistant prostate cancer (CRPC) patients have bone metastases and their disease state strongly develop the early resistance to hormonal therapy and chemotherapy [1]. From the viewpoint of cell biology, epithelial-to-mesenchymal transition (EMT) is an essential mechanism for tumor aggressiveness and metastasis [2]. Aggressive epithelial cancer cells can stimulate embryonic processes, which obtained in epithelial plasticity and change from a polarized, epithelial phenotype to a mesenchymal phenotype with increased motility. Numerous biological alterations, including expression of mesenchymal biomarkers, induction of angiogenesis, increased resistance to apoptosis and marked production of extracellular matrix components, occur in epithelial cancer cells throughout the course of EMT [3,4].

NEDD9 (neural precursor cell-expressed developmentally

downregulated protein 9) is a core member of the multiple docking protein Cas (Crk-associated substrate) family including BCAR1/p130Cas and CASS4/HEPL, which have been implicated as signaling mediators of diverse biological processes including adhesion, motility, cell cycle, mitosis, apoptosis, anoikis, and tumorigenesis [5-7]. We previously reported that NEDD9 is a crucial player in regulating tumor aggressiveness and invasion, which are triggered by the treatment with TGF- β , in some prostate cancer cell lines [8].

On the other hand, zoledronic acid (ZA), a major nitrogen-containing bisphosphonate, has been broadly used for the treatment of PCa bone metastases owing to potent inhibition of osteoclast activity and survival [9,10]. ZA also has been reported to have the potential of cytotoxic and cytostatic effects on prostate cancer cells [11-13], while those in depth molecular mechanisms remain unclear. In this study, we examined whether ZA can regulate the EMT process and tumor

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invasion by the control of NEDD9 as a target molecule in prostate cancer cells.

Materials and Methods

Cell culture and reagents

A human prostate cancer cell line, PC-3, was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). PC-3 cells were maintained in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/mL of penicillin and 100 µg/mL of streptomycin (Gibco, New York, NY, USA) at 37°C in a humidified atmosphere containing 5% CO₂. TGF-β was purchased from Cell Signaling Technology (Beverly, MA, USA) and dissolved in citric acid (pH 3.0). The concentration of stock solution was 50 µg/mL and the final concentration was 10 ng/mL for all the experiments. Zoledronic acid monohydrate was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in sterile milli-Q water. The concentration of stock solution was 250 mM. MG132 was also obtained from Sigma-Aldrich and dissolved in DMSO. The final concentration of stock solution was 2.5 mM and the final concentration was 0.5 µM for all the experiments.

Antibodies

Mouse monoclonal anti-NEDD9(ab18056), anti-β-actin(ab8226), and rabbit polyclonal anti-Snail+Slug (ab63371) antibodies were obtained from Abcam (Cambridge, UK). Rabbit polyclonal anti-vimentin (#5741), anti-E-cadherin (#3195) antibodies were from Cell Signaling Technology (Beverly, MA, USA). Mouse monoclonal anti-polyubiquitin antibody was purchased from MBL life science (Woburn, MA, USA).

Western blotting

Cells were harvested and whole-cell lysates were prepared using PRO-PREP protein extraction solution (iNtRON Biotechnology, Gyeonggi-do, Korea) in accordance with the manufacturer's instructions. Protein concentrations of samples were determined by the bicinchoninic acid protein assay (BioRad, Hercules, CA, USA). Protein samples (10 µg of each protein) were treated at 55°C for 20 min in 2% SDS-treating solution containing 5% β-mercaptoethanol and separated in 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with Tris-buffered saline (TBS) containing 0.05% Tween 20 and 5% non-fat dried milk and probed overnight at 4°C with primary antibodies under manufacturer-recommended conditions. Immunoblots were washed with TBS containing 0.05% Tween 20 and 1% non-fat milk and incubated with secondary antibodies conjugated with horseradish peroxidase against mouse IgG or rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. Immunoreactivity proteins were visualized using the ECL detection system (Pierce, Rockford, IL, USA). Each western blot analysis was performed in triplicate.

Transwell invasion assay

In vitro cell invasion assay was conducted using Matrigel invasion chambers (8 µm pore size; BD Biosciences Pharmingen, San Diego, CA, USA). Cells were allowed to grow to subconfluency (~75-80%) and serum-starved for 24 h. After detachment with trypsin, cells were washed with PBS, resuspended in serum-free medium and 500 µl cell suspension (4 × 10⁴ cells/mL) was added to the upper chamber. Complete medium was added to the bottom wells of the chambers. For the screening, after 24 h the cells that had not infiltrated were removed

from the upper face of the filters using cotton swabs, and the cells that had infiltrated were fixed with 5% glutaraldehyde solution and stained with Giemsa stain solution. Images of four different ×100 fields were captured from each membrane and the number of invading cells was counted.

Immunoprecipitation

The immunoprecipitation (IP) method was performed in accordance with the manufacturer's protocol of ImmunoCruz™ Immunoprecipitation / Western Blots (Santa Cruz Biotechnology, Santa Cruz, CA, USA). A total cell lysate was prepared in 1 ml of RIPA buffer with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Next, to preclean whole cell lysate, 50 µl of the preclean matrix (sc45056) obtained from Santa Cruz Biotechnology was added to 1 ml of whole cell lysate (25% v/v) in a 1.5 mL microcentrifuge tube, followed by incubation for 30 min at 4°C while rotating. After microcentrifugation at maximum speed for 30 s at 4°C, a desired supernatant of IP matrix was transferred to a new microcentrifuge tube. For the formation of IP antibody to IP matrix complex, 50 µl of suspended IP matrix, 5 µg of anti-NEDD9 antibody described above and 500 µl of PBS were added in a microcentrifuge tube. Then, the tube containing a mixture was incubated overnight at 4°C on a rotator. After incubation of the anti-NEDD9 antibody with the specific IP matrix, pellet matrix was formed via microcentrifugation at maximum speed for 30 s at 4°C. The supernatant was carefully aspirated and discarded. Next, the pelleted matrix was washed 2 times with 500 µl of PBS, each time the fore-mentioned centrifugation and aspiration were repeated. After the final wash of the anti-NEDD9 antibody with the specific IP matrix, 1 mg of the total cellular lysate was transferred to the pelleted matrix and incubated overnight at 4°C on a rotator. After incubation of the matrix and lysate, the pellet was kept through microcentrifugation at maximum speed for 30 s at 4°C. The supernatant was aspirated carefully and discarded. The pellet matrix was washed 4 times with RIPA buffer, each time the same centrifugation and aspiration were repeated. After final wash, the supernatant was carefully aspirated and discarded. The immunoprecipitate was resuspended in 40 µl of the sample buffer (sc-24945), followed by the boiling for 3 min. 5 µl of solubilized samples were loaded onto 15% SDS-polyacrylamide gels. Then, Western blotting was performed in accordance with the manufacturer's protocol of ImmunoCruz™ Immunoprecipitation / Western Blots.

Statistical analysis

Statistical analysis was performed by Ekuseru-Toukei' 2012 (Social Survey Research Information Co., Ltd., Tokyo, Japan). Data were expressed as mean ± S. D. Student's *t*-test was used to calculate the statistical significance of the experimental results of invasion assays.

Results

ZA suppresses the expressions of NEDD9 and EMT-associated biomarkers in PC-3 prostate cancer cells

As shown in Figure 1, the expression levels of NEDD9 and mesenchymal markers such as vimentin and Snail-Slug were down-regulated by the exposure to ZA in a dose-dependent manner in PC-3 prostate cancer cells. On the contrary, the expression of E-cadherin, an epithelial marker, markedly increased in the same condition. The changes in expressions of these proteins were obviously detected under the condition with more than 50 µM ZA. However, the cytotoxicity of ZA was severe under the exposure to 100 µM ZA as shown in the lower

panel of Figure 1 (i.e., small amount of β actin). Thus, we selected the condition of 50 μ M ZA in the followed experiments.

ZA strongly inhibits the TGF- β -triggered EMT process in PC-3 cells

In PC-3 prostate cancer cells, the addition with ZA (50 μ M) clearly reversed the TGF- β -induced EMT (namely mesenchymal-to-epithelial transition; MET), concomitant with the suppression of both the increase in the expression of vimentin and the decrease in that of E-cadherin, respectively (Figure 2A).

ZA markedly suppresses the TGF- β -induced tumor-cell invasion

Under the same condition as shown in Figure 2B, the treatment with ZA significantly inhibited PC-3 cell invasion compared with the control cells (Figure 2B; lane 1 vs. 2). Furthermore, ZA completely repressed cell invasion enhanced by the TGF- β treatment, as showing that there is no significant difference between lane 2 and 4 in Figure 2B.

Proteasome inhibitor overcomes ZA-induced down-regulation of NEDD9 expression

The previous report evaluated that NEDD9 is ubiquitinated by AIP4, a specific E3 ligase, and subsequently degraded by 26S proteasome [14]. As shown in Figure 3, a proteasome inhibitor MG132 clearly blocked the reduction of NEDD9 expression caused by the treatment with ZA. Based on this result, we speculated the possibility of ZA effect on the ubiquitination-proteasome system (UPS) targeted NEDD9 expression.

ZA induces the ubiquitination of NEDD9

In the immunoprecipitation assay using anti-NEDD9 antibody, the expression of poly-ubiquitinated NEDD9 was detected in PC-3 cells treated with ZA (Figure 4; lane 1 and 2). Moreover, addition with ZA markedly enhanced the poly-ubiquitination against NEDD9 under the block of degradation of NEDD9 using MG132 (Figure 4; lane 3 and 4).

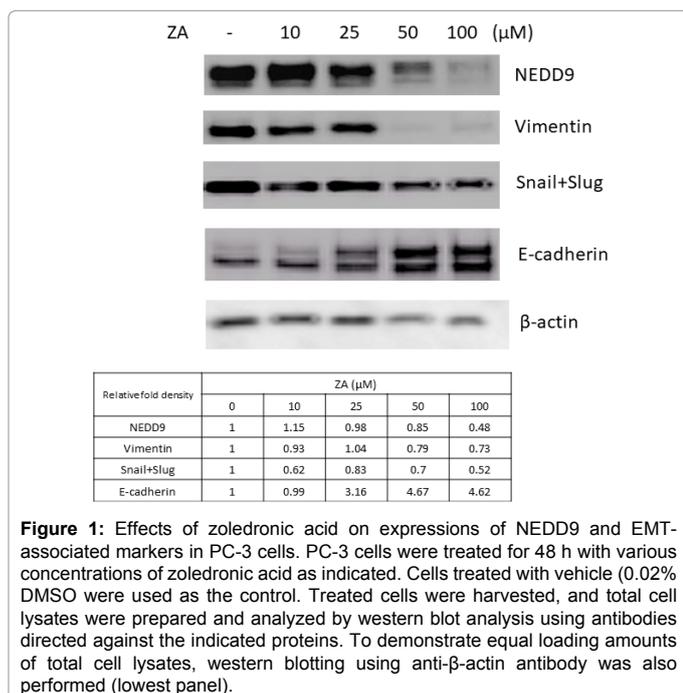


Figure 1: Effects of zoledronic acid on expressions of NEDD9 and EMT-associated markers in PC-3 cells. PC-3 cells were treated for 48 h with various concentrations of zoledronic acid as indicated. Cells treated with vehicle (0.02% DMSO) were used as the control. Treated cells were harvested, and total cell lysates were prepared and analyzed by western blot analysis using antibodies directed against the indicated proteins. To demonstrate equal loading amounts of total cell lysates, western blotting using anti- β -actin antibody was also performed (lowest panel).

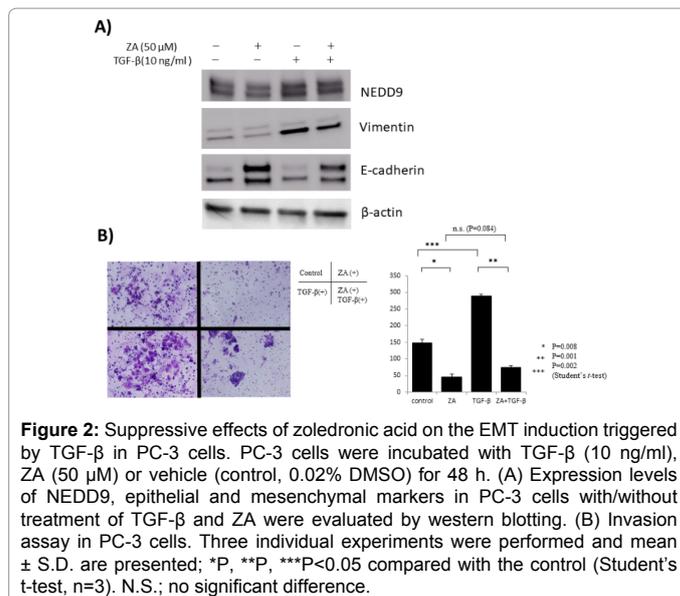


Figure 2: Suppressive effects of zoledronic acid on the EMT induction triggered by TGF- β in PC-3 cells. PC-3 cells were incubated with TGF- β (10 ng/ml), ZA (50 μ M) or vehicle (control, 0.02% DMSO) for 48 h. (A) Expression levels of NEDD9, epithelial and mesenchymal markers in PC-3 cells with/without treatment of TGF- β and ZA were evaluated by western blotting. (B) Invasion assay in PC-3 cells. Three individual experiments were performed and mean \pm S.D. are presented; *P, **P, ***P<0.05 compared with the control (Student's t-test, n=3). N.S.; no significant difference.

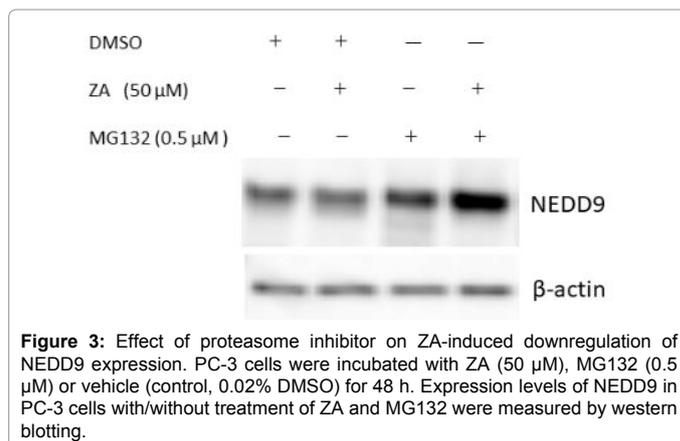


Figure 3: Effect of proteasome inhibitor on ZA-induced downregulation of NEDD9 expression. PC-3 cells were incubated with ZA (50 μ M), MG132 (0.5 μ M) or vehicle (control, 0.02% DMSO) for 48 h. Expression levels of NEDD9 in PC-3 cells with/without treatment of ZA and MG132 were measured by western blotting.

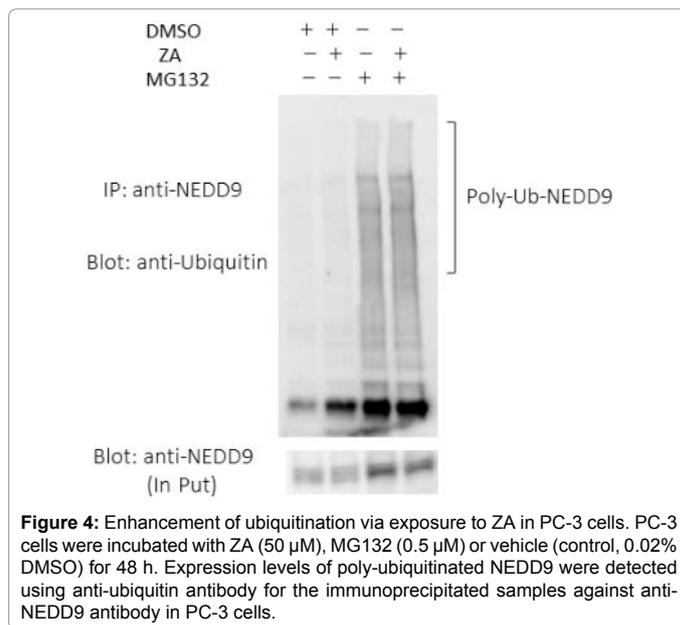


Figure 4: Enhancement of ubiquitination via exposure to ZA in PC-3 cells. PC-3 cells were incubated with ZA (50 μ M), MG132 (0.5 μ M) or vehicle (control, 0.02% DMSO) for 48 h. Expression levels of poly-ubiquitinated NEDD9 were detected using anti-ubiquitin antibody for the immunoprecipitated samples against anti-NEDD9 antibody in PC-3 cells.

Discussion and Conclusion

Zoledronic acid (ZA), a third-generation bisphosphonate agent, has been shown to be a compound to treat prostate cancer bone metastasis and reduce skeletal-related events (SREs) in the clinical settings. Based on several basic researches, the bone-modifying agents (BMAs) including some bisphosphonates and a RANK-L (receptor activator of nuclear factor- κ B-ligand) inhibitor are thought to be a potent option for not only inhibiting the activation of osteoclasts but also preventing the tumor burden through the disturbance in the mitogen-based crosstalk among tumor, stromal and immune cells in the microenvironment of distant metastases [11,15]. In particular, our group reported that the nitrogen-containing bisphosphonates such as pamidronate and zoledronic acid exerted significant inhibition of bone cell-induced androgen receptor transcriptional activity in LNCaP prostate cancer cells [11]. On the other hand, the previous reports demonstrated the biological effects of ZA to reduce the EMT axis in some malignancies such as breast and prostate cancers [16,17].

NEDD9 has been reported to be a key molecule that can regulate tumor aggressiveness, such as EMT, invasion, resistance to chemodrugs and dedifferentiation to stem cell via some down-stream cascades in cancer cells [5,7,8,18]. Regarding the turnover of NEDD9 expression, it was previously reported that NEDD9 is initially phosphorylated (targeted residue; serine 369) by a kinase sensitive to Hesperadin, followed by the 26S proteasomal degradation of ubiquitinated NEDD9 catalyzed by the specific ubiquitin E3 ligase, atrophin-1-interacting protein 4 [14,19].

In this study, we focused on the biological action of ZA against the EMT and tumor invasion by regulating the NEDD9 expression in PC-3 prostate cancer cells. First, we initially elucidated that the treatment with ZA induced the reduction in the expressions of NEDD9 and its downstream EMT molecules (e.g., Snail/Slug). Simultaneously, ZA clearly inhibited tumor cell invasion triggered by TGF- β , a strong effector for EMT, in a boyden chamber assay. Additionally, we speculated the possibility that ZA affects the ubiquitin-proteasome system (UPS) pathway specific for regulating NEDD9 protein. As shown in Figure 3 and 4, we confirmed that the expression of NEDD9 is reduced by the treatment with ZA across the enhanced poly-ubiquitination and the subsequent degradation via 26S proteasome in the PC-3 cells. To our knowledge, it is a first report to show the negative regulation of NEDD9 expression induced by the exposure to ZA.

Some clinical evidences that BMAs, such ZA and a RANKL inhibitor (i.e., denosumab), have strong effects on prolonged overall survival of CRPC patients with bone metastases and the period without the skeletal-related events (SREs) via marked inhibition of activity or formation of osteoclasts [9,20]. BMAs also have been reported to have anti-tumor effects on prostate cancer cells directly and indirectly in the basic experiments [11,12,15], while there are few clinical evidence that the administration of these agents can exert the significant suppression of tumor burden in PCa patients. Furthermore, concerning the possibility of preventing bone metastasis of PCa via the administration of BMA, there are no beneficial data except for the significant delay in onset of bone metastases when denosumab was compared with placebo in CRPC patients without bone metastasis [21,22]. However, the conclusion cannot be drawn because those clinical trials have severe heterogeneity about the enrolled patients' background and the treatment schedules of some bisphosphonates and denosumab. From the results of this study, ZA is thought to have a high potential of inhibiting PCa cell invasion and metastasis, in concomitant with suppression of NEDD9 expression via the UPS-mediated degradation.

However, there is a limitation of no data regarding the effect of ZA on the cells with knockdown of NEDD9 expression. Our group previously demonstrated that knockdown of NEDD9 expression robustly induced the suppression of cell invasion [8]. Regrettably, we could not detect the biological effects on the cells under such a severe condition. In a future trial, the protective effect of ZA on bone metastasis may be clarified in high-risk M0 PCa patients, who are monitored by a modern imaging apparatus (e.g., 68Ga-PSMA/PET-CT, Whole body diffusion weighted MRI) with greater sensitivity to detect oligo bone metastases.

Competing Interests

The authors declare they have no conflict of interest.

Authors' Contributions

TT and KM conceived and designed the study. TT and KM performed the experiments. TT drafted the manuscript. TT, KM and TN assisted in performing data interpretation. TT and TN reviewed the manuscript for submission. All authors approved the final draft of the manuscript.

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