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Original Article

# Inhibition of the RANK/RANKL signaling with osteoprotegerin prevents castration-induced acceleration of bone metastasis in castration-insensitive prostate cancer

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ABSTRACT

Androgen deprivation therapy (ADT) for patients with metastatic or locally advanced prostate cancer reduces bone mineral density by stimulating receptor activator of nuclear factor kappa-B (RANK) signaling in osteoclasts. The involvement of the RANK/RANKL signaling in ADT-induced acceleration of bone metastasis in castration-insensitive prostate cancer was examined in a murine model using osteoprotegerin (OPG). Male Balb/c nude mice were divided into three groups: the non-castration, castration, and castration + OPG groups. PC-3M-luc-C6 was injected into the left ventricle of the mice. Recombinant OPG was injected intravenously twice weekly in the castration + OPG group. *In-vivo* imaging system (IVIS®) determined that the prevalence and photon counts of bone metastasis in the castration group were significantly higher than that in the non-castration and castration + OPG groups. The mean number of RANKL-positive osteoblasts and the mean serum RANKL level in the castration group were significantly higher than those in the non-castration group. RANKL-enhanced activation of osteoclasts was attenuated in the castration + OPG group. These results suggest that the mechanisms of RANK/RANKL signaling are involved in the ADT-induced acceleration of bone metastasis in castration-insensitive prostate cancer.



## Introduction

Androgen deprivation therapy (ADT), via luteinizing hormone-releasing hormone agonists/antagonists or castration, is the gold-standard therapy for patients with metastatic or locally advanced prostate cancer [1]. ADT is initially effective in approximately 70%–80% of patients with hormone naïve prostate cancer [2]. However, virtually all patients with hormone-sensitive prostate cancer (HSPC) receiving ADT eventually develop castration-resistant prostate cancer (CRPC) [1]. In addition, ADT has been demonstrated to have several adverse effects, including the reduction of bone mineral density (BMD) [3]. Reportedly, around 45% of patients with prostate cancer receiving ADT develop osteoporosis [4].

ADT reportedly increased the levels of receptor activator of nuclear factor kappa-B ligand (RANKL) in rat serum and bone marrow [5], which caused a reduction in BMD due to osteoclast activation [6]. Conversely, osteoclast inhibition by recombinant osteoprotegerin (OPG), a decoy receptor for RANKL, was shown to prevent the establishment of bone metastases in a murine model of prostate

*Abbreviations:* ADT, androgen deprivation therapy; ANOVA, analysis of variance; AR, androgen receptor; BMAs, bone-modifying agents; BMD, bone mineral density; CRPC, castration-resistant prostate cancer; CT, computed tomography; ELISA, enzyme-linked immunosorbent assay; HSPC, hormone-sensitive prostate cancer; OPG, osteoprotegerin; RANK, receptor activator of nuclear factor kappa-B; RANKL, receptor activator of nuclear factor kappa-B ligand; TRAP, tartrate-resistant acid phosphatase.

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cancer [7]. Recently, bone-modifying agents (BMAs)—such as the bisphosphonate zoledronic acid (ZOL) and the fully human RANKL monoclonal antibody denosumab, which inhibits osteoclastogenesis and bone resorption—have been used to treat patients with prostate cancer bone metastases [8,9]. Smith et al. reported that denosumab was associated with a reduced incidence of new vertebral fractures in men receiving ADT for non-metastatic HSPC [10]. Moreover, they reported that denosumab significantly increased bone metastasis-free survival in men with non-metastatic CRPC [11]. In a murine model, Ottewell et al. recently showed that ADT triggered the growth of disseminated PC3 cells to form bone metastases and its prevention by ZOL [12]. However, the involvement of the receptor activator of nuclear factor kappa-B (RANK)/RANKL signaling in the ADT-induced acceleration of bone metastasis was not examined.

In this study, we examined the ADT-induced acceleration of bone metastases due to osteoclast activation and BMD reduction in a murine bone metastasis model of human castration-insensitive prostate cancer cell PC3. Furthermore, we investigated whether OPG instead of denosumab attenuates the ADT-induced acceleration of bone metastasis by inhibiting the RANK/RANKL signaling.

## Materials and methods

### *Animals*

All procedures involving animal experimentation described in this study were approved by the Institutional Review Board and Animal Research Committee of the Akita University School of Medicine. All subsequent animal experiments adhered to the "Regulation for Animal Experimentation" of the University which is in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. Male retired breeder (aged over 24 weeks) Balb/c nu/nu mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and randomized into three groups: the non-castration, castration, and castration + OPG groups. One mouse was placed in each cage, and the food was available *ad libitum*. All mice were euthanized under anesthesia using isoflurane.

### *Measurement of bone mineral density*

The bones of the mice were scanned, and BMD was measured using micro-computed tomography (CT; R.mCT2; Rigaku Co., Tokyo, Japan, and LCT-200; Hitachi-Aloka Medical Ltd., Tokyo, Japan, respectively). The mean total and cancellous BMD were calculated in 20 slices (240 μm/slice) to the proximal edge of the tibia perpendicular to the long axis using software installed on the micro-CT system (LCT-200; Hitachi-Aloka Medical Ltd.).

### *Cell culture*

The bioluminescent human prostate cancer cell line PC-3M-luc-C6 Bioware® (#119270; PerkinElmer, Inc., Waltham, MA, USA), which constitutively expresses luciferase and was authenticated by short tandem repeat analysis, was purchased on March 2011 and cultured for xenografting. The generation of this cell line was reported previously [13]. The cell line was maintained in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum at 37 °C in a 5% $CO_2$-humidified incubator. PC-3M-luc-C6 cells were used in *in-vivo* murine experiments within six passages of purchase.

### *Left-ventricular injection*

Anesthetized animals were injected percutaneously with $3 \times 10^6$ PC-3M-luc-C6 cells suspended in 100 μL sterile Dulbecco's phosphate-buffered saline (DPBS) into the left ventricle of the heart. The left-ventricular injection was performed 10 min after an intraperitoneal injection of 150 mg/kg D-luciferin (#1043; Promega Corp., Madison, WI, USA) in DPBS. The method of left-ventricular injection was previously described [13–15]. Briefly, a 27-gauge needle with a 1-mL tuberculin syringe was inserted into the second intercostal space, approximately 3 mm to the left of the sternum [13]. The mice were placed in an *in-vivo* imaging system (IVIS® Lumina LT; Xenogen/STTARR, Toronto, ON, Canada) and imaged ventrally under anesthesia. The successful left-ventricular injection was indicated by systemic bioluminescence distributed throughout the animal. Mice with evidence of a successful injection were enrolled into the experiment. Mice showing bilateral lung or paracardiac bioluminescence were excluded from the experiment.

### *Bioluminescent imaging*

*In-vivo* bioluminescent imaging was performed using the IVIS® imaging system as previously described [13,15]. Briefly, 10 min prior to imaging, animals received an intraperitoneal injection of the substrate D-luciferin. Animals were anesthetized using 2% isoflurane during imaging and placed on a stage inside the camera box. Bioluminescent signals were detected for 3 min by the IVIS® imaging system and electronically displayed using Living Image® software installed on the IVIS® imaging system. Regions of interest were selected around the tumor sites and quantified in photon counts/second.

### *Castration and tail-vein injection*

Bilateral orchiectomy (surgical castration) was performed on day −2 relative to the left-ventricular injection of tumor cells as previously described [16]. Mice in the castration + OPG group received a tail-vein injection of OPG without anesthesia twice weekly from day −1 relative to the left-ventricular injection. Mice were injected with 2 mg/kg recombinant mouse OPG/TNFRSF11B Fc chimera (#459-MO; R&D Systems, Inc., Minneapolis, MN, USA) in 200 μL sterile DPBS using a 26-gauge needle with a 1 mL tuberculin syringe into the tail vein.

### *Immunohistochemistry*

The lower limbs, including the femur, patella, and tibia, were obtained from mice at euthanasia. The specimens were fixed in 10% formalin and decalcified using 12% ethylenediaminetetraacetic acid for 72 h. The specimens were then paraffin-embedded and cut into sections 5-μm thick perpendicular to the long axis of the femur at the level of 2 mm proximal to the distal edge. The sections were stained using a primary antibody against tartrate-resistant acid phosphatase (TRAP; #sc-30833; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), which is a marker for osteoclasts, diluted 25 times and a rabbit polyclonal antibody against RANKL (#ab9957; Abcam, Cambridge, UK), which is a marker for osteoblasts, diluted 50 times overnight at 4 °C, and secondary anti-goat and -rabbit antibodies (#414341 and #414351; Nichirei Biosciences Inc., Tokyo, Japan), followed by counterstaining with hematoxylin. Osteoclasts were identified as TRAP-positive multinuclear (>three nuclei) cells on the surface of the trabecular bone matrix and counted using light microscopy on the inner edge of the cortical bone. RANKL-positive osteoblasts were identified as RANKL-positive mononuclear cells on the surface of the trabecular bone matrix and counted.

### *Measurement of serum levels*

Blood samples were collected from the left ventricle using a 24-gauge needle with a 1-mL tuberculin syringe under anesthesia at euthanasia. The collected blood was centrifuged at 3000 revolutions per minute for 10 min, and serum was stored at −80 °C until analysis. Levels of the serum testosterone, RANKL, and TRAP5b, which is expressed in high amounts by bone-resorbing osteoclasts [17], were measured using a testosterone enzyme-linked immunosorbent assay (ELISA) kit (#ab108666; Abcam), a mouse TRANCE/RANKL/TNFSF11 Quantikine ELISA Kit (#MTR00; R&D Systems, Inc.), and a mouse TRAP5b ELISA assay kit (#DS-SBTR103; Immunodiagnostic Systems, Boldon, UK), respectively."

### *Statistical analysis*

The chi-squared test was used to compare the prevalence of bone metastases. The Mann–Whitney *U* test, Student's *t*-test, and a one-way repeated-measures analysis of variance (ANOVA) covariance model were used to determine between-group differences. All statistical analyses were performed using SPSS version 22.0 (IBM Corp., Armonk, NY, USA), and p values < 0.05 were considered statistically significant.

## Results

### *Creating a murine bone metastasis model of CRPC*

To create the murine model of CRPC, anesthetized 6-week-old Balb/c nu/nu mice were injected percutaneously with $3 \times 10^6$ PC-3M-luc-C6 cells suspended in 100 μL sterile DPBS into the left ventricle 2 days after castration. The successful left-ventricular injection was confirmed by the systemic distribution of bioluminescence throughout the body of each mouse (Fig. 1A). IVIS® imaging, using intraperitoneal D-luciferin injection, was performed to detect metastases 3 weeks after ventricular injection (Fig. 1B). To distinguish bone metastases from IVIS®-positive metastases in other organs, three-dimensional bone imaging was performed using micro-CT (R.mCT2; Rigaku Co.), and osteolytic bone metastatic lesions were detected in IVIS®-positive areas (Fig. 1C). Bone metastatic lesions were excised at euthanasia and stained with hematoxylin and eosin for histologic evaluation. The histologic analysis