Pathol. Oncol. Res. (2014) 20:747–754
DOI 10.1007/s12253-014-9756-z

RESEARCH

Characterization of the Attenuation of Breast Cancer Bone Metastasis in Mice by Zoledronic Acid Using ^{99m}Tc bone Scintigraphy

Chunyu Wu · Shunfang Yang · Zhenping Sun · Xianghui Han · Yiyi Ye · Sheng Liu

Received: 30 May 2013 / Accepted: 25 February 2014 / Published online: 8 March 2014

Abstract Metastatic breast cancer often metastasizes to bone. The purposes of the study were (1) to evaluate the use of ^{99m}Tc-MDP bone scintigraphy for detection of metastatic bone lesions, and (2) to determine the efficacy of zoledronic acid in mice with breast cancer bone metastasis. All tumor-bearing mice were analyzed with radionuclide bone scintigraphy, X-ray, and histological analysis. The metastatic bone tissue was also harvested and analyzed by western blotting and real-time qPCR. Interestingly, zoledronic acid significantly decreased both the tumor burden and the incidence of bone metastasis in mice. In addition, histomorphometric, stereological, and molecular biology analyses demonstrated that zoledronic acid may function to inhibit breast cancer cell growth in the bone microenvironment and regulate the function of osteoblasts and osteoclasts in tumor-bearing mice. Finally, the attenuation of breast cancer bone metastasis using zoledronic acid can be accurately characterized by ^{99m}Tc bone scintigraphy in mice.

C. Wu · Z. Sun · S. Liu
Department of Breast Surgery, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China

S. Yang
Department of Nuclear Medicine, Shanghai Chest Hospital, Shanghai JiaoTong University, Shanghai 200030, China

X. Han · Y. Ye · S. Liu
Pharmacology Laboratory of Traditional Chinese Medicine, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, China

S. Liu (✉)
Department of Breast Surgery and Pharmacology Laboratory of Traditional Chinese Medicine, Long Hua Hospital, Shanghai University of Traditional Chinese Medicine, 725 South Wanping Road, Shanghai 200032, China
e-mail: sliu_tcm@163.com

Keywords Bone metastasis · Breast cancer · ^{99m}Tc-MDP bone scintigraphy · Zoleronic acid

Introduction

The most common cancer in women is breast cancer and unfortunately, this type of cancer frequently metastasizes to bone. Chen et al. reported that there were more than 280,000 breast cancer cases in China in 2007 [1]. In China alone, the number of breast cancer cases in women 55–69 years old is estimated to grow to 2.5 million cases by the year 2021 [2]. Breast cancer continues to be the predominant cause of death in women in Eastern and Southeastern Asian [1]. Metastatic breast cancer often metastasizes to bone. One report suggested that ~80 % of all advanced breast cancer patients developed bone metastases [3]. The clinical outcome of these secondary tumors is destruction of the bone, bone pain, fractures, hypercalcemia, nerve compression syndrome, declines in mobility and performance, which results in a severely reduce the quality of life [3–5].

Zoledronic acid, a bisphosphonate, is used in the treatment of bone metastases and osteoporosis and helps reduce the skeletal complications. Zoledronic acid inhibits cellular proliferation, induces apoptosis in cultured cancer cells, and importantly interferes with adhesion of metastatic cells to the bone matrix. This results in the inhibition of cell migration and invasion as well as preventing angiogenesis [6–11]. Yoneda et al. have discovered that bisphosphonates can inhibit bone metastases by MDA-231 breast cancer through the promotion of not only apoptosis in osteoclasts but also apoptosis of the MDA-231 cells [12]. MDA-231 has the ability to develop metastases in bone, brain, ovary, and adrenal glands. However, a derivative cell line, MDA-231BO, almost exclusively metastasizes to bone. MDA-231BO cells are separated from MDA-231 cells by culturing the metastatic bone cells in

sequential passages in nude mice and by culturing metastatic cells obtained from bone metastases in vitro [13]. MDA-231BO cells produce more parathyroid hormone-related protein than MDA-231. Furthermore, the sensitivity of MDA-231BO and its parental MDA-231 cells to stimulation of TGF-β and insulin-like growth factor I is quite different. Currently, no studies have investigated the direct effects of zoledronic acid on exclusively bone metastasis induced by MDA-231BO cells in mice.

The use of X-rays for the detection of bone metastases in clinical is common, however, it has limitations in detecting and diagnosing early stage bone metastases in small animals. In fact, El-Abdaimi et al. demonstrated that in certain cases X-rays did not detected bone metastasis but the metastases were discovered through histological studies [14, 15]. Bone scintigraphy is an established, widely available, and noninvasive imaging modality for the detection of bone metastases. Additionally, it provides visualization of the entire skeletal [16]. Bone scintigraphy has been estimated to detect malignant bone lesions several months earlier than X-rays [17]. ^{99m}Tc-MDP bone scintigraphy is a common method used to detect bone metastasis in patients. We previous established using ^{99m}Tc-MDP bone scintigraphy in mice that lung adenocarcinoma bone metastases can be detected and therefore, bone scintigraphy is more advantageous than X-rays analysis in the detection of early stage bone metastases [18]. Although radionuclide scintigraphy is widely used to detect human bone metastasis, the diagnosis and detection of bone metastasis using scintigraphy in small animal has not been fully established. In this study, ^{99m}Tc-MDP bone scintigraphy was used to detect human breast cancer bone metastatic sites in mice and to evaluate the efficacy of zoledronic acid in treating the bone metastasis.

Materials and Methods

Materials

Zoledronic acid (purity >99 %) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products, China. L-15 medium and fetal bovine serum were obtained from Gibco (Grand Island, NY). Antibodies against osteoprotegerin (OPG), macrophage colony stimulating factor (M-CSF), receptor activator of nuclear factor kappa-B ligand (RANKL) and parathyroid hormone related peptide (PTHrP) were obtained from Santa Cruz Biotechnology Biotechnology (Santa Cruz, CA). An antibody against interleukin-8 (IL-8) was provided by Abcam Technology (Abcam, MA). The other chemicals were purchased from Sigma-Aldrich (Saint Louis, Missouri) unless otherwise indicated.

Animals

Female BALB/c *nu/nu* mice (20±2.0 g; Shanghai Cancer Institute of Shanghai Jiaotong University; license number 2008–0043 SYXK) were housed in a temperature-controlled (24±2 °C) room with a regular 12-h light/dark cycle. After acclimatization for 1 week, 14 animals were randomly assigned to two experimental groups. All experiments were performed in accordance with the national regulations for animal experimentation approved by the Shanghai Laboratory Animal Science Administration Commission of Shanghai Municipality.

Cell Lines and Cell Culture

MDA-231BO cells, a kindly gift from Dr. Toshiyuki Yoneda, Osaka University, were cultured in L-15 medium supplemented with 10 % (v/v) fetal bovine serum at 37 °C in a 5 % CO_2 humidified environment. Cells were washed several times and placed in sterile PBS shortly before implantation. Though repeatedly injecting cancer cells into the left ventricle and isolating tumor cells from bone metastasis lesions, we finally acquired an exclusive bone metastatic subclone.

Intracardiac Injection

Intracardiac injection was operated described previously with little modification [18]. Briefly, cell were resuspended at 10^6/ml concentrated in PBS. The suspended cells (0.1 ml) were injected into the mice left cardiac ventricle using 29G needles (Terumo, Tokyo, Japan).

Radionuclide Bone Scintigraphy Analysis

Bone scintigraphy analysis was performed according to our previous work [18].

Radiographic Imaging

Conventional radiographs were obtained with a Philips Optimus Bucky Diagnost TS (Philips Healthcare, Eindhoven, Netherlands) X-ray System. Bone metastases were determined on radiographs 2–8 weeks after the inoculation of tumor cells. The X-ray tube voltage was fixed at 40 kVp, the current at 2 mA and the exposure time at 3 s.

Histopathology

Bone metastatic lesions observed by radio-nuclide scintigraphy and radiography were cut and sent for HE stain. Histopathology analysis was performed according to our previous work [19]. Briefly, cancer cells were identified and the percentage of cancer cells per high-power field-of-view (400×

magnification) was calculated. Two pathologists independently observed all histological sections. Sections that the two pathologists generated substantially different results for (>5 % discrepancy) were reviewed again until agreements were reached.

Real-Time qPCR

Metastatic bone lesions were harvested and stored in liquid N_2. Frozen tissue (0.2–0.25 g) was homogenized with a pestle and total RNA was extracted using TRIZOL (Promega, Madison, WI) according to themanufacturer's instructions. Real-time PCR was performed according to previous work [20]. Primers were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China) and their sequences were: 5′-GGTCGGAGTCAACG GATTTG-3′ (sense) and 5′-ATGAGCCCCAGCCTTCTCCA T-3′ (anti-sense) for GAPDH; 5′ACA TGA CTT CCA AGC TGG CCG T3′(sense) and 5′CCT CTT CAA AAA CTT CTC CAC AAC3′(anti-sense) for IL-8; 5′ AGC AGG AGT ATC ACC GAG GA 3′(sense) and 5′ TAT CTC TGA AGC GCA TGG TG 3′(anti-sense) for M-CSF; 5′ ATG CAG CGG AGA CTG GTT CAG 3′ (sense) and 5′TTC TAG TGC CAC TGC CCA TTG 3′(anti-sense) for PTH-rP; 5′CTT CGT GCC TTG ATG GA 3′ (sense) and 5′TTG GGA AAG TGG GAT GT3′ (anti-sense) for OPG; 5′ACC AAG ATG GCT TCT ATT ACC 3′ (sense) and 5′ TCC CTC CTT TCA TCA GGT TAT 3′ (anti-sense) for RANKL. The cycling conditions included an initial 3-min polymerase activation at 94 °C followed by 40 cycles at 94 °C for 30 s, with annealing temperature of 52.1 °C, 52.1 °C, 58.4 °C, 43.8 °C, and 43.8 °C for OPG, RANKL, IL-8, M-CSF, and PTH-rP respectively for 40 s and 72 °C for 30 s. Relative quantities of expression of the genes of interest in different samples were calculated by the comparative Ct (threshold cycle) value method using GAPDH as the reference gene [21].

Western Blotting

Protein extraction and western blotting were performed according to previous work with little modification [20]. Briefly, equal amounts of proteins (20 μg) were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blocked with 2 % BSA and then incubated with appropriate primary antibodies overnight at 4 °C. β-Actin was used as a loading control. Band intensities were measured using imageanalysis software (NIH Image).

Statistical Analysis

All results are presented as mean ± standard deviation. Two-tailed analysis of variance (ANOVA) and Fisher's exact tests were used to determine the statistical significance. A p value of <0.05 was considered significant for all tests.

Results

Radionuclide Bone Scintigraphy and X-ray Imaging

To evaluate the use of scintigraphy for metastatic bone lesions in small animals, tumor-bearing mice were subjected to both radionuclide bone scintigraphy and X-ray analysis for the identification of osseous metastases. Although not statistically significant ($p=0.192$, Fisher test), whole-body bone scintigraphy, which was confirmed by histomorphometry is more sensitive and accurate than X-ray analysis at 8 weeks post tumor inoculation (Fig. 1a–d). The positive index of bone metastasis (PI), the total number of bone metastasis in mice expressed as a percentage of the number of mice was used to classify the levels of bone metastases. Accordingly, the metastatic colonies identified by bone scintigraphy and X-ray analyses in seven mice have a PI score of 171.4 % and 71.4 %, respectively (Fig. 1e). In addition, the pinhole bone scintigraphy analysis is more sensitive than whole-body bone scintigraphy and X-ray analysis (Fig. 1a–c).

Zoledronic Acid Decreased Bone Metastasis

Bone scintigraphy imaging with confirmation using histomorphometry showed bone metastasis in 2/7 mice in the zoledronic acid treatment group (28.6 %), and 7/7 mice (100 %) in the vehicle group (Fig. 2b–e). On the other hand, X-ray analysis (not statistically significant) showed osseous metastasis in 1/7 mice in the zoledronic acid treatment group (14.3 %), and 4/7 mice (57.1 %) in the vehicle group (Fig. 2a/e). The PI of bone scintigraphy results showed osseous metastasis colonies in 2/7 mice in zoledronic acid group (28.6 %), and 12/7 mice (171.4 %) in the vehicle group (Fig. 2f) with similar results also confirmed by X-ray analysis (Fig. 2f).

Histopathology

The breast cancer metastatic lesions extensively infiltrated the bone marrow cavities of the vehicle treated. Multiple local tumor growths of densely clustered tumor cells were seen in the marrow cavities in these mice. The tumor cells have enlarged nucleoli and numerous mitotic features (Fig 3a.*a*). In the mice treated with zoledronic acid, there was a reduction in the tumor burden and the bone marrow cavities were partially preserved (Fig. 3a.*b*). In addition, zoledronic acid significantly decreased tumor infiltration and reduced by ~60 % the tumor cells in the metastatic lesions when compared to the vehicle-treated mice (Fig. 3b).

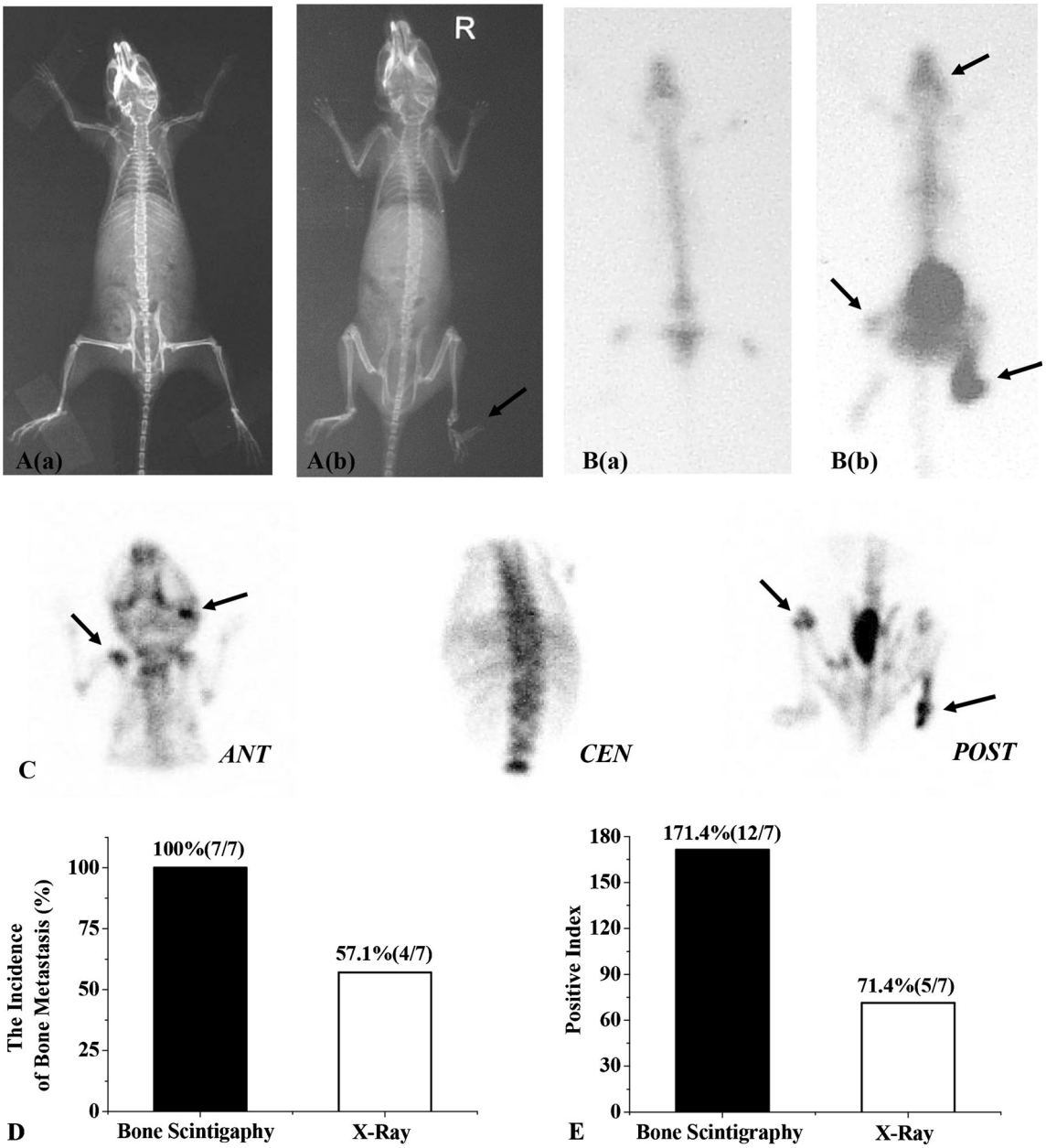
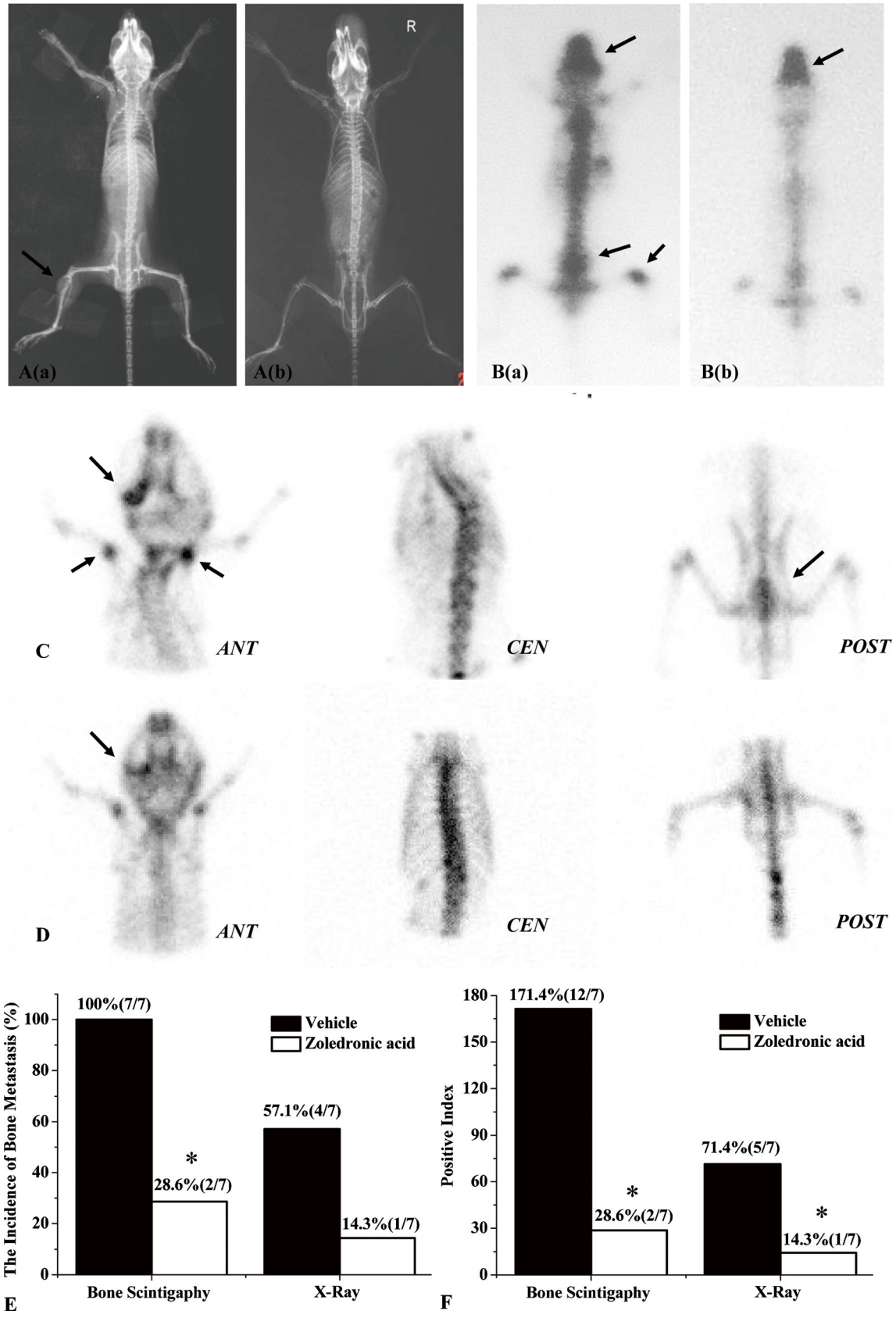


Fig. 1 X-ray and bone scintigraphy images from normal and experimental bone metastasis mice. **a** X-ray images: (*a*) normal mice and (*b*) experimental bone metastasis mice. **b** Whole-body bone scintigraphy images: (*a*) normal mice and (*b*) experimental bone metastasis mice. **c** The pinhole bone scintigraphy images. **d** The incidence of bone metastasis. **e** The quantitative analysis of PI value. The positive index of bone metastasis (PI), the total number of bone metastasis lesions (femurs, spines, mandibles or ribs) in mice expressed as a percentage of the number of mice. The black arrows indicate the bone metastasis sites

Expression of Genes Associated with Bone Metastasis

The metastatic bone lesions were harvested and analyzed by RT-qPCR and western blotting to determine the gene products associated with bone metastasis in both vehicle and zoledronic acid treatments. Zoledronic acid increased OPG gene expression and decreased IL-8, M-CSF, PTHrP and RANKL gene expression in osseous metastasis tissues at both the mRNA and protein levels (Fig. 4).

Fig. 2 Zoledronic acid significantly attenuated bone metastasis. **a** Whole-body X-ray images: (*a*) vehicle group and (*b*) zoledronic acid group (200 μg/kg, twice weekly, i.p. injection). **b** Whole-body bone scintigraphy images: (*a*) vehicle group and (*b*) zoledronic acid group. The pinhole bone scintigraphy images in vehicle group (**c**) and zoledronic acid group (**d**). **e** The incidence of bone metastasis, * $p<0.05$, assessed by the Fisher exact test. **f** The quantitative analysis of PI value, * $p<0.05$, assessed using Fisher exact test. The *black arrows* indicate the bone metastasis sites


A(a)
A(b)
R
B(a)
B(b)
C
ANT
CEN
POST
D
ANT
CEN
POST
The Incidence of Bone Metastasis (%)
100%(7/7)
28.6%(2/7)
57.1%(4/7)
14.3%(1/7)
Vehicle
Zoledronic acid
Bone Scintigaphy
X-Ray
E
Positive Index
171.4%(12/7)
28.6%(2/7)
71.4%(5/7)
14.3%(1/7)
Vehicle
Zoledronic acid
Bone Scintigaphy
X-Ray
F

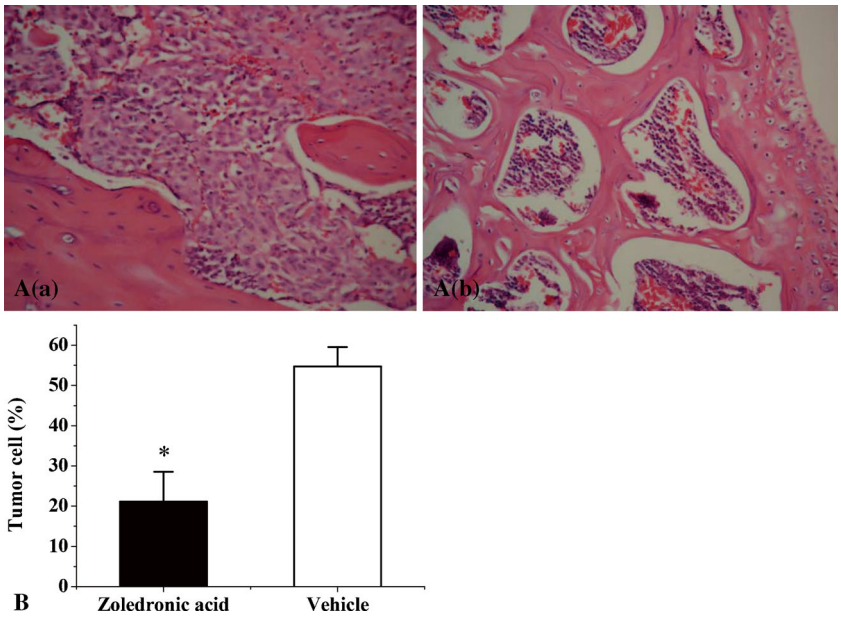


Fig. 3 The results of pathological examination. **a** Representative histology of the osseous metastases: (*a*) vehicle group (n=3), (*b*) zoledronic acid group (n=3). **b** Tumor burden in bone metastases lesions was measured by quantitative histomorphometry. Data are means ± SD, * p<0.05, analysis by ANOVA

Discussion

In this study, we observed bone destruction, accumulation of tumor cells, and the well known genes associated with bone metastasis in tumor-bearing mice. We were able to develop an exclusive bone metastasis model using human breast cancer cell line subclone MDA-231BO and injecting these cells into the intracardiac region in mice. Additionally, the sensitivity and accuracy of radionuclide bone scintigraphy is sensitive than X-ray analysis in detecting bone metastasis, especially early stage metastasis. In our mouse model of metastatic bone cancer, zoledronic acid attenuated the human breast cancer metastasis to bone.

Although radionuclide scintigraphy had been widely used to detect human bone metastasis, the diagnosis and detection for bone metastasis by bone scintigraphy in small animal is less well characterized. In this report, bone scintigraphy trended towards being more sensitive and accurate than X-ray analysis 8 weeks post tumor inoculation. Metastatic colonies identified in seven mice had a PI score of 171.4 % for bone scintigraphy and 71.4 % for X-ray analysis. Although bone scintigraphy has a great PI score, the combination of

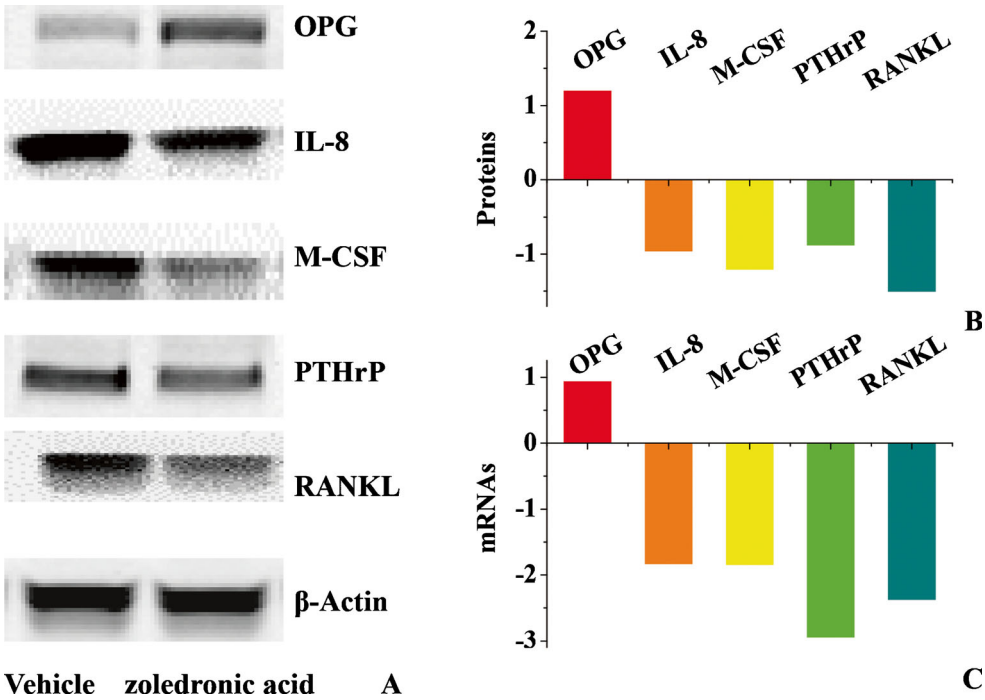


Fig. 4 mRNA and protein expression of metastasis-associated genes. Three representative mice bone tissues were acquired and prepared for RT-qPCR and western blotting analysis. **a** Representative imaging of protein expression by western blotting with β-actin as a loading control. **b** Quantitative results of protein expression. **c** Quantitative results of mRNA expression by RT-qPCR

both X-ray and bone scintigraphy imaging improved the diagnostic accuracy. The results from this study correlate well with our previous finding that bone scanning scintigraphic provides an enhanced anatomic localization and improves the diagnostic accuracy in the detecting of bone metastasis [18, 19].

In the past decade, zoledronic acid has become the most widely used treatment for breast cancer patients with bone metastases. This drug not only reduces the risk of skeletal-related events in bone metastases, but also presents to be the most potency drug in its class for treating metastatic breast cancer [22–24]. In addition to its role in suppressing bone metastases, preclinical data have demonstrated that zoledronic acid can also suppress liver and lung metastases in a breast cancer mouse model [8]. It also has antiproliferative, apoptotic, and cytostatic effects against cancer cells both in vitro and in vivo [7, 11, 25]. Another study indicated that zoledronic acid strongly inhibits angiogenesis in both bone and prostate tissues in a murine model [26]. Recently, Insalaco and colleagues showed that zoledronic acid treatment reduced the migration of cancer cells through the modulation of the attachment of tumor cells to the extracellular matrix proteins [7]. In this work, histomorphometric, stereological, and molecular analyses illustrated that the progression of metastatic breast cancer in bone was significantly decreased by zoledronic acid treatment. This treatment reduced the incidence of bone metastasis and the numbers of bone metastatic lesions compared to the vehicle group. Moreover, zoledronic acid impacted the gene expression in osseous metastasis tissues with an increase in OPG expression and a decrease IL-8, M-CSF, PTHrP and RANKL. These data suggested that zoledronic acid might be exerting its actions by inhibiting breast cancer cell growth in the metastatic microenvironment and regulating the functions of both osteoblasts and osteoclasts in the tumor-bearing mice. However, further investigations are needed to understand how zoledronic acid regulates osteoblast and osteoclast activity and how the tumor cells, osteoblasts and osteoclasts interact in bone metastasis microenvironment in the mouse model.

In conclusion, we have established a mouse model for exclusive bone metastasis based on the intracardiac injection of the subclone MDA-MB231BO and have analyzed the metastases using ^{99m}Tc-MDP bone scintigraphy. Remarkably, zoledronic acid significantly attenuated bone metastasis in this model.

Acknowledgments This research was supported by The National Natural Science Foundation of China (No. 30772811, No. 81102597 and No. 81173269), "Shu Guang" project supported by Shanghai Municipal Education Commission and Shanghai Education Development Foundation (No. 08GG12) and Sponsored by Program of Shanghai Subject Chief Scientist (No. 10XD1404000).

Conflict of Interest We have no conflict of interest to declare.

References

1. Chen WQ, Zeng HM, Zheng RS, Zhang SW, He J (2012) Cancer incidence and mortality in China, 2007. Chin J Cancer Res 24:1–8
2. Linos E, Spanos D, Rosner BA, Linos K, Hesketh T, Qu JD et al (2008) Effects of reproductive and demographic changes on breast cancer incidence in China: a modeling analysis. J Natl Cancer Inst 100:1352–1360
3. Lipton A, Uzzo R, Amato RJ, Ellis GK, Hakimian B, Roodman GD et al (2009) The science and practice of bone health in oncology: managing bone loss and metastasis in patients with solid tumors. J Natl Compr Cancer Netw Suppl 7:S1–S29
4. Roodman GD (2004) Mechanisms of bone metastasis. N Engl J Med 350:1655–1664
5. Anderson KC, Alsina M, Bensinger W, Biermann JS, Chanan-Khan A, Cohen AD, et al (2010) NCCN clinical practice guidelines in oncology: multiple myeloma, 2010, v.2. Available at: http://www.24hmb.com/UpLoad/Editor/2010/1/4/2010010471312017.pdf
6. Luo KW, Ko CH, Yue GG, Lee MY, Siu WS, Lee JK et al (2013) Anti-tumor and anti-osteolysis effects of the metronomic use of zoledronic acid in primary and metastatic breast cancer mouse models. Cancer Lett 339:42–48
7. Insalaco L, Di Gaudio F, Terrasi M, Amodeo V, Caruso S, Corsini LR et al (2012) Analysis of molecular mechanisms and anti-tumoural effects of zoledronic acid in breast cancer cells. J Cell Mol Med 16:2186–2195
8. Hiraga T, Williams PJ, Ueda A, Tamura D, Yoneda T (2004) Zoledronic acid inhibits visceral metastases in the 4T1/luc mouse breast cancer model. Clin Cancer Res 10:4559–4567
9. Jeong J, Lee KS, Choi YK, Oh YJ, Lee HD (2011) Preventive effects of zoledronic acid on bone metastasis in mice injected with human breast cancer cells. J Korean Med Sci 26:1569–1575
10. Koto K, Horie N, Kimura S, Murata H, Sakabe T, Matsui T et al (2008) Clinically relevant dose of zoledronic acid inhibits spontaneous lung metastasis in a murine osteosarcoma model. Cancer Lett 274:271–278
11. Rachner TD, Singh SK, Schoppet M, Benad P, Bornhäuser M, Ellenrieder V et al (2010) Zoledronic acid induces apoptosis and changes the TRAIL/OPG ratio in breast cancer cells. Cancer Lett 287:109–116
12. Hiraga T, Williams PJ, Mundy GR, Yoneda T (2001) The bisphosphonate ibandronate promotes apoptosis in MDA-MB-231 human breast cancer cells in bone metastases. Cancer Res 61:4418–4424
13. Yoneda T, Willams PJ, Hiraga T, Niewolna M, Nishimura R (2001) A bone-seeking clone exhibits different biological properties from the MDA-MB-231 parental human breast cancer cells and a brain-seeking clone in vivo and in vitro. J Bone Miner Res 16:1486–1495
14. El-Abdaimi K, Ste-Marie LG, Papavasiliou V, Dion N, Cardinal PE, Huang D et al (2003) Pamidronate prevents the development of skeletal metastasis in nude mice transplanted with human breast cancer cells by reducing tumor burden within bone. Int J Oncol 22:883–890
15. Even-Sapir E (2005) Imaging of malignant bone involvement by morphologic, scintigraphic, and hybrid modalities. J Nucl Med 46:1356–1367
16. Hamaoka T, Madewell JE, Podoloff DA, Hortobagyi GN, Ueno NT (2004) Bone imaging in metastatic breast cancer. J Clin Oncol 22:2942–2953
17. Padhani A, Husband J (1998) Bone metastases. In: Husband JES, Reznek RH (eds) Imaging in oncology oxford. Isis Medical Media Ltd, U.K., pp 765–787
18. Yang S, Dong Q, Yao M, Shi M, Ye J, Zhao L et al (2009) Establishment of an experimental human lung adenocarcinoma cell line SPC-A-1BM with high bone metastases potency by 99mTc–MDP bone scintigraphy. Nucl Med Biol 36:313–321

19. Wu C, Sun Z, Ye Y, Han X, Song X, Liu S (2013) Psoralen inhibits bone metastasis of breast cancer in mice. Fitoterapia 91:205–210
20. Chen M, Ye Y, Ji G, Liu J (2010) Hesperidin upregulates heme oxygenase–1 to attenuate hydrogen peroxide-induced cell damage in hepatic L02 cells. J Agric Food Chem 58:3330–3335
21. Mandal MN, Vasireddy V, Jablonski MM, Wang X, Heckenlively JR, Hughes BA et al (2006) Spatial and temporal expression of MFRP and its interaction with CTRP5. Invest Ophthalmol Vis Sci 47:5514–5521
22. Coleman RE, Marshall H, Cameron D, Dodwell D, Burkinshaw R, Keane M et al (2011) Breast-cancer adjuvant therapy with zoledronic acid. N Engl J Med 365:1396–1405
23. Costa L (2007) Bisphosphonates: reducing the risk of skeletal complications from bone metastasis. Breast 16:S16–S20
24. Gallo M, Luca AD, Lamura L, Normanno N (2012) Zoledronic acid blocks the interaction between mesenchymal stem cells and breast cancer cells: implications for adjuvant therapy of breast cancer. Ann Oncol 23:597–604
25. Niikura N, Liu J, Hayashi N, Palla S, Tokuda Y, Hortobagyi G et al (2012) Retrospective analysis of antitumor effects of zoledronic acid in breast cancer patients with bone-only metastases. Cancer 118: 2039–2047
26. Fournier P, Boissier S, Filleur S, Guglielmi J, Cabon F, Colombel M et al (2002) Bisphosphonates inhibit angiogenesis in vitro and testosterone-stimulated vascular regrowth in the ventral prostate in castrated rats. Cancer Res 62:6538–6544