Validating of the pre-clinical mouse model for metastatic breast cancer to the mandible

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ABSTRACT

Metastatic breast carcinoma has a great tendency to spread to the mandible. It is concomitantly associated with bone destruction, food intake disorder, and a poorer prognosis. Appropriate animal models need to be developed for a better understanding of the mechanisms underlying the metastatic process of breast cancer cells to mandible and to test the effects of potential lead compounds. Here, we assessed the metastasis model of intracardiac injection using luciferase-transfected metastatic breast cancer cells (MDA-MB-231[Luc+]) by determining the incidences of metastasis, μCT images, and histopathological results. A high bioluminescence signal mainly detected mandibular lesions with less frequent distal femora and proximal tibiae lesions. Extensive mandibular bone destruction occurred in nude mice grafted with metastatic breast cancer cells. This type of animal model might be a useful tool in assessing therapeutic implications and the efficacy of anti-cancer drugs for osteolytic cancers.

Keywords: Breast cancer. Mandible. Osteolysis. Xenograft. Luminescence.

INTRODUCTION

Bone is one of the most common sites for metastasis[29]. Bone metastases represent a major complication of malignant tumors. Metastatic tumors to the mandibles not a uncommon phenomenon and account for approximately 1% of all malignant oral tumors[27]. Metastatic tumors in the mandible are more frequently reported than those in the oral mucosa. In particular, breast carcinoma metastasizes to the mandible three times as often as any other malignant tumor[1]. However, the pathogenesis of the metastatic process in the mandible remains unclear. Metastatic tumors to the oral region are challenging and difficult to diagnose because of their relative infrequency. Therefore, they should be considered in the differential diagnosis of inflammatory and reactive lesions that are common to the oral region.

In the meantime, most of the currently available metastatic animal models of human breast tumor xenografts using orthotopic transplantation have only a limited success rate in most trials. The intra-tibia inoculation of breast cancer as a cancer-bone metastasis model fails to address certain aspects of the clinical situation and does not precisely reflect the characteristics of metastatic breast cancer[8,13]. Appropriate animal models are currently insufficient for the research before clinical trials. Thus, a feasible and adequate metastatic animal model needs to serve as a suitable tool to help precisely understand the molecular mechanism of mandible bone metastasis of breast cancer and to develop new therapeutic agents against metastatic breast cancer to bone.

In this study, we assessed the metastasis model of intracardiac injection using luciferase-transfected metastatic breast cancer cells, demonstrated its value as a useful *in vivo* tool for metastatic breast cancer to mandible bone, and examined the

histopathological aspect. This type of animal model approach might have the additional advantage of potentially accelerating the biological discovery process.

MATERIAL AND METHODS

Material and reagents

Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), fetal bovine serum (FBS), antibiotic-antimycotic mixture containing 100 U/ml penicillin and 100 µl/ml streptomycin, phosphate-buffered saline (PBS), and 0.25% trypsin-EDTA were purchased from Gibco BRL Co. (Rockville, MD, USA). D-Luciferin potassium salt was obtained from Goldbio Technology (St. Louis, MO, USA). DMSO and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents used in this research were of analytical grade.

Cell culture

The human mammary carcinoma cell line MDA-MB-231 was obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in DMEM supplemented with 10% FBS at 37°C under a humidified atmosphere of 5% CO_2.

Luciferase vector construction

To generate the lentiviral particles with lentiviral vector-based luciferase, firefly luciferase gene from *Photinus* pyralis was amplified by polymerase chain reaction (PCR)-based methods using pTAL-Luc vector (Clontech, Palo Alto, CA, USA) as a template, followed by subcloning into the pLenti6/V5 Directional TOPO Cloning vector in ViraPower™ Lentiviral Expression Systems (Invitrogen, Carlsbad, CA, USA). pLenti6/V5-Luc plasmid was subjected to DNA sequencing analysis to confirm a successful construction. Lentivirus particles were produced by cotransfecting the 293FT producer cell line with pLenti6/V5-Luc plasmid and the ViraPowerTM Packaging Mix. The cells were transducted using 2×10^7 lentiviral particles with transduction enhancer Polybrene at 10 mg/ml to establish luciferase-transfected MDA-MB-231 (MDA-MB-231[Luc+]) stable cells. Blasticidine (10 µg/ml) was added to select stably transduced cells. Blasticidine-resistance clones exhibited the V5 epitope detection against anti-V5 antibody by western blot analysis and revealed the maximum level of luciferase activity using a microplate spectrofluorometer (Molecular Devices, Palo Alto, CA, USA).

Animals

Female Balb/c *nu/nu* mice (5 weeks of age) were purchased from Nara Bio Inc. (Seoul, Korea) and maintained at 20-22°C on a 12 h light/dark cycle.

All animal studies were performed in accordance with experimental protocols that were approved by the animal ethics committee of Yonsei University College of Dentistry.

Animal model of the metastatic breast cancer to the mandible

Mice were anesthetized by intramuscular injection of a mixture with 30 mg/kg Zoletil (Verbac, Carros, France) and 10 mg/kg xylazine (Bayer Korea, Seoul, Korea). MDA-MB-231[Luc+] cells (1×10^6 cells/0.1 ml in HBSS) were injected into the left cardiac ventricle of nude mice with a 26-1/2 gauge needle according to the modification of the method[3]. Metastatic progression in nude mice was shown with bioluminescence imaging on 6 weeks after intracardiac injection. Then all animals were euthanized by CO_2 asphyxiation and histological analysis was performed.

Bioluminescent imaging

Bioluminescent *in vivo* imaging was detected with a highly sensitive CCD camera mounted in a light-tight specimen box (IVIS-200™, Xenogen, Caliper Life Sciences, Alameda, CA, USA). Animals were given the substrate D-Luciferin potassium salt by intraperitoneal injection of 150 mg/kg in Dulbecco's Phosphate Buffered Saline and then they were anesthetized with 1-3% isoflurane gas. Ten min after the D-Luciferin injection, mice were placed onto the warmed stage inside the light-tight camera box with continuous exposure to 1-2% isoflurane gas.

µCT analysis

The mandibles of the nude mice were scanned using a Skyscan 1076 high-resolution µCT with 100 Kv, 140 mA current, rotation step 0.6° and camera pixel size 35 mm (SkyScan, Antwerpen, Belgium). NRecon and CT analyzer (CTAn) software (SkyScan) were used to analyze the structure of the sample. Two-dimensional images were used to generate three-dimensional (3-D) reconstructions with the software. The bone volume (BV) was calculated from the processed images shown in 3-D image using CTAn software with the same diameter given.

Hematoxylin and eosin (H&E) staining

The tissues were fixed in 10% buffered formalin solution and embedded in paraffin. Tissue sections (4 µm thickness) were sectioned using a Leica 2135 microtome, deparaffinized in xylene, rehydrated through an alcohol series, and stained with H&E for histopathology. Then, tissues were dehydrated and mounted.

Statistical analysis

The statistical analysis was conducted using InStat™ statistical software (GraphPad Software, Inc., San Diego, CA, USA), and results are expressed as mean±standard error. The statistical significance of differences between groups was analyzed via repeated measures of Student's paired and unpaired *t*-tests. Asterisks were used to graphically indicate statistical significance. *P* values of <0.05 were considered significant.

RESULTS

Mouse model for metastatic breast cancer to the mandible

MDA-MB-231[Luc+] cells were inoculated into the left ventricle of mice and the metastatic progression of breast cancer was tracked through bioluminescence imaging using an IVIS Imaging System (Figure 1). Metastasis to mandible were detected in all mice injected with MDA-MB-231[Luc+] cells (n=250). A high level of total photon flux was observed following the injection of MDA-MB-231[Luc+] cells (*P*<0.001). Multiple organ metastases were frequently observed in distal femora and proximal tibiae. The high bioluminescence signal was also mainly observed in mandibles.

Mandible bone analysis using μCT

The μCT images afforded a nondestructive visualization of the changes that occurred over the entire mandible. Osteolytic lesions of mice were analyzed by radiography at 6 weeks after cancer cell injection. On the mediolateral radiograph, an extensive bone destruction was observed around the mandibular bone in mice injected with MDA-MB-231Luc+ cells (Figure 2A). Osteolytic lesions occurred in both cancellous and cortical bones on vertical projection (slice) (Figure 2A) and 3-D reconstruction images (Figure 2B). The bone volume was determined from the processed images shown in Figure 2B using CTAn software with the same diameter given as where bioluminescence signal and osteolytic site were overlapping (Figure 2C). The total bone volume was decreased about 28.9% in mice injected with MDA-MB-231Luc+ cells. The H&E staining that tumour cells infiltrated into the mandibular region and thereby induced bone destruction (Figure 2D).

DISCUSSION

Carcinomas are malignant neoplasms derived from epithelial cells which are surrounded by stroma that interacts with cancer cells to modulate cell growth, migration, invasion, and tissue-specific gene expression. Recently studies have shown that interactions of tumor cells with their adjacent microenvironment are intimately associated with tumor growth and the development of metastasis. Therefore, appropriate *in vivo* animal models are required to study interactions between tumor cells and their environment. In addition, a feasible animal model is critical to test the effects of potential lead compounds.

Cancer is a complex disease in which cells develop aberrant proliferation and survival. The progression of metastasis severely increasis morbidity and eventually mortality. Metastatic tumors to the oral region are infrequent and account for approximately 1% of all malignant oral tumors[27]. However, metastatic tumors to the mandibular bone are not a rare phenomenon. Many
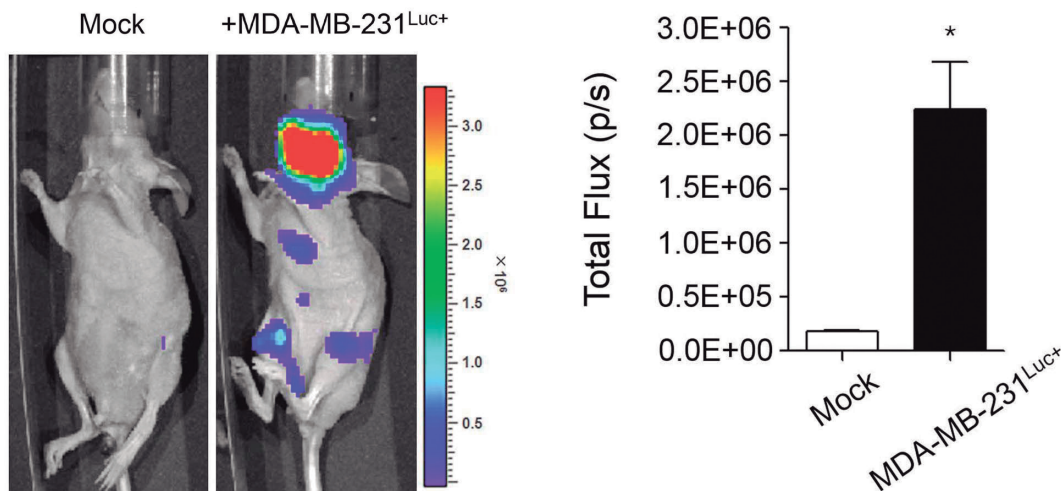

Figure 1- Mouse model for metastatic breast cancer to the mandible. The growth and part of the metastatic spread (left) was detected by bioluminescence imaging after the injection of cancer cells. The formed metastases were quantified by measuring total photon flux *per* second (right). Data are expressed as the mean±standard error (SEM). *P<0.001 versus Mock group

cases with metatastic breast carcinoma to the mandible have been reported and their metastases are well documented. Metastases in the oral region can occur in oral soft tissues or the mandibular bone. Metastatic tumors in the mandibular bone are more frequently reported than those in the oral mucosa. Primary sites are usually the breast, lung, intestine, ovaries, prostate, thyroid, kidney, testes, uterine cervix, and adrenal glands[9,11,12,14,15,17,19,23,24,27,30-32]. Many authors reported that breast cancer metastasis to the oral cavity is more frequent for a disseminated tumor to involve the mandible rather than the maxilla[1,21]. Breast carcinoma is commonly osteolytic with an indistinct outline, but it is osteoblastic in 10% and mixed in type in another 10% of cases. Hormone therapy and sterilization may often result in the conversion of osteolytic lesions to the osteoblastic type[2]. Taken together, breast carcinoma has a great tendency to be spread by metastasis, thus it is a common clinical problem associated with bone destruction and a poorer prognosis. Breast carcinoma metastasizes to the mandibles three times as often as any other malignant tumor[22]. Also it has been reported that

periodontal diseases are associated with breast cancer[28]. Therefore, a metastasis of an aggressive breast cancer to the mandible has a tendency to increase. However, the complete understanding is lacking about the process how breast cancer metastasizes to the mandibles with the most frequency at the cellular level. Consequently, diversified insights into the molecular mechanism of breast cancer metastasis to the mandible are necessary to devise preventive and therapeutic approaches. An appropriate animal model might have an additional advantage to understand the mechanisms underlying the metastatic process and to test the effects of potential lead compounds.

Due to the technical complication, there is a low success rate in most trials due to the susceptibility of bacterial contamination and the weak cellular activity of primary tumors as opposed to metastatic tumors[7,20]. Human tumor xenografts using the orthotopic transplantation of histologically intact tissues of several carcinomas have not been adopted for a metastatic animal model although these models represent the entire environment that occurs during cancer progression[16]. Thus,
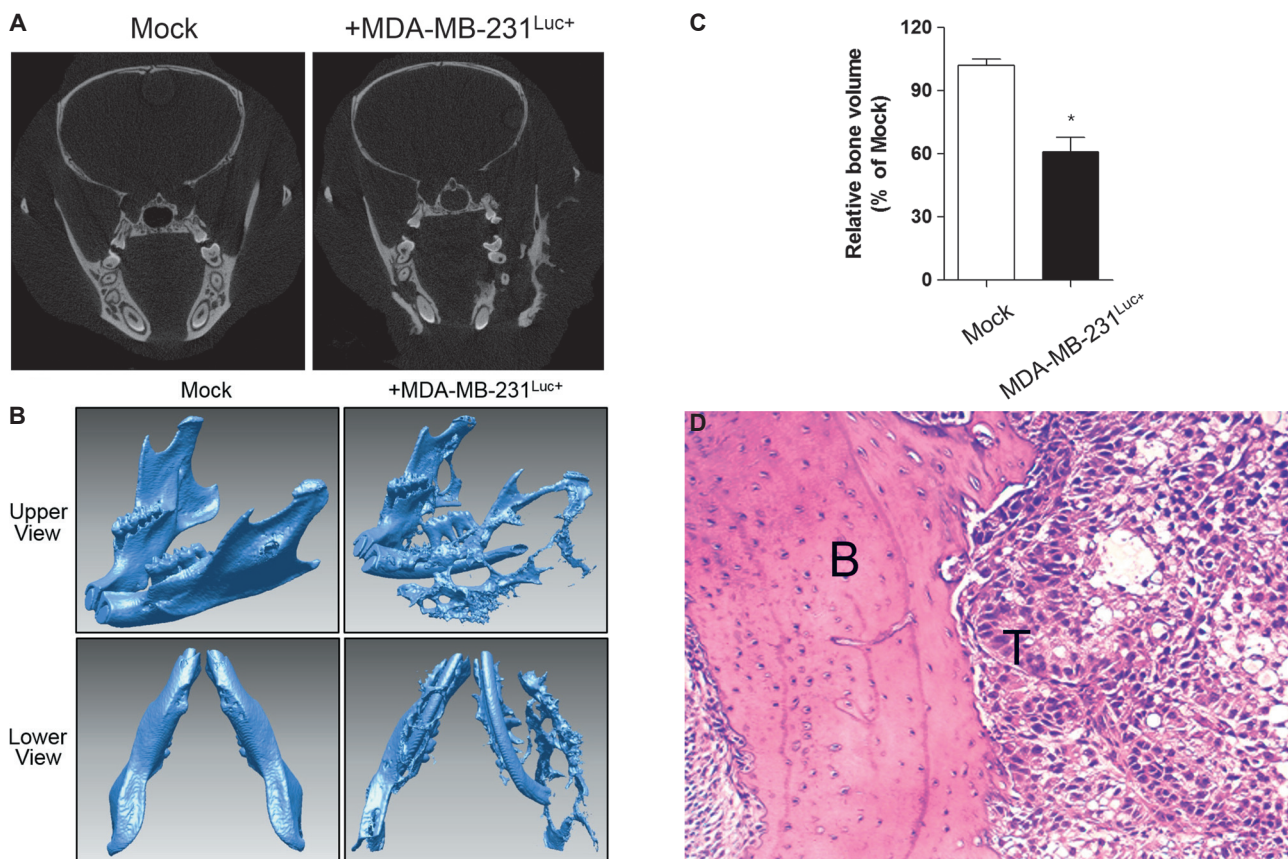

Figure 2- Mandible bone analysis. Radiographic images of mandibles in mice were scanned by μCT at 6 weeks. The osteolytic lesions were analyzed on vertical projection (slice) (A); and 3-D reconstruction images (B; upper and lower view each). The bone volume was determined from processed images shown in 3-D images (B); with the same given diameter (C). Data are expressed as the mean±standard error (SEM). *P<0.01 versus Mock group. Hematoxylin & eosin staining around the mandibular region was performed and the tumor-induced osteolytic site was observed. (original magnification ×100) (B; bone, T; tumor) (D)

xenograft models have been used to study the development and metastasis of human carcinomas. The xenograft animal model with a cultured cancer cell line is technically simple and reflects more precisely the morphology and character of their respective original tumor. Nevertheless, there is no single animal model that precisely replicates all of the events and circumstances that occur during cancer progression. The most widely used model for cancer development and behavior research is the flank model. However, not all transplanted cancer cells develop tumors at an atopic site due to the different cancer environments and no spontaneous metastasis does occur[4]. Also in case of a mammary fat pad xenograft, breast cancer development has shown a low success rate in most trials due to the technically limited occurrence of mammary glands in neonatal mice. Also, an intra-tibia inoculation of breast cancer is frequently applied as a cancer-bone metastasis model. In this model, cancer cells are inserted extra-capsulary through the tibial crest, epiphysis, and growth plate and then cells are sequentially injected into the bone marrow space. The intra-tibia model does not precisely reflect the characteristics of bone-invasive OSCC[8,13]. Thus, it fails to address certain aspects of the clinical situation. This model is not suitable to elucidate the cellular and molecular mechanisms of metastatic breast cancer to mandible and could not serve as suitable tool in the development of new therapeutic agents against metastatic breast cancer to bone.

Triple-negative breast cancer (TNBC), as defined by the absence of estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2/neu) expression, can be particularly aggressive, and more likely to recur than other subtypes of breast cancer. MDA-MB-231 cell is triple-negative and preferentially metastasizes to the bone[5]. Particularly, metastatic MDA-MB-231 breast cancer cells have a clinical tendency to be spread to mandible. Breast carcinoma metastasizes to the mandibles three times as often as any other malignant tumor[12]. In many types of animal models including tibia, MDA-MB-231 cells led to osteolytic bone destruction, reproducibly. Therefore, MDA-MB-231 cell is widely used for TNBC studies. However, a complete understanding of how breast cancer metastasizes to the mandibles with the most frequency at the cellular level is lacking.

Above all, in this study, we tried to find a proper animal model of metastatic breast cancer to the mandibular bone. Experimental metastases refer to the injection of tumor cells directly to the systemic circulation. Metastatic breast cancer cells have a great tendency to be spread to bone including mandible. Therefore, research groups have been adopting the intracardiac injection method for osteolytic metastasis model[17,18,25,26].

Although this animal model accurately reflects the bone disease portion of the clinical disease, the complete mechanism of how breast cancer metastasizes to the mandibles with the most frequency at the cellular level is not clear. The *active hematopoietic* tissue is known as *red* bone *marrow.* Bones with red marrow are the favored sites for metastatic deposits[17]. Mainly in elderly persons, jawbones have little active marrow, while remnants of hematopoietic active marrow are detected in the posterior areas of the mandible and the hematopoietically active sites attract metastatic tumor cells. Meanwhile, the oral soft tissues have a rich network of capillaries which can entrap malignant cells. Proliferating capillaries display an uneven basement membrane, thereby increasing the penetration of tumor cells. In addition, inflammation plays an aggravative role in attracting metastatic cells to the gingiva[9]. In this study, firefly luciferase-expressing metastatic breast cancer cells (MDA-MB-231[Luc+]) were injected into the left cardiac ventricle producing uniform mandibular bone metastases with complete success rate by *ex vivo* imaging and histological analysis. Osteolytic lesions were widely observed in both cancellous and cortical bones. The subsequent histological evaluation of tissue sections from the mandible confirmed the spontaneous tumor metastasis. In animal models, bioluminescent imaging is a useful and sensitive method for detection of primary cancer growth and for tracking the metastasis in animal models[6,10]. In addition, luciferase-expressing cells permit the real-time tracking of tumor sites in living animals.

CONCLUSIONS

This study verified the reproducible xenograft model of metastatic breast cancer to mandibular bone using intracardiac injection with MDA-MB-231[Luc+]. The development of breast cancer to mandible mouse model remains a challenge in the preclinical evaluation of therapies for human breast cancer. Now, the current xenograft model serves as suitable research tool and this application offers the preclinical assessment of anticancer therapies *in vivo*.

CONFLICT OF INTEREST STATEMENT

No conflict of interests.

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