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Metastatic human breast cancer to the spine produces mechanical hyperalgesia and gait deficits in rodents

Rachel Sarabia-Estrada, DVM, PhD^{a,+}, Alejandro Ruiz-Valls, M.D.^b, Hugo Guerrero-Cazares, PhD.^a, Ana M. Ampuero, B.S.^b, Ismael Jimenez-Estrada, PhD.^c, Samantha De Silva, B.S.^b, Lydia J. Bernhardt, B.S.^b, C. Rory Goodwin, M.D., Ph.D.^b, A. Karim Ahmed, BS^b, Yuxin Li, M.D.^d, Neil A. Phillips, M.S.^b, Ziya L. Gokaslan, M.D.^e, Alfredo Quiñones-Hinojosa, M.D.^a, and Daniel M. Sciubba, M.D.^{b,+}

^aDepartment of Neurological Surgery, Mayo Clinic, Jacksonville, FL, USA

^bDepartment of Neurosurgery, The Johns Hopkins University School of Medicine, Baltimore, MD, USA

^cPhysiology, Biophysics and Neurosciences, Research Center and Advanced Studies, IPN, Mexico City, MEXICO

^dDepartment of Neurosurgery, Jinan General Hospital of PLA, Jinan, 250031, CHINA

^eDepartment of Neurosurgery, The Warren Alpert Medical School of Brown University Providence, Rhode Island, USA

Abstract

BACKGROUND CONTEXT—Metastases to the spine are a common source of severe pain in cancer patients. The secondary effects of spinal metastases include pain, bone fractures, hypercalcemia, and neurological deficits. As the disease progresses, pain severity can increase until it becomes refractory to medical treatments and leads to a decreased quality of life for patients. A key obstacle in the study of pain-induced spinal cancer is the lack of reliable and reproducible spine cancer animal models. In the present study we developed a reproducible and reliable rat model of spinal cancer using human derived tumor tissue to evaluate neurological decline using imaging and behavioral techniques.

PURPOSE—The present study outlines the development and characterization of an orthotopic model of human breast cancer to the spine in immunocompromised rats.

STUDY DESIGN/SETTING—This is a basic science study.

METHODS—Female immunocompromised rats were randomized into three groups: Tumor (n=8), RBC3 mammary adenocarcinoma tissue engrafted in the L5 vertebra body; Sham (n=6),

*Co-corresponding Authors: Daniel M. Sciubba, MD, 600 North Wolfe Street, Meyer 7-109, Baltimore, MD 21287, Phone (410) 955-4424; Fax (410) 502-3399, dsciubb1@jhmi.edu. Rachel Sarabia-Estrada, DVM, PhD, 4500 San Pablo Rd, Griffin 1-113, Jacksonville, FL, Phone (904)-953-0120; Fax (904)-953-0742, Sarabia-Estrada.Rachel@mayo.edu.

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surgery performed but no tumor engrafted, and Control (n=6, naive rats, no surgery performed) groups. To evaluate the neurological impairment due to tumor invasion, functional assessment was done in all rodents at day 40 after tumor engraftment using locomotion gait analysis and pain response to a mechanical stimulus (Randal-Sellitto test). Bioluminescence (BLI) was used to evaluate tumor growth *in vivo* and cone beam computed tomography (CBCT) was performed to evaluate bone changes due to tumor invasion. The animals were euthanized at day 45 and their spines were harvested and processed for H&E staining.

RESULTS—Tumor growth in the spine was confirmed by bioluminescence imaging and corroborated by histological analysis. CBCT images were characterized by a decrease in the bone intensity in the lumbar spine consistent with tumor location on BLI. On H&E staining of tumor-engrafted animals, there was a near-complete ablation of the ventral and posterior elements of the L5 vertebra with severe tumor invasion in the bony components displacing the spinal cord. Locomotion gait analysis of tumor-engrafted rats showed a disruption in the normal gait pattern with a significant reduction in length ($P=.02$), duration ($P=.002$) and velocity ($P=.002$) of right leg strides and only in duration ($P=.0006$) and velocity ($P=.001$) of left leg strides, as compared to control and sham rats. Tumor-engrafted animals were hypersensitive to pain stimulus shown as a significantly reduced response in time ($P=.02$) and pressure ($P=.01$) applied when compared with control groups.

CONCLUSION—We developed a system for the quantitative analysis of pain and locomotion in an animal model of metastatic human breast cancer of the spine. Tumor engrafted animals showed locomotor and sensory deficits that are in accordance with clinical manifestation in patients with spine metastasis. Pain response and locomotion gait analysis were performed during follow-up. The Randal-Sellitto test was a sensitive method to evaluate pain in the rat's spine. We present a model for the study of bone associated cancer pain secondary to cancer metastasis to the spine, as well as for the study of new therapies and treatments to lessen pain from metastatic cancer to the neuroaxis.

Keywords

pain; breast cancer; spine; metastasis; locomotion; gait

Introduction

Cancer patients will experience multiple distressing symptoms during the course of their illness. Pain has been recognized as a common burden for these patients, and it is clear that patients with advanced stages of cancer are in need of new pain management approaches. Breast cancer (BCa) is the second most common cause of cancer-related mortality for women and is the leading cause of morbidity, particularly at the end of life [1, 2]. BCa preferentially metastasizes to the axial skeleton affecting the lumbar vertebrae and pelvis, followed by the ribs, skull, and femur, causing significant and life-altering pain [3–5]. Spinal column metastases can result in intractable pain, pathological fractures, severe neurological deficits (from spinal cord and nerve compression) disability and severe deterioration in quality of life [6–8]. Treatments for spinal metastases are focused to improve neurologic function, alleviate pain, prevent or treat instability and prolong the patient's quantity and quality of life.

Pain is the most common symptom for patients with spine metastases. Pain can be localized, constant and have multiple origins: 1) biological tumor pain, that is associated with localized inflammation initiated by the immune response and intrinsic tumor mediators (a local pain exaggerated by palpation or percussion) [9–12]; 2) radicular pain, caused by compression of the individual nerve roots (typically follows a dermatomal distribution); or 3) mechanical pain (axial loading pain), product of the bone loss in the structural integrity of the vertebral column, manifesting after movement and positions that require axial spine loading, such as sitting or standing [3, 9–17]. When not related to movement, the pain is probably the result of periosteal stretching or a rise in the endosteal pressure and if the pain is relieved by rest then it is usually attributed to structural vertebral deformity [17]. Given the debilitating nature of tumor related pain, treatments and therapies are primarily focused to improve patient's quality of life. In order to more effectively treat these symptoms, improved models are necessary to better understand the molecular mediators of cancer pain in metastatic spine disease. *In vivo* animal models that recapitulate the human disease are critical for the understanding of pain associated with cancer in spine metastasis. [18]. Distinct animal models are currently used to mimic metastatic bone cancer. The majority of these models involve the injection of cancer cells directly into the intramedullary space of the femur or tibia (sarcoma, prostate and breast) [19–21], increasing bone destruction, and producing ongoing and stimulus-evoked pain behaviors. The most used tests are radiant heat paw-withdrawal for thermal sensitivity [22], von Frey monofilament test [23] for mechanical allodynia [24], and the study of pain-related nocifensive behaviors (hunching, vocalization, paw lifting, flinching or shaking) [25–27]. The implantation of cancer cells or tissue directly to the vertebral body provides an opportunity to study the single effect of the tumor without involvement of other metastases, and accurately correlates the neurological decline (motor and nociceptive) with the severity of bone destruction and/or spinal cord compression [28]. None of the current metastatic animal models using breast cancer describe the pain response after spine cancer invasion. In a previous report the successful generation of an orthotopic prostate cancer spinal metastases model following the local engrafting of PC in the L5 vertebral body, gait locomotion was negatively affected by tumor growth. Animal gait was negatively affected by tumor implantation, however no sensory evaluation was investigated in such study [28]. Our model has the advantage of allowing the investigation of the interaction between tumor cells and the bone-spinal cord microenvironment (gait deficits due to spinal cord compression). This animal model is easily reproducible and can be used to investigate well-established single metastasis. We now report an orthotopic animal model of human-derived breast cancer to the spine in rats. Following tumor engrafting, image studies and neurological evaluation to assess pain response and behavioral changes in the gait locomotion were performed. Our model will allow future testing of experimental therapies in a clinically relevant system, thus providing predictive models of treatment efficacy.

Material and Methods

Animals

Rats were housed in an Association for Assessment and Accreditation Laboratory Animal Care-accredited facility in compliance with the Guide for the Care and Use of Laboratory

Animals. The Institutional Animal Care and Use Committee at our institution approved all surgical and non-surgical procedures. 23 female, 5 week old, immunocompromised rats (Cr:NIH-RNU, from Charles River Laboratories, Frederick, MD) were maintained in standard environmental conditions, with free access to food and water. The rats were randomly allocated to different experimental groups.

Breast Cancer Cell Line

We utilized the RBC3 sub-clone that is specific to the bone. This cell line was derived from the commercial cell line MDA-MB-231 GFP/LUC+. RBC3 cells were cultured and expanded using Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 [DMEM/F12 + 10% FBS + 10% antimycotic/antibiotic]. Cells were maintained in a humidity incubator in an atmosphere of 5% CO₂ at 37°C [29].

Xenograft Establishment

To establish a subcutaneous breast cancer tumor, RBC3 cells [29] were trypsinized and centrifuged at 180-x g for 5 minutes at 4°C. Cells were counted and 2×10^6 cells were resuspended in serum free media and mixed with Growth Factor Reduced Matrigel (Corning^R, Corning, NY) at a 2:1 ratio, to a final volume of 200 µl [28]. The cell-matrigel mixture was subcutaneously injected in the left flank of 5 week-old immunocompromised rats (n=3, Cr:NIH-RNU, from Charles River Laboratories, Frederick, MD, USA, Figure 1A). The size of the tumor was monitored weekly and allowed to grow to a size of 2 cm². After this period, animals were euthanized and subcutaneous tumors were excised and minced to formulate 0.1 cm³ pieces that were used for the intravertebral tumor implantation (Figure 1B) as detailed in the following section.

Establishment of breast cancer in the lumbar spine

Once flank tumors were established, tumor was excised (Figure 1A). To establish the breast cancer model in the lumbar spine, twenty female immunocompromised rats (5 weeks old) were randomized into three surgical groups: Experimental group (E), rats implanted with a 0.1-cm³ pieces of RBC3 tumor (n=8); Sham group (S), rats receiving only a sham surgery, in which the vertebral body was only exposed and drilled, but no tumor was implanted (n=6); and Control group (C), rats left intact (n=6). The surgical approach to the L5 vertebra was performed as we have previously described [28, 30]. Briefly, rats were anesthetized and prepped under sterile conditions. The abdominal cavity was exposed through a superficial midline incision centered between the iliac crests and a small incision along the midline linea alba of the bilateral rectus abdominis muscle. A cotton-tipped applicator was used to mobilize adipose tissue and bowels to visualize the aorta, vena cava and finally the L5 vertebral body. The targeted vertebral body was drilled approximately 0.5 mm superior to the intervertebral disc (Figure 1B). Subcutaneous tumors were excised from carrier rats as previously described [28], and harvested for the intravertebral tumor implantation. Harvested tumors were cut into fragments of approximately 0.1 cm³ and placed into the drilled space in the vertebral body. The cavity was sealed with Geristore (Dual-Cure resin-ionomer; DenMat, CA, USA). The fascia was closed with a running 5-0 absorbable suture, and the skin was closed with surgical autoclips. Rats were assessed carefully after surgery and daily afterward, looking for signs of discomfort such as decreased spontaneous activity, lack of

mobility, hunched posture, decreased grooming and porphyrin eye/nose secretion, paresis, including abnormal gait or inability to stand on the hind limbs.

Behavioral Tests

The motor and nociceptive functions were evaluated at day 40 (D40) after tumor engrafting to determine the effects of the breast cancer tumor growth at a functional level. For all the behavioral evaluations, the rats were gently handled for 5 minutes during a 5-day period to minimize stress. All the behavioral tests, were performed in a quiet, red illuminated room and were carried out from 12:00 pm to 17:00 pm by a blinded researcher to avoid bias in the results.

Kinematic analysis of gait locomotion

To evaluate the functional effects of spine tumor growth, analysis of rat locomotion was performed as previously described [31, 32]. Briefly, each rat was trained to walk on a narrow Plexiglas tunnel (100 cm long x 10 cm wide x 14 cm high) to ensure locomotion in a straight line. Training sessions of 5 minutes per rat were used once a day in a minimum-noise room. Supreme mini-treats, chocolate flavor, (BioServ, Flemington, NJ, USA) were used as bait at the end of the runway. Free non-stop walking with minimum grooming was considered as a sign of the rats being habituated to the runway. The rats' hip, knee, and ankle (i.e. lateral malleolus) joints of both hind limbs were marked (Figure 3A). Unrestrained gait of non-tumor- and tumor-implanted rats were recorded with a high-definition digital video camera at a rate of 30 frames per second (fps). This recording rate allows the observation of hind limb motion and collects an accurate movement registry. Hind limb movements of each rat consisting of a three-stride cycle were videotaped and analyzed frame-by-frame using the Image J software (NIH, Bethesda, MD, USA). Coordinates for each articulation were introduced in the Walking Rats Software. The duration (in seconds) and length (in centimeters) of each stride were determined, and from these parameters, the stride speed was calculated (in cm/sec). These parameters were calculated and each rat's mean hind limb stride is reported using previous definitions of stride and elevation coordinates for hind limbs [28, 32, 33]. Briefly, the marked points in the rat's hind limb were tracked frame by frame, obtaining a two-dimensional coordinates (x, y) using the ImageJ software (NIH; Bethesda, MD, USA), then data is imported into Microsoft Office Excel (Microsoft) and further analyzed with pre-assembled Excel sheets to modeled body segments as rigid straight lines between the marked points. The kinematics of gait is then reconstructed from changes in the marked points located between consecutive frames, facilitating the generation of stick diagrams (superimposing modeled body segments of every frame) and spatial displacement plots. Angles and distances can be calculated directly by the software. Optical deformation of the image produced by the camera lens is determined and corrected by using an acrylic square (5 cm x 5 cm), which served as a bi-dimensional scale [34].

Mechanical spine pressure threshold test

The nociceptive withdrawal threshold was assessed by using the Randall-Selitto electronic algometer (IITC Digital Paw Pressure Meter, IITC Life Science, Woodland Hills, CA). Each rat in all groups received ~5 min of handling to get acclimated to manipulation; then the rat was carefully immobilized with the left hand, and the tester's right hand was used to

operate the algometer. The test consisted of the application of an increasing mechanical force, in which the tip of the device was applied onto the skin surrounding the lumbar area (between the tip of the hips corresponding to the site of tumor engrafting) [35]. Time of response (s) and pressure applied (g) was registered when the rat withdrew from the stimulus. Pressure was increased at 5g/sec and measurements were performed in triplicate and averaged for data analysis. Pressure-applied measurements were limited to a maximum force of 450 g (500 g is the maximum reliable measurement suggested by the manufacturer).

In vivo imaging of spine breast cancer

To determine tumor growth in the tumor engrafted rats, *in vivo* bioluminescence images were obtained as previously described [28, 29, 36] using the IVIS Spectrum System that has a cooled CCD camera to capture images of animals and tissues in a light-tight box. Before imaging, rats were anesthetized and D-luciferin was injected I.P., at a dose of 30 mg/kg and allowed to distribute for 5 min, then imaging of the rodents in the lateral decubitus position was done as previously described [4, 37]. Imaging times ranged from 5 seconds to 5 minutes, depending on the total tumor burden as a function of light emission from tumor cells. Shorter acquisition times were necessary at later time points to avoid saturation of pixels. Bioluminescent signal was captured as the absolute total flux (photons/steradian/cm²) emitted with a 5-minute integration time and plotted against time. Rats were imaged at day 10, 20, 30, 35 and 40 after tumor engrafting; dorsal views facing the camera were acquired [4]. Region of interest analysis was performed using Living Image Software to determine the light emitted (relative counts) from the spine region. The mean $\pm$ SD light emission over the time was plotted for each spine tumor engrafted rat. Rats engrafted with RBC3 tumors into the spines were imaged by cone beam computed tomography (CBCT) at D40 after tumor implantation using a SARRP (small animal research radiation platform, Xstrahl Life Sciences, Camberley, United Kingdom) as previously described [29, 36]. Images were acquired at 65 kVp and 0.7 mA using a 20 x 20-cm beam. CBCT images were used to identify bone lesions in the RBC3 rats after tumor engraftment [29, 36]. CBCT images were used to identify lytic lesions at day 40 after tumor engrafting in all the animals from T, C and S groups. Rats were anesthetized as previously and scanned in a sagittal plane position.

Histopathological analysis

At day 45, rats were euthanized according to our IACUC guidelines. Rats were injected I.P. with an overdose of sodium pentobarbital, 100 mg/kg. After euthanasia, postmortem analysis of the animals' spines was performed. Rats' spines were harvested and fixed in PFA 4% for 12 hours. After fixation, the spines were rinsed in running tap water, then freed of excess tissue and decalcified with hydrochloric acid for 8 hours. A second dissection was made to localize the tumor engrafted L5 vertebral body, and the entire segment was carefully trimmed to include only the segment immediately above and below the vertebral body of interest. The segments were then processed for dehydration, clearing, and infiltration with paraffin. Sagittal sections of tissue were obtained at a thickness of 15 μ m. Sections were dried, deparaffinized, and stained with hematoxylin and eosin (H&E) by standard laboratory protocols. An independent blinded pathologist then analyzed stained sections for the presence or absence of tumor cells.

Statistical analysis

A comparative analysis of the length, duration and velocity of strides in both right (R) and left (L) hind limbs was performed between tumor, control and sham groups by using one-way analysis of variance and Tukey post hoc test to compare the means. Alpha value was set at $P = 0.05$. Graph Pad Prism v6.0 software (GraphPad Software, San Diego, CA USA) was used for all the statistical analyses. Results for gait locomotion and nociceptive testing are reported as the mean $\pm$ standard error.

Results

Xenograft Characteristics in the flank

After 25 days of the subcutaneous injection of the breast cancer cells in the left flank, all the rats developed small and round-shaped tumors that reached 2 cm³. Upon gross macroscopic evaluation, these tumors showed an external capsule with a multilobular growth pattern, characteristic of mammary adenocarcinoma [29, 38]. Histologically, H&E of the tumor slides showed the classical histology of mammary adenocarcinoma.

Human derived metastatic breast cancer growth in the vertebral body of rodents

Immediately after euthanasia, the metastatic spine lesions of all tumor-engrafted rats were resected. Intact control and sham-treated rats demonstrated normal histology. On inspection, the tumor-bearing vertebral bodies were markedly osteolytic, demonstrating complete destruction in some areas of the bone and replacement with tumor cells. The tumor had a lighter appearance and a jelly-like consistency (Figure 5A, gross morphology). The tumor-bearing portion of the vertebral body was poorly demarcated, with some invasion into the surrounding musculature in two rats. A representative paraffin section from a tumor-engrafted rat, stained with H&E is shown in Figure 5B. In tumor-engrafted rats, there was a near-complete obliteration of the vertebral body (Figure 5A). The majority of the bone marrow and intradural space was filled with tumor cells (Figure 5B), which is a characteristic of the osteolytic phenotype of breast metastases.

Representative dorsal views are showed in the BLI images in Figure 2. Bioluminescence performed at Day 10 showed tumor growth in all animals that increased from Day 10 to Day 40 (Figure 2A). One rat died at Day 35 from tumor-related complications; another one developed hemiparesis of the left hind limb at Day 30. CBCT was performed at day 40; osteolytic lesions were observed (Figure 2B–C white arrows) with a decrease in the density of the bone in the lumbar spine consistent with the tumor location as demonstrated by the bioluminescence imaging (Figure C).

Human orthotopic breast cancer growth affected the locomotion and nociceptive response in rats

After 40 days of tumor engraftment, we determined the effect of the spine tumor on the locomotor behavior of rats. Analysis of the hind limbs revealed that control and sham group rats had a very well defined gait (Figure 3A–B) with clearly differentiated stance and swing phases, whereas tumor-engrafted rats (Figure 3C) exhibited clear gait disturbances in the left

and right hind limb movements. Significant gait deterioration was observed in tumor-engrafted rats compared with control or sham groups.

Stride length of the right hind limb in tumor-engrafted rats showed a significant decrease as compared was decreased in comparison to control and sham groups (TR: $2.5 \pm .36$ cm, CR: $3.0 \pm .53$ cm and SR: $3.0 \pm .56$ cm, $P = .02$) but no differences were observed in the left hind limb stride length (TL: $2.5 \pm .51$ cm, CL: $2.6 \pm .46$ cm and SL: $2.6 \pm .46$ cm, $P = .11$; Figure 3D). Stride duration of the right hind limb is larger in tumor rats than in control and sham rats (TR: $.46 \pm .09$ sec, CR: $.32 \pm .06$ sec and SR: $.37 \pm .09$ sec, respectively, $P = .002$) and in left hind limb strides (TL: $.46 \pm .13$ sec, CL: $.35 \pm .05$ sec and SL: $.34 \pm .03$ sec, $P = .0006$. Figure 3E). Accordingly to the latter, both right and left hind limb stride speed showed a noticeable decrement in tumor rats as compared with control and sham rats (Right hind limb, TR: 5.6 ± 1.3 cm/sec, CR: 9.8 ± 2.5 cm/sec, SR: 8.46 ± 3.3 cm/sec, respectively; $P = .001$. Left hind limb, TL: 5.83 ± 1.87 cm/sec, CL: 7.55 ± 1.80 cm/sec, SL: 8.42 ± 2.0 cm/sec, respectively; $P = .002$. Figure 3F).

When analyzing pain response results at Day 40 (Figure 4) from intact control and sham-treated animals we found that the maximum tolerated applied mechanical force was 435 ± 59 grams and 428 ± 71 grams, respectively with the spine pressure test, which was similar between these two groups ($P > .05$). Conversely, we found that the maximum tolerated applied mechanical force was 320 ± 58 grams in the tumor-engrafted rats that was statistically significantly different from the control and sham groups ($P = .01$) (Figure 4A). In terms of withdrawal time, the intact control group had a withdrawal time of 11 ± 8.2 sec, whereas the sham-treated animals had a withdrawal time of 11 ± 6.8 sec, which was not significant ($P > .05$) (Figure 4B). Comparably, the tumor-engrafted animals had a withdrawal time of 1.7 ± 0.82 sec, which was statistically significant ($P = .02$). In summary, there was a significant decrease in the latency and spine withdrawal threshold compared with sham and control rats, indicative of mechanical hyperalgesia following bone tumor invasion.

Discussion

Naturally occurring metastatic cancer in animals is unusual and unpredictable. In order to develop a practical and accurate animal model of bone metastasis, it is necessary to orthotopically inject or engraft cancer cells in a focal manner. In the present work, we show a human-derived metastatic breast cancer model in rat's spine. In this model, a metastatic lesion from the vertebral body of immunocompromised rats was able to invade, compress the spinal cord (Figure 5A–B), and cause gait impairment (Figure 3C–F), hyperalgesia, and allodynia 40 days after tumor engraftment (Figure 4A–B). Our model is an accurate and reliable representation to investigate bone-associated cancer pain derived from a solitary metastatic breast cancer mass to the spine with clinical and radiographical characteristics of human spinal metastatic breast cancer. As of today, some animal models have been developed to study metastases to the bone, but none of them have explored the pain response evoked by metastatic tumors. Other models of spine metastasis [30, 39–44] either by spontaneous bone metastasis [45, 46], or systemic injection of cancer cells [40] allow for the study of mechanisms that lead to the metastatic process. However, their variable and comorbidity nature do not allow a consistent assessment of tumor-induced pain. In our model,

we use human derived tissue engrafted in immunocompromised rats and the neurological decline was reproducible, as we have previously described in a similar model of human metastatic prostate cancer. The orthotopic spine breast cancer model used in this work to develop vertebrae metastasis, without compromising other organs, offers the advantage of demonstrating consistent and reproducible significant decline in pain response and locomotion parameters.

Tumor Morphology and imaging characteristics

We have described in our results that breast cancer engrafted in the vertebral body of athymic rats retained the human morphological and histological characteristics of metastatic human mammary adenocarcinoma. This is similar to the presentation in humans where metastatic breast cancer tumors range from localized or small tumors deposits to massive invasion, bone lysis, and vertebral collapse [47]. In addition, the radiological findings on computer tomography scans of our model show osteolysis signs similar to those observed in patients [47]. Further studies will focus on the correlation between tumor size and extension of osteolysis and neurological signs.

Clinical Evaluation

Patients with bone metastasis suffer from neurological abnormalities such as sensory loss, paraparesis, or paraplegia, related to pain [17]. Cancer pain is complex, pain treatment for patients with terminal disease is difficult and most of the times cannot be managed by systemic analgesics [48]. Bone metastasis tends to induce alterations in the skin sensation overlying sites of skeletal disease and increased sensitivity in response to warm stimuli or pinprick, heat hyperalgesia, and mechanical allodynia [49], similar to what we reported in our research.

Cancer pain due to spine metastasis is poorly characterized. Patients with spine tumors suffer from neuropathic pain and radicular pain during the course of their illness. Pain in the lower back is very common [50–53]. In our model we observed that the proliferation of tumor cells produced bone destruction of the trabecular and cortical bone. We evaluated the nociceptive response to a mechanical, since the major cutaneous receptors types are found in the skin [54], the mechanical hyperalgesia observed in the tumor implanted rats could be explained by the compression produced by the BCa tumor invasion into the spinal cord canal.

Motor dysfunction is another symptom observed in patients with spine tumors [55]. In current models the clinical evaluation is done by observational findings or by semi-quantitative rating scales (BBB modified scale) after tumor engraftment, progression of paraparesis until paraplegia [30, 40, 41, 56–58]. In our model we implemented a quantitative methodology to evaluate gait impairment in tumor implanted rats and demonstrated that stride length, velocity and time in the left hind limbs was affected; in some rats the right hind limb had a compensatory role by supporting more weight during locomotion. Interestingly, compared with the control and sham groups, the tumor-engrafted rats exhibited a decrease in the stride lengths in the left hind limb due to the decrease in the length in the stance and swing phases. In patients the majority of abnormalities of pathologic gait are

observed during the stance phase, since whole body weight is then being supported by one leg [59, 60]. In our model, we observed abnormalities in the swing and stance phase in the tumor-engrafted rats. Similarly to other orthotopic cancer models [28, 61], in our control rats, during the stance phase, the dorsiflexion in the ankle reached its peak near the mid stance phase, and then plantar flexion was observed until the end of this phase. Histological findings in this experiment produce similar compression findings found in humans leading to myelopathy that present severe pain and severe motor deficits that leading to gait deterioration [62–66]. In summary, this breast cancer metastatic model to the spine constitutes an advance in the study of this malignant disease. It settles the base for further studies to improve our understanding of the neurological deficits that occurs when breast cancer and other metastatic tumors affect the spine. Understanding the pathophysiology of metastatic breast cancer will help to develop therapeutics and treatments that might lead to a decrease in morbidity and mortality in these patients.

Conclusions

We have developed a model of metastatic spine cancer for the quantitative analysis of pain and function in an animal model. Rats were followed for 40 days using imaging techniques to evaluate tumor progression and bone integrity. Tumor engrafted rats showed locomotor and sensory deficits that are in accordance with the clinical manifestation in patients with spine metastasis. Our intraspinal preclinical model of a single metastasis represents a reliable method to evaluate experimental therapeutic approaches.

References

1. Esteva FJ, Valero V, Pusztai L, Boehnke-Michaud L, Buzdar AU, Hortobagyi GN. Chemotherapy of metastatic breast cancer: what to expect in 2001 and beyond. *The oncologist*. 2001; 6(2):133–46. [PubMed: 11306725]
2. American Cancer Society. *Cancer Facts & Figures 2014*. Atlanta: American Cancer Society; 2014. Available from: <http://www.cancer.org/acs/groups/content/%40research/documents/webcontent/acspc-042151.pdf>
3. Briasoulis E, Karavasilis V, Kostadima L, Ignatiadis M, Fountzilas G, Pavlidis N. Metastatic breast carcinoma confined to bone: portrait of a clinical entity. *Cancer*. 2004; 101(7):1524–8. [PubMed: 15316943]
4. Cowey S, Szafran AA, Kappes J, et al. Breast cancer metastasis to bone: evaluation of bioluminescent imaging and microSPECT/CT for detecting bone metastasis in immunodeficient mice. *Clin Exp Metastasis*. 2007; 24(5):389–401. [PubMed: 17541709]
5. Mantyh P. Bone cancer pain: causes, consequences, and therapeutic opportunities. *Pain*. 2013; 154(Suppl 1):S54–62. [PubMed: 23916671]
6. Mercadante S. Malignant bone pain: pathophysiology and treatment. *Pain*. 1997; 69(1–2):1–18. [PubMed: 9060007]
7. Gokaslan ZL. Spine surgery for cancer. *Curr Opin Oncol*. 1996; 8(3):178–81. [PubMed: 8804815]
8. Smith HS, Barkin RL. Painful boney metastases. *Am J Ther*. 2014; 21(2):106–30. [PubMed: 23344095]
9. Kassamali RH, Ganeshan A, Hoey ET, Crowe PM, Douis H, Henderson J. Pain management in spinal metastases: the role of percutaneous vertebral augmentation. *Ann Oncol*. 2011; 22(4):782–6. [PubMed: 20966180]
10. Coleman RE. Skeletal complications of malignancy. *Cancer*. 1997; 80(8 Suppl):1588–94. [PubMed: 9362426]

11. Coleman RE. Metastatic bone disease: clinical features, pathophysiology and treatment strategies. *Cancer Treat Rev.* 2001; 27(3):165–76. [PubMed: 11417967]
12. Coleman RE. Clinical features of metastatic bone disease and risk of skeletal morbidity. *Clin Cancer Res.* 2006; 12(20 Pt 2):6243s–9s. [PubMed: 17062708]
13. Sciubba DM, Petteys RJ, Dekutoski MB, et al. Diagnosis and management of metastatic spine disease. A review. *J Neurosurg Spine.* 2010; 13(1):94–108. [PubMed: 20594024]
14. Ju DG, Yurter A, Gokaslan ZL, Sciubba DM. Diagnosis and surgical management of breast cancer metastatic to the spine. *World J Clin Oncol.* 2014; 5(3):263–71. [PubMed: 25114843]
15. Laufer I, Sciubba DM, Madera M, et al. Surgical management of metastatic spinal tumors. *Cancer Control.* 2012; 19(2):122–8. [PubMed: 22487974]
16. Wong M, Pavlakakis N. Optimal management of bone metastases in breast cancer patients. *Breast Cancer (Dove Med Press).* 2011; 3:35–60. [PubMed: 24367175]
17. Colbert SD, Ramakrishna S, Harvey JR, Brennan PA. Metastases in the cervical spine from primary head and neck cancers: current concepts of diagnosis and management. *Br J Oral Maxillofac Surg.* 2016
18. Cossigny D, Quan GM. In vivo animal models of spinal metastasis. *Cancer Metastasis Rev.* 2012; 31(1–2):99–108. [PubMed: 22090011]
19. Yanagisawa Y, Furue H, Kawamata T, et al. Bone cancer induces a unique central sensitization through synaptic changes in a wide area of the spinal cord. *Mol Pain.* 2010; 6:38. [PubMed: 20602757]
20. Muralidharan A, Smith MT. Pathobiology and management of prostate cancer-induced bone pain: recent insights and future treatments. *Inflammopharmacology.* 2013; 21(5):339–63. [PubMed: 23918298]
21. Bloom AP, Jimenez-Andrade JM, Taylor RN, et al. Breast cancer-induced bone remodeling, skeletal pain, and sprouting of sensory nerve fibers. *J Pain.* 2011; 12(6):698–711. [PubMed: 21497141]
22. Hargreaves K, Dubner R, Brown F, Flores C, Joris J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain.* 1988; 32(1):77–88. [PubMed: 3340425]
23. Bonin RP, Bories C, De Koninck Y. A simplified up-down method (SUDO) for measuring mechanical nociception in rodents using von Frey filaments. *Mol Pain.* 2014; 10:26. [PubMed: 24739328]
24. Pacharinsak C, Beitz A. Animal models of cancer pain. *Comp Med.* 2008; 58(3):220–33. [PubMed: 18589864]
25. Lindsay TH, Jonas BM, Sevcik MA, et al. Pancreatic cancer pain and its correlation with changes in tumor vasculature, macrophage infiltration, neuronal innervation, body weight and disease progression. *Pain.* 2005; 119(1–3):233–46. [PubMed: 16298491]
26. Mogil JS, Crager SE. What should we be measuring in behavioral studies of chronic pain in animals? *Pain.* 2004; 112(1–2):12–5. [PubMed: 15494180]
27. Sevcik MA, Jonas BM, Lindsay TH, et al. Endogenous opioids inhibit early-stage pancreatic pain in a mouse model of pancreatic cancer. *Gastroenterology.* 2006; 131(3):900–10. [PubMed: 16952558]
28. Sarabia-Estrada R, Zadnik PL, Molina CA, et al. A rat model of metastatic spinal cord compression using human prostate adenocarcinoma: histopathological and functional analysis. *Spine J.* 2013; 13(11):1597–606. [PubMed: 23810458]
29. Zadnik P, Sarabia-Estrada R, Groves ML, et al. A novel animal model of human breast cancer metastasis to the spine: a pilot study using intracardiac injection and luciferase-expressing cells. *J Neurosurg Spine.* 2013; 18(3):217–25. [PubMed: 23259542]
30. Mantha A, Legnani FG, Bagley CA, et al. A novel rat model for the study of intraosseous metastatic spine cancer. *J Neurosurg Spine.* 2005; 2(3):303–7. [PubMed: 15796355]
31. Luque Contreras D, Jimenez Estrada I, Martinez Fong D, et al. Hindlimb claudication reflects impaired nitric oxide-dependent revascularization after ischemia. *Vascul Pharmacol.* 2007; 46(1):10–5. [PubMed: 17011243]
32. Sarabia-Estrada R, Banuelos-Pineda J, Osuna Carrasco LP, et al. Aberrant gastrocnemius muscle innervation by tibial nerve afferents after implantation of chitosan tubes impregnated with

- progesterone favored locomotion recovery in rats with transected sciatic nerve. *J Neurosurg.* 2015; 123(1):270–82. [PubMed: 25679274]
33. Lopez Ruiz JR, Osuna Carrasco LP, Lopez Valenzuela CL, et al. The hippocampus participates in the control of locomotion speed. *Neuroscience.* 2015; 311:207–15. [PubMed: 26597762]
 34. Razgado-Hernandez LF, Espadas-Alvarez AJ, Reyna-Velazquez P, et al. The transfection of BDNF to dopamine neurons potentiates the effect of dopamine D3 receptor agonist recovering the striatal innervation, dendritic spines and motor behavior in an aged rat model of Parkinson's disease. *PLoS One.* 2015; 10(2):e0117391. [PubMed: 25693197]
 35. Bordet T, Buisson B, Michaud M, et al. Specific antinociceptive activity of cholest-4-en-3-one, oxime (TRO19622) in experimental models of painful diabetic and chemotherapy-induced neuropathy. *J Pharmacol Exp Ther.* 2008; 326(2):623–32. [PubMed: 18492948]
 36. Wong J, Armour E, Kazanzides P, et al. High-resolution, small animal radiation research platform with x-ray tomographic guidance capabilities. *Int J Radiat Oncol Biol Phys.* 2008; 71(5):1591–9. [PubMed: 18640502]
 37. Burch S, Bisland SK, Wilson BC, Whyne C, Yee AJ. Multimodality imaging for vertebral metastases in a rat osteolytic model. *Clin Orthop Relat Res.* 2007; 454:230–6. [PubMed: 16924176]
 38. Molina CA, Sarabia-Estrada R, Gokaslan ZL, et al. Delayed onset of paralysis and slowed tumor growth following in situ placement of recombinant human bone morphogenetic protein 2 within spine tumors in a rat model of metastatic breast cancer. *J Neurosurg Spine.* 2012; 16(4):365–72. [PubMed: 22264176]
 39. Amundson E, Pradilla G, Brastianos P, et al. A novel intravertebral tumor model in rabbits. *Neurosurgery.* 2005; 57(2):341–6. discussion -6. [PubMed: 16094165]
 40. Arguello F, Baggs RB, Duerst RE, Johnstone L, McQueen K, Frantz CN. Pathogenesis of vertebral metastasis and epidural spinal cord compression. *Cancer.* 1990; 65(1):98–106. [PubMed: 2293874]
 41. Cossigny DA, Mouhtouris E, Dushyanthen S, Gonzalvo A, Quan GM. An in vivo mouse model of intraosseous spinal cancer causing evolving paraplegia. *J Neurooncol.* 2013; 115(2):189–96. [PubMed: 23955595]
 42. Miki T, Yano S, Hanibuchi M, Sone S. Bone metastasis model with multiorgan dissemination of human small-cell lung cancer (SBC-5) cells in natural killer cell-depleted SCID mice. *Oncol Res.* 2000; 12(5):209–17. [PubMed: 11417746]
 43. Takahashi M, Ogawa J, Kinoshita Y, Takakura M, Mochizuki K, Satomi K. Experimental study of paraplegia caused by spinal tumors: an animal model of spinal tumors created by transplantation of VX2 carcinoma. *Spine J.* 2004; 4(6):675–80. [PubMed: 15541702]
 44. Tatsui CE, Lang FF, Gumin J, Suki D, Shinjima N, Rhines LD. An orthotopic murine model of human spinal metastasis: histological and functional correlations. *J Neurosurg Spine.* 2009; 10(6):501–12. [PubMed: 19558282]
 45. Lelekakis M, Moseley JM, Martin TJ, et al. A novel orthotopic model of breast cancer metastasis to bone. *Clin Exp Metastasis.* 1999; 17(2):163–70. [PubMed: 10411109]
 46. Rosol TJ, Tannehill-Gregg SH, LeRoy BE, Mandl S, Contag CH. Animal models of bone metastasis. *Cancer.* 2003; 97(3 Suppl):748–57. [PubMed: 12548572]
 47. Jonsson B, Petren-Mallmin M, Jonsson H Jr, Andreasson I, Rauschnig W. Pathoanatomical and radiographic findings in spinal breast cancer metastases. *J Spinal Disord.* 1995; 8(1):26–38. [PubMed: 7711367]
 48. Jeon YS, Lee JA, Choi JW, et al. Efficacy of epidural analgesia in patients with cancer pain: a retrospective observational study. *Yonsei Med J.* 2012; 53(3):649–53. [PubMed: 22477012]
 49. Scott AC, McConnell S, Laird B, Colvin L, Fallon M. Quantitative Sensory Testing to assess the sensory characteristics of cancer-induced bone pain after radiotherapy and potential clinical biomarkers of response. *Eur J Pain.* 2012; 16(1):123–33. [PubMed: 21658980]
 50. Boriani S, Biagini R, De Iure F, Andreoli I, Lari S, Di Fiore M. Low back pain in tumors: diagnosis and treatment. *Chir Organi Mov.* 1994; 79(1):93–9. [PubMed: 8076483]
 51. Chaichana KL, Pendleton C, Wolinsky JP, Gokaslan ZL, Sciubba DM. Vertebral compression fractures in patients presenting with metastatic epidural spinal cord compression. *Neurosurgery.* 2009; 65(2):267–74. discussion 74–5. [PubMed: 19625904]

52. Sciubba DM, Gokaslan ZL. Diagnosis and management of metastatic spine disease. *Surg Oncol*. 2006; 15(3):141–51. [PubMed: 17184989]
53. Sciubba DM, Gokaslan ZL, Suk I, et al. Positive and negative prognostic variables for patients undergoing spine surgery for metastatic breast disease. *Eur Spine J*. 2007; 16(10):1659–67. [PubMed: 17486376]
54. Hoheisel U, Taguchi T, Treede RD, Mense S. Nociceptive input from the rat thoracolumbar fascia to lumbar dorsal horn neurones. *Eur J Pain*. 2011; 15(8):810–5. [PubMed: 21330175]
55. Harel R, Angelov L. Spine metastases: current treatments and future directions. *Eur J Cancer*. 2010; 46(15):2696–707. [PubMed: 20627705]
56. Hibberd C, Cossigny DA, Quan GM. Animal cancer models of skeletal metastasis. *Cancer Growth Metastasis*. 2013; 6:23–34. [PubMed: 24665205]
57. Wang L, Rahman S, Lin CY, et al. A novel murine model of human renal cell carcinoma spinal metastasis. *J Clin Neurosci*. 2012; 19(6):881–3. [PubMed: 22516545]
58. Zibly Z, Schlaff CD, Gordon I, Munasinghe J, Camphausen KA. A novel rodent model of spinal metastasis and spinal cord compression. *BMC Neurosci*. 2012; 13:137. [PubMed: 23116234]
59. Lehmann JF, Condon SM, de Lateur BJ, Smith JC. Gait abnormalities in tibial nerve paralysis: a biomechanical study. *Arch Phys Med Rehabil*. 1985; 66(2):80–5. [PubMed: 3970662]
60. Winter DA. Biomechanics of normal and pathological gait: implications for understanding human locomotor control. *J Mot Behav*. 1989; 21(4):337–55. [PubMed: 15136250]
61. Sarabia-Estrada R, Ruiz-Valls A, Sha RS, et al. TMOD-35. Primary and recurrent sacral chordoma affects the hind limbs motor and nociceptive function in rats: an orthotopic spine model. *Neuro-Oncology*. 2016; 18(suppl 6):vi214.
62. Hirschfeld A, Beutler W, Seigle J, Manz H. Spinal epidural compression secondary to osteoblastic metastatic vertebral expansion. *Neurosurgery*. 1988; 23(5):662–5. [PubMed: 3200401]
63. Liu YS, Liu SB, Li DF, Fan HT, Huai JY, Guo J. An exploration on the radiological features associated with motor deficits in patients with metastatic epidural spinal cord compression. *Eur Rev Med Pharmacol Sci*. 2015; 19(2):274–9. [PubMed: 25683941]
64. Loblaw DA, Laperriere NJ. Emergency treatment of malignant extradural spinal cord compression: an evidence-based guideline. *J Clin Oncol*. 1998; 16(4):1613–24. [PubMed: 9552073]
65. Rodriguez M, Dinapoli RP. Spinal cord compression: with special reference to metastatic epidural tumors. *Mayo Clin Proc*. 1980; 55(7):442–8. [PubMed: 7382554]
66. Sundaresan N, Scher H, DiGiacinto GV, Yagoda A, Whitmore W, Choi IS. Surgical treatment of spinal cord compression in kidney cancer. *J Clin Oncol*. 1986; 4(12):1851–6. [PubMed: 2431111]

Figure 1.

Human orthotopic breast cancer model in the spine of immunocompromised rats. A) RBC3 cells were injected in the flank of 5-week old female rats, after 3 weeks subcutaneous tumors developed. B) Illustrations showing the engrafting procedure in the L5 vertebral body with subcutaneous tumor tissue from donor rats.

Figure 2.

RBC3 Tumor growth imaging. A. Bioluminescence image from a rat with tumor showing tumor signal after 10 and 40 days of tumor engraftment. B. Small animal CBCT images from a naive, sham and tumor engrafted rat that shows osteolysis of the ventral and posterior elements of the L5 vertebra body (white arrows). C. CBCT axial image of the L5 vertebra body from a tumor rat.

Figure 3.

Breast metastatic tumor to the spine induces gait locomotion alterations in rats. A. Anatomical reference points in the hind limb joints. B–C. Superimposed images of four consecutive stride cycles on a graphic representation to evaluate hind limb joints. The tumor group suffered from an extended period of stance and a decrease in the swing phase D. Gait parameters evaluated at 40 days after tumor implantation showed a significance decrease in d, stride length right, $*p=.02$; stride length left, $*p=.11$. E. Stride duration right, $*p=.002$; stride duration left $*p=.0006$ stride velocity right, $*p=.002$; F. Stride velocity left $*p=.001$.

Figure 4.

Breast metastatic tumor to the spine induces mechanic hyperalgesia in rats. Time withdrawal and grams of force was evaluated. Mechanic nociceptive response was significantly reduced in pressure (A) and time (B) in rats with tumor compared with control rats *P 0.05. Pressure applied, *p=.01; Withdrawal response, *p=.02.

Figure 5.

Spine breast cancer tumors invade the vertebral body in the tumor-engrafted rats. A. Panel shows the L5 vertebra with severe tumor invasion in the vertebral bony components; spinous processes are affected by tumor growth. Spinal cord is totally displaced by the tumor invasion into the epidural space. B. H& E stain of rat's 5th lumbar vertebrae. sc=spinal cord; t=tumor cells; sp, spinous process; * bone trabeculae in the vertebral body (vb).