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ORIGINAL PAPER

Establishment and characterization of xenograft models of human neuroblastoma bone metastasis

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Abstract
Objects To improve the therapy of advanced neuroblastoma (NB), it is critical to develop animal models that mimic NB bone metastases. Unlike the human disease, NB xenograft models rarely metastasize spontaneously to bone from the orthotopic site of primary tumor growth.
Methods Single-cell suspensions of SY5Y, KCNR NB cells were injected directly into the femur of nude mice. Radiological and histological analyses and immunohistochemistry analyses were performed to characterize these osseous NB models. SY5Y and KCNR result in osteolytic responses.
Results We have detected osteoprotegerin, receptor activator of nuclear factor kappa B ligand, parathyroid hormone-related protein, and endothelin-1, proteins associated with bone growth and osteolysis, and C-X-C chemokine receptor type 4 (CXCR4) involved in tumor growth and tumor cell migration in the NB cells grown in the bone.
Conclusions These animal models can be used to study biological interactions, pathways, and potential therapeutic targets and also to evaluate new agents for treatment and prevention of NB bone metastasis.

Keywords Neuroblastoma · Bone metastasis · Osteolytic response · Animal model

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Introduction

Neuroblastoma (NB) is the most commonly diagnosed neuroendocrine extracranial solid cancer in childhood and infancy. NB accounts for 8–10 % of all childhood cancers and for approximately 15 % of cancer deaths in children [4, 13, 23]. It is associated with poor prognosis because of its ability to regress spontaneously, transform, or show aggressive behavior [2, 16, 17]. Approximately 70 % of NB patients present metastatic dissemination at diagnosis, and the bone, bone marrow, and liver are the most common metastatic sites [7]. Patients with metastatic NB experience frequent bone metastasis (56 %) [5]. NB leads in a high percentage of patients to metastases with the radiographic appearance of osteoclastic reactions [7, 19]. The consequences of bone metastasis are often devastating. Osteolytic metastases can cause severe pain, pathologic fractures, life-threatening hypercalcemia, spinal cord compression, and other nerve compression syndromes [20]. Despite the overall osteoclastic nature of NB bone metastasis, a number of studies suggest that simultaneous upregulation of osteoblastic bone metastasis occurs [24]. Bone metastasis is a complex process, and there exists a close interaction between metastatic tumor cells and the unique environment of the bone and bone marrow. Many mechanisms controlling this process remain unknown, although certain key factors and specific pathways responsible for bone metastasis have been identified [1, 11, 14, 24].

A classic review by Ara and DeClerck [1] provides an in-depth discussion of such factors and their potential relationship to NB bone metastases. Generation of suitable in vivo models is critical for understanding the interactions between NB cells and the bone microenvironment. Although much effort has been expended in the production of new NB bone metastasis xenografts, as a useful alternative, attention often was focused on bone invasion characteristic using bone invasion model via direct injection of tumor cells in bone

marrow cavity, which ideally mimics biochemical interactions of NB cells with the bone environment.

The propensity of NB to metastasize to bone is not well understood at the mechanistic level. After NB cells infiltrate the bone marrow, there are at least two possible modes of engraftment. One is purely mechanical and involves trapping of NB cells by filtration through the marrow. The other is biological and is derived from the seed and soil hypothesis of Paget, which holds that specific interactions between the NB cells and the bone marrow microenvironment are required for growth to occur [28].

In this model, the NB cells express proteins associated with osteoclast maturation and differentiation and osteolytic response. Mechanism of bone invasion/metastasis in NB is still unclear. NB tumors have been reported to express receptor activator of nuclear factor kappa B ligand (RANKL) which directly stimulates osteoclasts. Through the production of parathyroid hormone-related protein (PTHrP) which is stimulated by BDNF produced by osteoblasts, RANKL expression by osteoblasts is enhanced, which results in further activation and maturation of osteoclasts. In the absence of expression of RANKL, NB cells can interact with bone marrow mesenchymal stem cells or tumor-associated macrophages that secrete SDF-1, MMPs, and OAFs like IL-6, IL-8, and MIP-1 β [1, 6, 31].

Herein, we report on the direct injection of two NB cell lines (SY5Y and KCNR) into the femur of nude mice and characterization of these osseous NB models. Histology indicates that both SY5Y and KCNR yield osteolytic responses. We also examined expression of various representative proteins associated with bone growth and osteolysis in these osseous NB models.

Materials and methods

Cell lines and xenografts

SY5Y and KCNR cell lines were purchased from the American Type Culture Collection (Rockville, MD). Cells were maintained in complete medium comprising Iscove's modified Dulbecco's medium (BioWhittaker, Walkersville, MD) supplemented with 3 mM L-glutamine (Gemini Bioproducts, Inc., Calabasas, CA); 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml of selenous acid (ITS Culture Supplement; Collaborative Biomedical Products, Bedford, MA); and 20 % heat-inactivated fetal bovine serum (Omega Scientific, Tarzana, CA). All cell lines used were less than passage 30. The cell lines were cultured devoid of antibiotics in a humidified atmosphere of 5 % CO_2/95 % air. All cell lines tested negative for mycoplasm and were not selected for drug resistance in vitro.

Preparation of murine animal

Female 4–6-week-old athymic BALB/c (nu/nu) mice were obtained from Charles River, Wilmington, MA. Cages with laminar-flow air delivery system (Lab Products, Seaford, DE) and filter cage bonnets on polycarbonate microisolator cages lined with autoclaved bedding were used to maintain an aseptic environment. Autoclaved and acidified (pH=4–6) water and autoclaved standard Purina mouse chow were provided ad libitum. Mice were exposed to 12-h cycles of light and dark. All procedures were performed in compliance with the Shengjing Hospital of China Medical University Institutional Animal Care and Use Committee.

Establishment of xenograft models of NB bone invasion and metastasis

To establish invasive bone tumor models by direct injection, we used female athymic BALB/c (nu/nu) mice. Group comprised five mice for both NB cell lines. Inhalational anesthesia was used as described previously [25]. A small incision (10 mm) was made along the right knee, and the patellar tendon and muscle were split longitudinally to expose the distal femur. NB cells were suspended in serum-free L-15 (calcium- and magnesium-free) medium without fetal bovine serum (FBS) at a concentration of 20×10^6 cells/ml, A 30-gage needle attached to a Hamilton 10-μl syringe (Hamilton, Reno, NV) was inserted ~3 mm into the distal end of the femur using a 26-gage needle stabilized with a drill holder, and 2 μl cell suspension in L-15 medium (without FBS) containing 2×10^5 tumor cells was injected into the bone marrow cavity. After the procedures, animals were placed on a body temperature controller and monitored carefully for sign of discomfort until fully recovered. The study was carried out in accordance with the institutional ethical guidelines and was approved by the Medical Ethics Committee of China Medical University.

Determination of NB bone invasion and metastasis from radiographs

All animals were performed on radiographs weekly. Inhalational anesthesia was used to provide the few minutes needed to complete X-ray imaging procedure. A Faxitron MX-20 small animal X-ray device (Faxitron X-ray Corp., Wheeling, IL) and mammography computed radiography cassettes with high-resolution screens and high-detail single-emulsion mammography film and screens (Fuji EC-MA cassette, Fuji Photo Film Co., Japan) were used. Animals were placed on optimal position to ensure imaging of the caudal portion of them, including both hind legs.

A grading system for bone lesions in mice was established to provide quantitative scoring of the bone invasion

and metastasis. The system defines four grades: grade 1, a normal bone when compared with the contralateral femur; grade 2, the presence of asymmetric, nonprogressive radiolucent lesions limited to the distal femur or those associated with the trauma of intraosseous injection; grade 3, asymmetrically osteolytic and progressive radiolucencies extending beyond the distal femur; and grade 4, the presence of a pathological fracture of the bone or a breach in the bone cortex. Each radiograph was determined by two independent radiologists. The time to form a grade 4 lesion or the highest grade lesion when animal was sacrificed was used as an end point to evaluate tumor progression and invasive ability of NB cell lines.

Histological analyses of NB xenografts

For histological analysis, the bone samples were dissected en bloc with acetabulum, soft tissues, and muscles and were processed by fixation in 10 % formalin containing 2 % sucrose. Samples were then decalcified in 0.37 % unbuffered formaldehyde containing 5.5 % EDTA (pH 6.0–6.5) for 1–2 weeks and embedded in paraffin. Analysis was performed on 5-μm sagittal sections of femurs. H&E staining was used for histological verification of tumor cells invasiveness in the bone.

Histological presentations were classified into four types for accurate evaluation of the extent of bone invasion and metastasis: grade 1, neither bone nor bone marrow invasion; grade 2, invasion confined in bone marrow cavity; grade 3, invasion extending outside the bone area, but underneath bone membrane; and grade 4, invasion beyond the bone membrane and infiltrate into soft tissues, even muscle. All images were evaluated by two independent pathologists. The time to develop the highest grade lesion when mouse was killed was used to determine tumor cell invasiveness capability of NB cell lines.

Immunohistochemistry

The Medical Ethics Committee of China Medical University approved our experimental protocols. For immunohistochemistry, bone samples were processed by fixation in 10 % neutral buffered formalin for 24 h. Samples were then decalcified in 10 % formic acid and embedded in paraffin. Staining was performed to determine expression of osteoprotegerin (OPG), RANKL, PTHrP, endothelin-1 (ET-1), and CXCR4. Staining was performed on 5-mm sections which were deparaffinized in xylene and rehydrated through graded alcohols. Washes were with phosphate-buffered saline which was blocked by incubation with 0.3 % hydrogen peroxide in phosphate-buffered saline for 10 min. To minimize nonspecific binding in the xenograft tissues, we used a serum block which contained horse, goat, and chicken serum (5 % each) for 1 h. The primary antibodies were diluted in the serum-blocking solution. Antigen retrieval was performed only for the anti-PTHrP antibody, based on the manufacturer's recommendations. Primary antibody information and conditions used are summarized in Table 1. Control slides were treated in the same way with the exception that rabbit IgG or preimmune serum was used instead of primary antibodies. Biotinylated secondary anti-rabbit antibodies were detected with the ABC kit (Vector Laboratories, Burlingame, CA) and DAB as substrate. Slides were counterstained with hematoxylin, and coverslipped with Histomount (Zymed, South San Francisco, CA). Samples were counted as positive when >10 % of tumor cells were positive.

Statistical analysis

Statistical analyses were done using SPSS 17.0. Data graphed with error bars represent mean and SD. A two-sided Student's *t* test was used to determine the significance of any differences.

Results

To determine whether local injection in the femur could cause osteolytic lesions in bone invasion models, SY5Y and KCNR cells were either injected into the femur of five athymic BALB/c (nu/nu) mice, respectively. Animals were observed daily, X-rayed weekly, and subsequently sacrificed when a grade 4 lesion was presented in the radiographs, or the presence of significant discomfort was noted. The SY5Y and KCNR cells injected into the femur both exhibited tumor invasiveness in the bone radiographically (Fig. 1), and both of them produced macroscopically lytic expansible lesion with soft tissue abnormalities (Fig. 2).

Histological evaluation showed that SY5Y and KCNR cells in the femur resulted in osteolytic lesions; cortical shafts were destroyed, and eroded surfaces were observed. Histological examination of the invaded femur showed large numbers of osteoclasts lining and eroding the bone. No new bone formation was observed (Fig. 3).

To evaluate the osseous NB xenografts as models of human NB bone metastasis, we characterized the xenografts with regard to the presence of a number of proteins commonly expressed in human metastases and known to play important roles in bone invasion and metastasis. We detected OPG, RANKL, PTHrP, ET-1, and CXCR4 immunoreactivity in all xenografts in the bone. Comparison of staining intensity of bone versus contralateral normal bone showed that expression of all these proteins appeared to be upregulated in NB cells in the bone environment (data not shown) strikingly, except CXCR4. Representative staining

Table 1 Antibodies and conditions used in detection of immunoreactivity

Antigen	Manufacturer	Type	Concentration (mg/ml)	Antigen retrieval	Conditions
OPG	Chemicon	Rabbit serum	1:2,500	None	Overnight, 4 °C
RANKL	Calbiochem	Rabbit polyclonal	16	None	1 h, room temperature
PTHrP	Calbiochem	Rabbit polyclonal	4	Neuraminidase, 37 °C, 30 min	Overnight, 4 °C
ET-1	Chemicon	Rabbit polyclonal	9	None	Overnight, 4 °C
CXCR4	Calbiochem	Rabbit polyclonal	6	None	Overnight, 4 °C

of SY5Y and KCNR in the femur is presented in Fig. 3. Control staining with unrelated antibodies (preimmune serum or rabbit IgG) was uniformly negative.

Our immumohistostaining results also showed that strong positivities of PTHrP, RANKL, OPG, and ET1 were presented in KCNR and SY5Y cell lines growing tumors. PTHrP immunoreactivity was detected in tumor cells as well as in osteocytes. However, PTHrP, OPG, RANKL, ET-1, and CXCR4 had different expression intensities. Very few positive cells of CXCR4 were found in KCNR and SY5Y cells growing bone metastatic xenografts. The positive expression ratios of PTHrP and RANKL are higher than that of OPG and ET-1 significantly ($p<0.001$). Osteoblasts and osteoclasts can be found in the KCNR and SY5Y cell lines growing bone invasive microenvironment (Fig. 4).

Discussion

NB is the most common extracranial solid tumor in childhood and the most frequently diagnosed neoplasm during infancy [13]. It accounts for 6–10 % of all childhood cancers and 15 % of all cancer-related mortalities in childhood [4, 23]. NB is derived from embryonic neural crest cells that form the peripheral sympathetic nervous system and have a high potential to migrate [10, 27]. It is remarkable for its broad spectrum of clinical behaviors, including spontaneous regression, maturation, or aggressive progression [29]. Despite a tremendous progress in the therapeutic strategy, NB is still associated with poor prognosis and remains a challenge with an unpredictable clinical course and dismal overall outcome for advanced stage disease [9, 10]. Metastasis at diagnosis is common in patients with NB, and sites most frequently involved include the bone marrow, bone, lymph nodes, liver, and intracranial and orbital sites [26]. NB rarely metastasizes to the lungs or the brain. Bone metastasis is the second most common site of metastasis in NB and is observed in 56 % of the patients of metastatic NB [5]. Metastatic NB has a high mortality rate; therefore, understanding the mechanism by which tumor cells invade and metastasize to bone will be of great benefit in applying more effective therapies to control the progression and metastasis of tumor cells to bone and will further help to develop an animal model that more ideally mimics tumor metastasis process in patients [32].

Several factors accounting for the predilection of metastases for bone include high blood flow in areas of red marrow, adhesive molecules derived from tumor cells that bind them to marrow stromal cells and bone matrix, and adhesive interactions that cause the tumor cells to increase the production of angiogenic factors and bone-resorbing factors. Also, it is an important reason that bone is a large repository for immobilized growth factors. These factors are released and activated during bone resorption and further

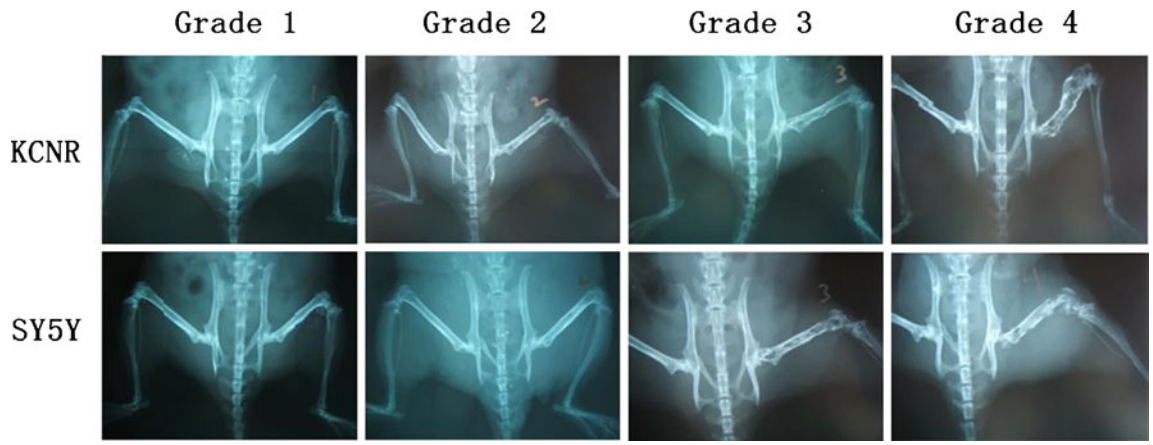

Fig. 1 Radiographs of all four grades were presented. Representative radiographs of the femurs obtained 4 weeks after KCNR and SY5Y tumor cells implantation. The KCNR and SY5Y cells injected into the femur both exhibited tumor invasiveness in the bone radiographically

Fig. 2 Gross pictures or representative images of mice implanted by KCNR and SY5Y cells into femur. Both of them produced macroscopically lytic expansible lesion with soft tissue abnormalities

enhance tumor growth in bone [18, 21]. Metastasis is a highly complex and organized process that consists of multiple but interrelated steps. Three stages are involved in tumor metastasis mechanism including the local invasion to surrounding tissues, tumor cell migration after access to the circulation, and sequent colonization at the distant metastasis site. "Home" to bone specifically as a common metastasis site is determined by a close interaction between some metastatic tumor cells and the unique microenvironment of the bone and bone marrow; on the other hand, it also represent the metastatic potential for bone of different NB cell lines [28].

Establishment of preclinical models provided us a promising solution for studying the bone metastasis mechanism. Ideally, animal models of NB bone metastasis would parallel the human disease in all aspects, but it has proven to be very difficult to develop models of spontaneous NB bone metastasis that mimics the trafficking and biochemical and biological characters of the human disease because of the fact that many NB cell lines do not develop bone lytic lesions until the xenograft model death from disseminated disease at organs other than bone, which prevented us from monitoring the progression of bone lesions and studying the metastasis mechanism of NB cell lines [25, 30]. As a useful alternative for studying biochemical interactions of NB cells with the bone environment, we have explored models wherein NB cells are directly injected into the femur bone marrow cavity of nude mice. Using these models, we observe useful morphological and biochemical parallels with NB growth in bone. However, since the bone metastases are experimentally induced by direct intrafemur injection, these models cannot be used in studies of early metastatic events, such as migration, extravasation, etc. This is an important limitation on the use of these models for the study of trafficking and migration of NB, but the results reported herein suggest that this has little impact on the value of the models for investigation of the interactions between NB and bone. This claim is partly based on the assumption that common expression of factors known to be

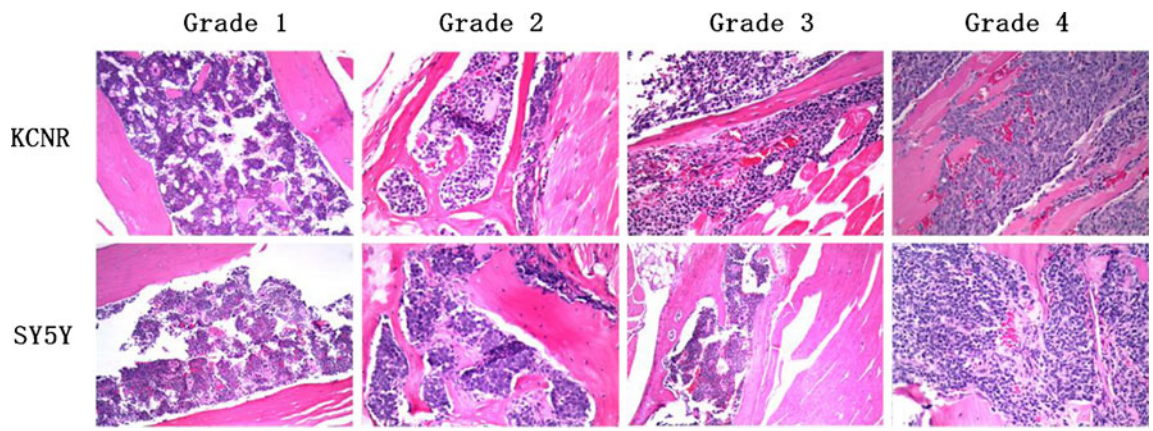

Fig. 3 Hematoxylin-and-eosin (H&E) staining pictures of all four grades were presented, showing H&E staining of KCNR and SY5Y in the femur with an increase in osteoclasts lining the bone (×200). Tumor cells infiltrated into bone and bone membrane, even into muscle

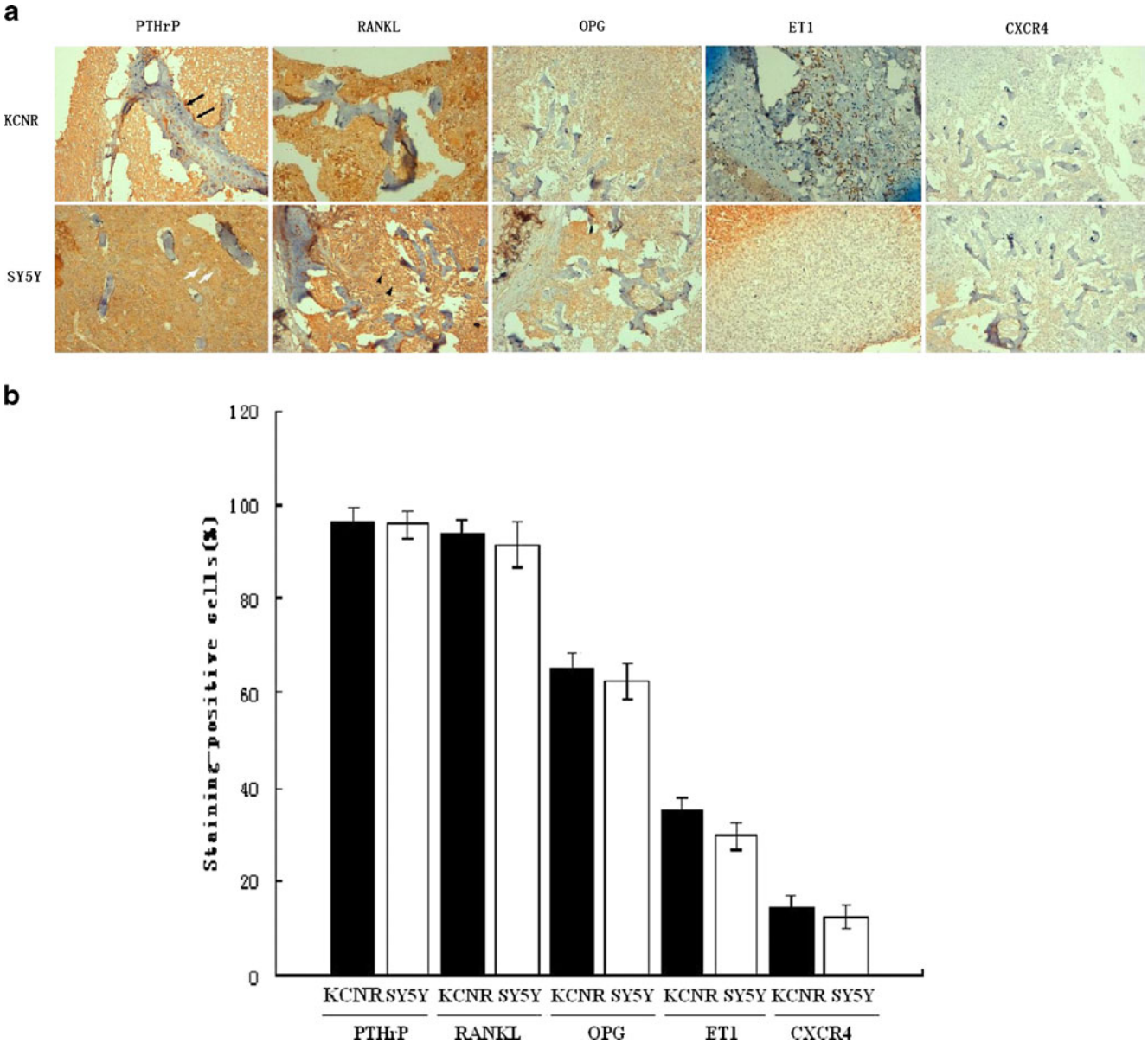

Fig. 4 Immunohistochemistry staining of the presence of PTHrP, OPG, RANKL, ET-1, and CXCR4 immunoreactivity in osteolytic osseous-NB models is presented. **a** Representative histologic sections stained for PTHrP, OPG, RANKL, ET-1, and CXCR4 in tumors obtained in mice 4 weeks after injection of KCNR and SY5Y cells in the femur (×200). *Black arrows* indicate osteoclasts; *white arrows*, staining-positive NB cells. **b** Quantitative analysis of the percentage of staining-positive tumor cells of KCNR and SY5Y cell lines. *Columns* indicate the mean percentage of staining-positive cells in 12 histologic sections; *bars*, SD

involved in bone invasiveness by both NB metastatic cells in our xenografts implies a commonality of pathways giving rise to this expression.

In our study, NB cells injected directly into the femur of nude male mice are capable of proliferating and stimulating osteolytic reaction of mouse bone. The SY5Y and KCNR cells injected into the femur both exhibited tumor invasiveness in the bone radiographically and pathologically, and both of them produced macroscopically lytic expansible lesion with soft tissue abnormalities.

Clinical NB bone metastases exhibit various morphologies [1, 22, 24], and it is desirable to have models of all of the types that can be discretely identified. This has led us to repeat the process of generating and characterizing xenografts and testing them in the osseous NB models, in order to produce a range of murine morphologies corresponding to the observed human heterogeneity. This may make it possible to find biochemical correlates of the morphologies.

To evaluate the osseous NB model for study of biochemical interactions between NB cells and bone, we examined

the expression of various representative proteins involved in bone osteolytic response and reported to be associated with NB progression in the bone. In the osseous NB models, we observed immunoreactivity of OPG and RANKL in all xenografts tested. We observed lower levels of OPG in the osteolytic intrafemur tumors and contrary higher intensities of RANKL staining. Since the ratio between OPG and RANKL is important in determining the number of osteoclasts and level of osteoclast activity, we hypothesize that the low expression of OPG and high expression of RANKL in NB cells play a role in activation of the osteoclastogenesis cascade, leading to the osteolytic effects of these cells in vivo. We have recently shown that OPG and RANKL are expressed by NB cells in patient specimens, and that their expression is upregulated in NB cells present in bone compared to primary tumors and soft tissue metastases, and also, that serum levels of OPG are upregulated in NB patients with bone metastasis [8]. We have also shown that all of the femur xenografts express PTHrP and ET-1, and that expression of these proteins by these cells appears to be upregulated in the bone environment versus contralateral normal bone, again showing behavior parallel to that of clinical NB. Furthermore, the immunoreactivity of PTHrP and RANKL is higher than that of OPG and ET-1 significantly. Together, these results indicated that PTHrP and RANKL may play a more important role in bone invasion and metastasis microenvironment. We also investigated another representative protein involved in the bone metastasis mechanism, such as CXCR4, and we found that the expression of CXCR4 in femur xenografts is downregulated compared to contralateral normal bone strikingly, and the immunoreactivity of CXCR4 is lower than those of other proteins associated with bone invasion significantly. These results further proved that CXCR4 promotes primary and secondary tumor growth and the migration, but not the invasion in the bone invasion/metastasis mechanism [3, 12, 15].

Conclusions

We report herein the generation and characterization of osseous NB models. We have shown that the characteristics of the osseous NB xenografts tested realistically mimic key aspects of human NB bone metastasis. These models are therefore useful for investigation of the interactions between NB and bone cells to delineate the mechanisms and pathways involved in formation of NB bone metastases and the effects of investigational drugs on this aspect of the disease.

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