NPNT promotes early-stage bone metastases in breast cancer by regulation of the osteogenic niche

Dongsheng Wang¹,², Chenglong Zhao³,⁴, Liangliang Gao³,⁴, Yao Wang², Xin Gao¹, Liang Tang¹, Kun Zhang¹, Zhenxi Li⁵, Jing Han⁶,⁷, Jianru Xiao⁸,⁹

¹ Spinal Tumor Center, Department of Orthopedic Oncology, Changzheng Hospital, The Second Military Medical University, No.415 Fengyang Road, Huangpu District, Shanghai, China
² Department of orthopedics, Shanghai Keyuan Orthopedic Hospital, Shanghai, China
³ Jiangsu Key Laboratory for Functional Substance of Chinese Medicine, Jiangsu Collaborative Innovation Center of Chinese Medicinal Resources Industrialization, Stake Key Laboratory Cultivation Base for TCM Quality and Efficacy, School of Pharmacy, Nanjing University of Chinese Medicine, Nanjing, China

ABSTRACT

Patients with breast cancer are often afflicted by bone metastases, while the establishment and growth of bone metastases depend on interaction between cancer cells and the host environment. Moreover, osteoblasts, which play a vital role in cancer cells survival and colonization, can form an osteogenic niche in early stage of bone metastases. Also, it is widely accepted that there is a genetic determinant during bone metastases. Nephronectin (NPNT) is an extracellular matrix protein which has shown biological activities in breast cancer metastases and osteoblasts differentiation. But the role of NPNT in mediating breast cancer bone metastases remains elusive. In the present study, we revealed that up regulation of NPNT is associated with incidence of bone metastases. What’s more, NPNT could significantly enhance the tumor cell clone formation but not proliferation and migration. We further demonstrated that NPNT significantly enhance osteoblast differentiation and tumor adhesion. Thus, we proposed that cancer secreted NPNT may be a novel marker with potential value of prediction and diagnosis of breast cancer bone metastases.

1. Introduction

Breast cancer is one of the most common cancers in women worldwide, while patients with breast cancer are often afflicted by bone metastases [1]. The bone is a preferred site of breast cancer metastases, with the incidence of 65%−75% [2]. Normally, bone metastases can cause several symptoms such as fractures, spinal cord compression, severe pain, hypercalcemia, and bone marrow aplasia. Current therapies, including tumor targeted chemo-/radio-/endocrine therapies, and bone remodeling therapies with denosumab and bisphosphonates have showed some effects in alleviating bone metastases associated symptoms [3,4]. Unfortunately, these treatments are insufficient to relieve metastases burden and the median survival after diagnosis of overt skeletal metastases is as low as 2 years [5,6]. Therefore, it is in urgent will to develop more effective strategies to prevent and reduce bone metastases.

Bone stroma cells contain osteoblast cells, osteoclast cells, and hematopoietic stem cells. When cancer cells home to the bone, interactions between bone stroma cells and cancer cells could moderate bone homeostasis, which can further promote tumor cells colonization, survival, dormancy and/or proliferation in this environment [7]. It has been suggested that in the formation of metastatic lesions, an osteogenic process occurred in the early-stage of bone colonization [8]. Osteoblasts could secrete CXCL12 to promote CXCR4 positive tumor cells migrate to the bone [9]. Besides, micro metastases predominantly reside in an osteogenesis niche, which was mediated by heterotypic adherens junction (hAJs) involving cancer-derived E-cadherin and osteogenic N-cadherin [10]. All these clues indicated the crucial role of osteoblasts in cancer cell seeding and growth in bone.

Nephronectin (NPNT) is an extracellular matrix protein originally identified in the embryonic kidney [11]. Structurally, it has an MAM (meprin, A5 protein and receptor protein tyrosine phosphatase) domain, five EGF-like domains, and an RGD integrin binding motif [12]. Previous studies have reported that NPNT could participate and regulate the process of cell adhesion, differentiation, spreading and survival [13]. When it comes to the bone, NPNT is proved to promote
osteoblast differentiation via the epidermal growth factor-like repeats [14]. Recent studies revealed the potential role of NPNT in certain types of cancer. Eckhardt et al. [15] reported that knockdown of NPNT in high metastases 4T1.2 mammary tumor caused a significant reduction of metastases to spine, lung and kidney. Steigedal et al. [16] reported that granular cytoplasmic staining was associated with poor prognosis in breast cancer patients. However, the role of NPNT in breast cancer bone metastases remains elusive.

In current study, we identified that NPNT was upregulated in breast cancer tissues compared to normal breast tissues. Patients with higher NPNT expression are prone to form bone metastases. Knockdown of NPNT in breast cancer cells decreased the cell colony formation and adhesion to osteoblasts. Conditioned medium (CM) from NPNT knockdown cancer cells inhibits osteoblasts differentiation. Collectively, our data showed the functional role of NPNT during bone metastases and indicated the potential prognostic value in prediction and diagnosis of breast cancer bone metastases.

2. Material and methods

2.1. Expression profile microarray

The mRNA level of NPNT in breast cancer tissues and normal was acquired from Oncomine (http://www.oncomine.org). The NPNT expression data in breast cancer metastases were downloaded from the Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo).

GSE12276 was included in the present study.

2.2. Cell lines

The human breast cancer cells, including MCF-10A, BT549, MDAMB-231, BT474, T47D, and MCF-7, were obtained from either the Type Culture Collection of the Chinese Academy of Science (Shanghai, China) or American Type Culture Collection (ATCC). All cells except MCF-10A were cultured in DMEM medium mingled with 10% fetal bovine serum (Gibco, Carlsbad, CA) and 1% penicillin-streptomycin. MCF-10A was cultured in MEBM (Lonza) containing insulin, EGF, hydrocortisone, and bovine pituitary extract. All the cells were cultured in a humidified incubator (Thermo Fisher Scientific, Inc) with 5% CO2 at 37 °C.

2.3. Lentivirus-based knockdown

The virus-based knockdown was conducted by using pklo.1 vector. The shRNA oligos targeting human NPNT were GCACAGGTCATGAA CACTTA (Sh1) and GCTGACATCAAGAGCGAATCA (Sh2). Virus was harvested from the supernatant of 293T cells 48 h post-transfection before being used to infect target cells (2 × 10⁶). The cells were not used for proliferation and migration assay or Western blot experiments until the cells were cultured without virus for 24 h.
2.4. Cell proliferation and clone formation assay

Cell proliferation analysis was performed by using MTS solution follow the manufacturer’s instruction. For the clone formation, breast cancer cells were plated in 6-well plates and cultured for 7 days. The colonies were fixed with 4% paraformaldehyde for 10 min at room temperature, stained with crystal violet for 10 min, washed 3 times with PBS and dried off. The number of colonies was counted under a microscope.

2.5. qRT-PCR

Total RNA was extracted from cells by using TRIzol (Invitrogen) and reverse-transcribed to cDNA by using PrimeScript™ RT reagent kit (Takara). Quantitative real-time PCR analysis was performed by using SYBR Green PCR Master Mix as suggested by the manufacturer. Data were collected and quantitatively analyzed on an MX3005p quantitative PCR system. The PCR primer used were as follows: NPNT forward 5’-TGGGGACAGTGCCAACCTTTCT-3’; reverse 5’-TGTGCTTACAGGGCGAGGCT-3’; ALP forward 5’-TGTTACTGCTGATCATTCCCACG-3’; reverse 5’-AATGTAGTTCTGCTCATGGACGCC-3’; Osterix forward 5’-GCTGCCCTACCTACCGCTG-3’; reverse 5’-GTTGCCCACTATTTGCCAAC-3’; OCN forward 5’-AGTCACCAACCACAGCATCC-3’; reverse 5’-TTTGTCCTTCCCTTCTGCC-3’; and β-actin forward 5’-GTACGGCAACACGAGTGGCT-3’, reverse 5’-GTTCATACCTCCTTGCTCGT-3’.

2.6. Transwell assay

Transwell assay was performed as previously reported [17]. The lower chambers of 24-well chemotaxis chambers (Corning, CA, USA) were filled with 600 µl medium containing 10% fetal bovine serum. Cancer cells were added into the upper chambers coated with serum-free medium. After 12 h incubation, migrated cells were fixed with 4% paraformaldehyde (PFA) and stained with 1% crystal violet. Images were taken using an Olympus inverted microscope and migrated cells were counted using Image-Pro Plus 6.0.

2.7. Mouse BMSCs isolation and osteoblast differentiation assay

Mouse bone marrow stromal cells (BMSCs) were flushed and isolated from C57/BL6 mice [18]. The cell suspension was cultured in modified α-MEM containing 10% FBS and 1% penicillin/streptomycin. Three days later, cells reached confluency and were ready for experiments. BMSCs were plated in 24-well plates with 1 × 10^5 cells each well. Then, cells were cultured in a mix of 90% osteoblast differentiation media (modified α-MEM containing 50 µg/ml L-ascorbic acid and 10 nM dexamethasone, 10 mM β-glycerophosphate and 10 mM ascorbic acid) and 10% FBS.

Fig. 2. Expression of NPNT in breast cancer tissues. (A) Immunohistochemical (IHC) staining was performed to validate the expression of NPNT in primary and bone metastatic samples. (Original magnification ×200(left), × 400(right)). (B) Patients with higher level of NPNT were more prone to form bone metastases ($x^2 = 5.743, p = 0.029$). (C) Kaplan–Meier analysis was used to compare bone metastases free survival with high level of NPNT and low level of NPNT in our patient cohort.
2.0 mM β-glycerophosphate) and 10% CM from (shctrl, shNPNT-1 or shNPNT-2) breast cancer cells. Medium was changed every 2d for a 7-day period. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature and then alkaline phosphatase (ALP) staining was performed by using FastRed (F4381; Merck KGaA), according to the manufacturer’s protocol.

2.8. Cell–cell adhesion assays

BMSCs were seeded at 20,000 cells per well in a 48 well plate and allowed to proliferation for 3 days. MCF-7 and T47D cells (expressing an empty vector control or shRNA to NPNT) were seeded at 20,000 cells per well and incubated for 1 h at 37 °C with 5% CO2 (before seeding, all cells were transfected with GFP+vector), cells were gently washed for three times with PBS to remove non-adherent cells, Images were taken by using a Leica DM 4000B photomicroscope. The number of GFP+ cells was counted [19].

2.9. Western blot

Western blot was performed as previously reported [20]. Protein was extracted from the cells of each group, and protein concentration was measured with a BCA kit. Equal amounts of protein samples were separated by SDS-PAGE and transferred to PVDF membrane. PBST solution containing 5% skim milk powder was used to block the membrane for 1 h. The membrane was incubated overnight at 4 °C with polyclonal anti-NPNT (1:500, Cosmo Bio, Carlsbad, USA), or anti-β-actin (1:12,000; Sigma, Deisenhofen, Germany). Then, the membrane was washed and monitored by immunoblotting using a DyLight 800–conjugated secondary antibody. The membrane was scanned by using a LI-COR Infrared Imaged Odyssey (Gene Company Limited).

2.10. Immunohistochemistry

Clinical tissues were obtained from patients who received surgeries at Changzheng hospital (Shanghai, China). Informed consents were obtained for using all human samples. Ethical consents were granted by the committees for ethical review of research involving Human Subjects of Second Military Medical University (Shanghai, China). Tissues were obtained and fixed with 4% paraformaldehyde and embedded with paraffin. Immunostainings were performed according to standard procedures. Staining was developed by using 3, 3′-diaminobenzidine/peroxidase substrate (brown precipitate). Slides were counterstained with hematoxylin. Images were taken by using a Leica DM 4000B photomicroscope.

2.11. Statistical analysis

Data were presented as the means ± SD of three independent experiments done in triplicate. Statistical analysis was performed by Student t test or one-way ANOVA. In all cases, differences between values were considered statistically significant when *P < 0.05.

3. Result

3.1. Bioinformatics analysis of NPNT expression in breast cancer tissues from public database

Public data (Oncomine) were utilized to detect the mRNA level of NPNT in normal breast tissues and invasive breast carcinoma tissues; it
was found that NPNT was significantly upregulated in tumor samples versus normal (Fig. 1A). We also revealed that mRNA levels were elevated in bone metastases versus bone free tissues in GSE12276 (probe 225911_at and 244747_at) dataset (Fig. 1B–C). We further examined the prognostic value of NPNT in breast cancer by Kaplan-Meier survival analysis. It was found (GSE12276 dataset) that NPNT mRNA expression correlated with bone metastases (Fig. 1D–E).

3.2. Expression of NPNT in breast cancer correlated with bone metastases

To validate the expression of NPNT in primary and bone metastatic samples versus normal (Fig. 1A). We also revealed that mRNA levels were elevated in bone metastases versus bone free tissues in GSE12276 (probe 225911_at and 244747_at) dataset (Fig. 1B–C). We further examined the prognostic value of NPNT in breast cancer by Kaplan-Meier survival analysis. It was found (GSE12276 dataset) that NPNT mRNA expression correlated with bone metastases (Fig. 1D–E).

3.3. Effects of NPNT on breast cancer cells proliferation, migration and clone formation

To examine NPNT expression in breast cancer cell lines, qRT-PCR and western blot were performed, which demonstrated that the expression of NPNT in MCF-7 and T47D was relatively high (Fig. 3A). Subsequently, MCF-7 and T47D cells were transfected with control (shCtrl) or NPNT (shNPNT-1 and shNPNT-2) shRNAs. NPNT dramatically decreased in knockdown groups (Fig. 3B).

To evaluate the effects of NPNT on breast cancer cells, several experiments were performed. By MTS assay, we found that knockdown of NPNT have little effect on cell proliferation (Fig. 3C). Additionally, transwell assay showed no significant effect on cell migration (Fig. 3D–E). However, knockdown of NPNT inhibited the clone formation ability on both MCF-7 and T47D cell lines ((Fig. 3F–G). These results demonstrated that NPNT may play an important role in tumor cell clone formation but not in proliferation and migration.

3.4. Knockdown of NPNT suppressed tumor induced osteoblast differentiation and reduced tumor adhesion to osteoblasts in vitro

Once cancer cells disseminated into the bone microenvironment, they firstly interact with MSCs and induce an osteogenic process, which promote early-stage bone colonization [5]. Therefore, we intended to study whether NPNT could promote breast cancers induced osteogenesis. BMSCs were separated and cultured with CM from indicated tumor cells. The alkaline phosphatase (ALP) staining was significantly attenuated when treated with CM from NPNT knockdown cells (Fig. 4A–B). Meanwhile, mRNA levels of ALP, osterix (Osx), and osteocalcin (Ocn) were all decreased in NPNT knockdown groups (Fig. 4C–E). Furthermore, we found that knockdown of NPNT reduced the adhesion of cancer cells to osteoblasts (Fig. 4F–I). These results demonstrated the potential role of NPNT in regulating osteoblasts differentiation and tumor adhesion. These in vitro data partially explained the important role of NPNT in breast cancer bone metastases.
4. Discussion

Bone is a preferred site of metastases for breast cancer [21]. It is well-recognized that there is a genetic determinant during tumor metastases. Gene expression profiling revealed that different organ-specific metastatic variants show distinct signatures [22]. Veer and colleagues identified a gene expression signature for breast cancer metastases consisting of 70 genes [23]. Kang et al reported a multigenic program mediating breast cancer metastases to bone, which demonstrated a “molecular signature” acquired by cancer cells within the primary tumor, allowing them to spread and colonize to the bone marrow [24]. The present study provides direct clinical evidence that NPNT was upregulated and correlated with breast cancer bone metastases and may be a potential biomarker.

The establishment and growth of bone metastases depend on interaction between cancer cells and the host environment. Cross talk within the bone may alter the cell properties and further moderate surrounding microenvironment [25]. Under conditions of normal bone homeostasis, there is a tightly regulated balance between osteoblasts, bone deposition and osteoclasts resorption. When cancer cells metastasizes to bone, this balance was disrupted. Osteoclasts were over activated by RANKL secreted by osteoblast, then, cathepsin K and other cysteine proteinases were secreted into the bone matrix, which resulted in bone matrix breakdown [26]. Recent studies showed osteoblasts play vital role for cancer cells survival and colonization to bone microenvironment [10]. It revealed an osteogenic feature after intralilac artery (IIA) injection of cancer cells to hind limbs in the early stages [5]. The number of osteoblasts was increased at the initiated stage of bone metastases after cardiac inoculation of MDA-MB-231 cells [27]. Co-injection of pre-osteoblastic MC3T3-E1 cells and MDA-MB-231 cells to mice showed a higher rate of tumor formation compared with injection of cancer cells alone [28]. In our experiment, we found that knockdown of NPNT decreased clone formation, but not proliferation and migration, of tumor cells. Tumor induced osteoblasts differentiation was suppressed when the expression of NPNT was downregulated. These results suggested that tumor derived NPNT may play a critical role in the osteogenic niche.

It is reported that breast cancer could secrete NPNT by microvesicles or exosomes to promote the adhesive ability of tumor cells and promote lung colonization [16]. The expression of NPNT could increase melanoma cell adhesion by binding with the receptor αβ1 [29]. Direct osteoblast interaction with extracellular matrix is mediated by a group of integrin receptors including Integrin αβ1 [30]. Our experiments showed that the ability of tumor formation was decreased while tumor adhesion to osteoblasts was also suppressed in NPNT knockdown cells. Thus, our findings indicated the important role of NPNT in breast cancer bone colonization and survival.

5. Conclusion

We demonstrated that NPNT was upregulated in breast cancer and correlated with bone metastases. Knockdown of NPNT in breast cancer cells decreased the cell clone formation and adhesion to osteoblasts. CM from NPNT knockdown cancer cells inhibit BMSCs cells differentiation. Taken together, our data identified the potential values of cancer secreted NPNT, serving as an important factor during bone metastases, in prediction and diagnosis of breast cancer bone metastases.

Funding

This work was supported by grants from National Key Research and Development Program of China (2016YFC0902100).

Supplementary materials

Supplementary article associated with this article can be found, in the online version, at doi:10.1016/j.jbo.2018.09.006.

References

[1] T. Hamaoka, et al., Bone imaging in metastatic breast cancer, J. Clin. Oncol. 22 (14) (2004) 2942–2953.
[2] Y. Kang, Dissecting tumor-stromal interactions in breast cancer bone metastasis, Endocr. Metab. (Seoul) 31 (2) (2016) 206–212.
[3] M.E. Sari, et al., Prognostic factors and treatment outcomes in surgically-staged non-invasive uterine clear cell carcinoma: a Turkish Gynecologic Oncology Group study, J. Gynecol. Oncol. 28 (4) (2017) e49.
[4] T.A. Guise, Breast cancer bone metastases: it’s all about the neighborhood, Cell 154 (5) (2013) 957–959.
[5] H. Zheng, Y. Kang, Cradle of evil: osteogenic niche for early bone metastasis, Cancer Cell 27 (2) (2015) 153–155.
[6] P.P. Major, et al., Natural history of malignant bone disease in breast cancer and the use of cumulative mean functions to measure skeletal morbidity, BMC Cancer 9 (2009) 272.
[7] A. Mishra, et al., Homing of cancer cells to the bone, Cancer Microenviron. 4 (3) (2011) 221–235.
[8] C. Tulett, O. Pettewell, The role of IL-1B in breast cancer bone metastasis, Endocr. Relat. Cancer 25 (7) (2018) R421–R434.
[9] A.H. Jinnah, et al., Emerging and established models of bone metastasis, Cancers (Basel) 10 (6) (2018) pii: E176.
[10] H. Wang, et al., The osteogenic niche promotes early-stage bone colonization of disseminated breast cancer cells, Cancer Cell 27 (2) (2015) 193–210.
[11] R. Brandenberger, et al., Identification and characterization of a novel extracellular matrix protein nephronectin that is associated with integrin alpha1beta1 in the embryonic kidney, J. Cell Biol. 154 (2) (2001) 447–458.
[12] S.E. Zimmerman, et al., Nephronectin regulates mesangial cell adhesion and behavior in glomeruli, J. Am. Soc. Nephrol. 29 (4) (2018) 1128–1140.
[13] Y. Sun, et al., The emerging role of NPNT in tissue injury repair and bone homeostasis, J. Cell Physiol. 233 (3) (2018) 1887–1894.
[14] K. Troppan, et al., Frequent down regulation of the tumor suppressor gene a20 in multiple myeloma, PLoS One 10 (4) (2015) e0123922.
[15] B.L. Eckhardt, et al., Genomic analysis of a spontaneous model of breast cancer metastasis to bone reveals a role for the extracellular matrix, Mol. Cancer Res. 3 (1) (2005) 1–13.
[16] T.S. Steigedal, et al., Nephronectin is correlated with poor prognosis in breast cancer and promotes metastasis via its integrin-binding motifs, Neoplasia 20 (4) (2018) 387–400.
[17] Z. Li, et al., Activator protein-2beta promotes tumor growth and predicts poor prognosis in breast cancer, Cell Physiol. Biochem. 47 (5) (2018) 1925–1935.
[18] Y. Zhang, et al., The effects of Runx2 immobilization on poly (epolin-caprolactone) on osteoblast differentiation of bone marrow stromal cells in vitro, Biomaterials 31 (12) (2010) 3231–3236.
[19] J.E. Noll, et al., SAMS1 is a tumor suppressor gene in multiple myeloma, Neoplasia 16 (7) (2014) 572–585.
[20] D.S. Wang, et al., Anxiolytic-like effects of translocator protein (TSPO) ligand ZBD-2 in an animal model of chronic pain, Mol. Pain 11 (2015) 16.
[21] C.J. Zhao, et al., Outcomes and prognostic factors for surgically treated patients with breast cancer spine metastases, J. Bone Oncol. 12 (2018) 38–43.
[22] L. Jin, et al., Breast cancer lung metastasis: molecular biology and therapeutic implications, Cancer Biol. Ther. (2016) 1–11.
[23] L.J. van’t Veer, et al., Gene expression profiling predicts clinical outcome of breast cancer, Nature 415 (6871) (2002) 530–536.
[24] Y. Kang, et al., A multigenic program mediating breast cancer metastasis to bone, Cancer Cell 3 (6) (2003) 537–549.
[25] C. Kan, et al., Cancer cell colonization in the bone microenvironment, Int. J. Mol. Sci. 17 (10) (2016).
[26] D. Granchi, et al., Molecular basis of osteoclastogenesis induced by osteoblasts exposed to wear particles, Biomaterials 26 (15) (2005) 2371–2379.
[27] H.K. Brown, et al., Location matters: osteoblast and osteoclast distribution is modified by the presence and proximity to breast cancer cells in vivo, Clin. Exp. Metastasis 29 (8) (2012) 927–938.
[28] T.M. Bodenstine, et al., Pre-osteoblastic MC3T3-E1 cells promote breast cancer growth in bone in a murine xenograft model, Chin. J. Cancer 30 (3) (2011) 189–196.
[29] S. Kuphal, S. Wallner, A.K. Bossenhoft, Loss of nephronectin promotes tumor progression in malignant melanoma, Cancer Sci. 99 (2) (2008) 229–235.
[30] A.M. Moursi, R.K. Globus, C.H. Damsky, Interactions between integrin receptors and fibronectin are required for calvarial osteoblast differentiation in vitro, J Cell Sci. 110 (Pt 18) (1997) 2187–2196.