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Research Paper

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

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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

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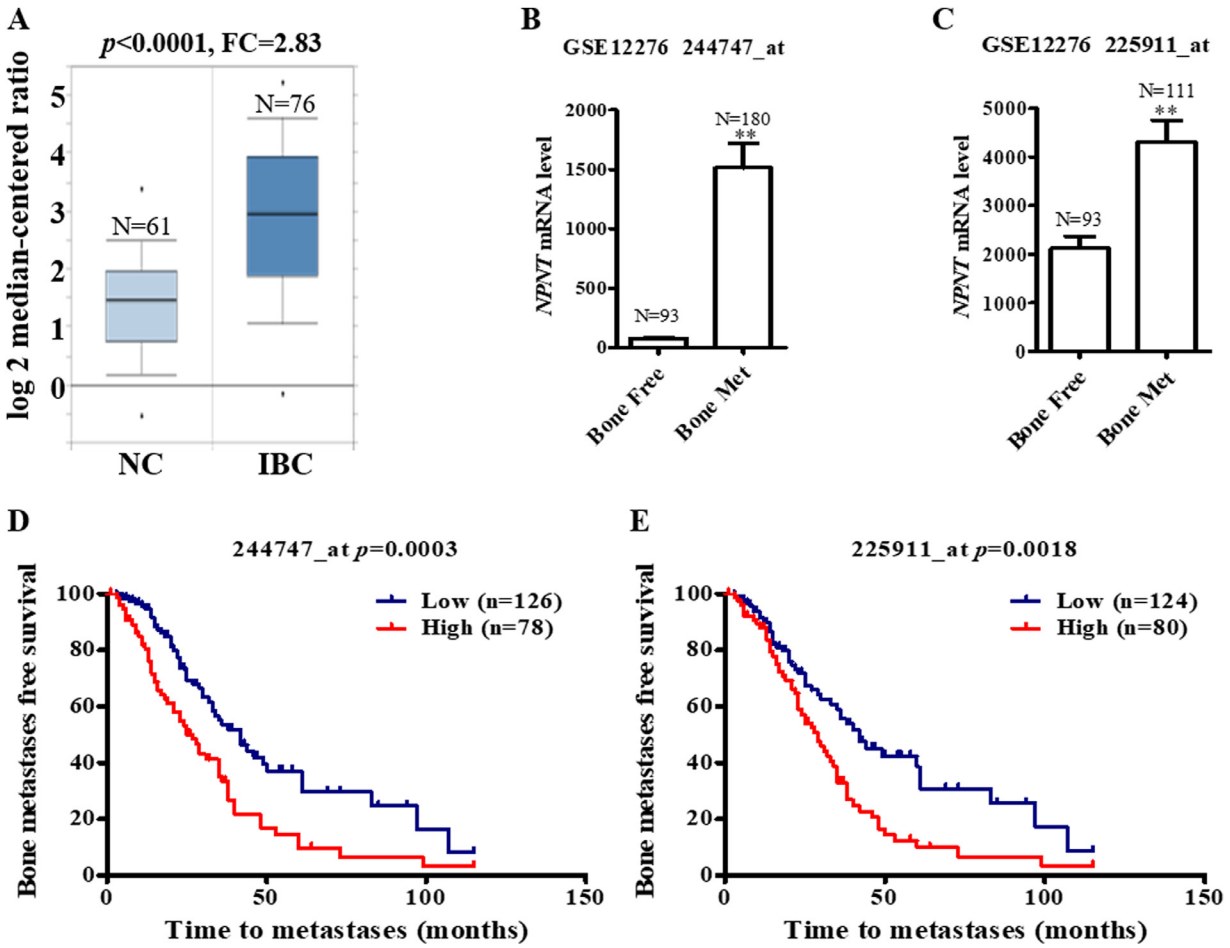

Fig. 1. *Bioinformatics analysis of NPNT expression in breast cancer tissues.* (A) Oncomine cancer database revealed that mRNA level of NPNT was upregulated in breast carcinoma tissues versus (v.s.) normal. (B–C) GSE12276 (probe 225911_at and 244747_at) dataset showed that mRNA level of NPNT was elevated in bone metastases versus bone free tissues. (D–E) Kaplan-Meier analysis was used to compare bone metastases free survival with high level of NPNT and low level of NPNT in GSE12276 dataset (Fig. 1D–E). *p* value was calculated on the basis of log-rank test. NC: normal breast tissue; IBC: Invasive breast carcinoma.

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 differention. 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, MDA-MB-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% CO_2 at 37 °C.

2.3. Lentivirus-based knockdown

The virus-based knockdown was conducted by using plko.1 vector. The shRNA oligos targeting human NPNT were GCACAGGTGCATGAA 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 (2×10^5). The cells were not used for proliferation and migration assay or Western blot experiments until the cells were cultured without virus for 24 h.