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Review

Bone metastasis in breast cancer: The story of RANK-Ligand

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Abstract The primary cellular mechanism responsible for osteolytic bone metastases is osteoclastic activation. Preclinical models have shown that breast cancer cells can produce parathyroid hormone-related protein (PTHrP), and other osteolytic molecules, which stimulate excessive osteoclastic bone resorption and establishment of osteolytic lesions. It has been shown that PTHrP by itself cannot directly induce osteoclastic activation, but it mediates its effect through the transactivation of RANK-ligand (RANKL) gene on stromal and osteoblastic cells. Accordingly RANKL up-regulation has been considered as a prerequisite in virtually all conditions of cancer induced bone destruction. Hence, therapeutic targeting of RANKL seems to be a rational approach to treat or even to prevent the process of bone metastases.

In this review, we will focus on the unique patho-physiological aspects related to the evolution of bone metastases in breast cancer, emphasizing the pivotal role of RANKL and some other key molecules in osteoclastic bone resorption. We will discuss the therapeutic interventions using bisphosphonates and RANKL inhibitors in patients with bone metastases and the outcome of this novel approach.

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Introduction

Approximately 65–75% of patients with advanced breast cancer will develop bone metastases, which result in bone destruction and skeletal-related events (SRE) [1]. During the last two decades, it has been shown that the process of osteolytic metastases depends essentially on osteoclast-mediated bone resorption rather than a direct destructive effect by the cancer cells [2]. This has promoted the use of bisphosphonates, which are potent inhibitors of osteoclastic bone resorption, in the treatment of almost all types of bone metastases [3,4]. Treatment with bisphosphonates (especially zoledronic acid) resulted in a significant decrease in the morbidity associated with bone metastases [5–7]. However, many patients will continue to develop SREs, calling for adopting novel strategies to manage these patients.

Perhaps, among the most important discoveries in the field of bone biology were those related to the role of the RANK-ligand/RANK/Osteoprotegerin (OPG) system in the regulation of osteoclastic function and bone remodeling [8,9]. This has led to a better understanding of the process of bone resorption, which is considered a primary step in the evolution, and progression of bone metastases.

Physiological bone remodeling

The adult skeleton is in a dynamic state of continuous coordinated cycles of bone resorption and bone formation, a process known as bone remodeling. In physiological bone remodeling, there is a well-balanced interplay between osteoclasts – dissolving old bones – and osteoblasts – laying down new bones – in order to maintain the structural bone integrity. Accordingly, the amount and contour of new bone deposition by osteoblasts are always equivalent to what has been resorbed by osteoclasts (balanced & coupled remodeling) [10].

Bone resorption by osteoclasts disintegrates collagen (which is the major organic component of osseous tissue), and releases calcium and growth factors from the bone matrix. Ninety percent of the proteins released consists of collagen degradation products; while the remaining 10% consists of the matrix cytokines and growth factors [11]. Importantly, bone matrix is considered as a storehouse for growth factors and cytokines. Large amount of transforming growth factor- β (TGF- β) and insulin growth factor (IGF) II along with fibroblast growth factor,

platelet-derived growth factor, and other cytokines are produced by the stromal and immune cells and are stored within the mineralized bone matrix [10,11]. These factors can mediate cellular interaction – in a paracrine fashion – with cancer cells, which is a critical step in the development and progression of bone metastases. Although termed “growth factors”, they do not necessarily induce a direct stimulation of cancer cell proliferation, as they can also indirectly promote angiogenesis and osteoclastogenesis, which in turn would remodel the skeleton to accommodate tumor growth [12,13].

Osteoclastogenesis

Osteoclastogenesis is an extremely complex process, which is predominately controlled by three members of the tumor necrosis factor (TNF) family of receptors and ligands known as RANK, RANKL and OPG [8,9,14].

RANK (Receptor Activator of Nuclear Factor- κ B) is a surface receptor mainly expressed on mature osteoclasts and their progenitors [14,15]. Its primary function is to induce osteoclastogenesis and control calcium metabolism [16]. While RANK expression has been primarily observed on osteoclasts, recent studies have demonstrated RANK expression on tumor cells, including breast cancer cell lines and tissue specimens of breast cancer patients which may suggest a role of this receptor in the migration and metastatic behavior of such cancer cells [17,18] as will be discussed later.

RANKL is a polypeptide that belongs to type II transmembrane proteins [9,14]. RANKL is found on the surface of osteoblasts and bone stromal cells but may be also found in a soluble form within the bone microenvironment. In the presence of low levels of macrophage colony-stimulating factor (M-CSF), RANKL can generate osteoclasts from hematopoietic cells [12,14]. It binds to its receptor RANK on preosteoclasts and mature osteoclasts. Signaling through RANK would then activate transcription factors such as nuclear factor κ B, which leads to the differentiation of osteoclast progenitors and limits apoptosis of mature osteoclasts [15,19,20]. Many other molecules like parathormone (PTH), parathyroid hormone related protein (PTHrP) 1, 25-dihydroxyvitamin D, TNF, interleukin (IL)-1 and IL-11 can also induce osteoclastogenesis [12]. A curious observation many years ago was the finding that osteoclasts do not express

receptors for these potent bone resorbing agents. Later works have shown that these bone resorbing agents are able to induce osteoclastic activation via the up-regulation of RANKL expression by osteoblasts, stromal cells and other immune cells (e.g. pre-B lymphocytes and activated T-cells) [20,21].

Osteoclastic suppression

Importantly, under physiological conditions, the stimulatory effects of RANKL on osteoclasts are opposed by another molecule known as OPG, which is also secreted by the osteoblasts and stromal cells [9,14,22]. In contrast to all other TNF receptor family members, OPG lacks the transmembrane and cytoplasmic domains and acts as a soluble decoy receptor specific for RANKL [22]. OPG competes with RANK for RANKL, thus it prevents the RANKL–RANK interaction on the osteoclast cell membrane. When RANKL binds to OPG, osteoclastogenesis is markedly inhibited leading to cessation of bone resorption [20,22].

Conceptually, the level of osteoclastogenesis and bone remodeling is primarily regulated by a very delicate balance in the RANKL/OPG ratio, such that a relative decrease in OPG results in excessive bone resorption whereas a relative increase in OPG inhibits resorption [14,15,23].

Breast cancer bone metastases

Breast cancer is a frank example of neoplasms that display an extraordinary affinity to grow in bone. The mechanisms underlying this osteotropism are complex and involve some peculiar characteristics of both the breast cancer cells and the bone matrix to which these tumors metastasize (soil and seed concept) [13]. Accordingly, breast cancer cells should possess certain properties that enable them to grow in bone, while the bone matrix provides the suitable microenvironment, which facilitates growth of these cells.

The bone matrix is considered unique among target tissues affected by cancer, as it is continuously enriched by bone-derived growth factors and cytokines attributed to osteoclastic activity during the physiological process of bone remodeling as discussed earlier. This would render the bone microenvironment as an appropriate fertile soil, which can attract and support the growth of circulating tumor cells, thus contributing to the pathogenesis of bone metastases [13,24].

Chemotaxis

Chemotaxis of circulating tumor cells and the stromal cells within the bone microenvironment is an essential component of bone metastasis. Bone resorption products such as type I collagen fragments, TGF β , and IGFs have been shown to stimulate chemotaxis of breast cancer cells [13,24,25,26].

More recently, the role of chemokine receptors in chemotaxis of breast cancer cells to the bone microenvironment has been described. It is well known that the circulating leukocytes and stem cells use chemokine receptors (CXCR4) for homing to the bone marrow, where an excessive amount of their corresponding chemokine; SDF-1 is present [27]. Importantly, the overexpression of CXCR-4 has been reported in around 30% of primary breast cancer cells, and it has been also shown to mediate the movement of malignant cancer cells to specific

organs especially bone where SDF-1 α is abundant [28,29]. In a more recently published trial CXCR4 expression in primary breast cancer was significantly associated with subsequent development of bone metastases in these patients [30].

Several studies have also implicated RANKL in the process of chemotaxis. As mentioned earlier, human breast cancer cell lines and tissue specimens are reported to express RANK protein on their surface and it has been suggested that RANKL may act as a chemotactic factor for these cancer cells [18]. The rich source of RANKL within the bone microenvironment would attract RANK expressing tumor cells to migrate to the bone. The correlation of high RANK expression with osteotropism in murine models was demonstrated across many tumor cell types, including breast cancer [18]. Santini et al. has recently provided the first clinical evidence of the role of RANK expression in primary tumors as a predictive marker of bone metastasis. In their study which included 93 patients with early breast cancer, those with “RANK-positive” tumors had a significantly higher rate of bone metastasis compared to patients with tumors, which had low or negative RANK expression [17]. These data suggest that investigating the RANK/RANKL pathway might open new venues in predicting bone recurrence and may also identify a subgroup of patients, which are at a higher risk of developing skeletal metastasis [31].

Growth of bone metastasis

It should be noted that unlike other tissues, bone is mainly composed of hard-mineralized tissue; hence it is more resistant to invasion and destruction by cancer cells compared to other metastatic sites [32]. Osteoclasts have been described as the most efficient cells to induce bone resorption “bone-resorbing machines” [12]. Therefore, in order to grow within the bone matrix, the cancer cells must possess the capacity to induce osteoclastic activation, which is the main cellular mechanism for cancer induced bone destruction [20,29]. Increased osteoclastic bone resorption would then provide the space in which cancer cells can grow and induce further molecular interactions with the different cytokines within the bone microenvironment.

Most evidence indicates that breast cancer cells can induce osteoclastic activation through the release of soluble mediators such as IL-1, IL-6, IL-8, prostaglandin E2, TNF and most importantly, PTHrP [2,32]. PTHrP, which is expressed in around 50% of primary breast cancers [33,34] has been shown to play a causal role in the pathogenesis of breast cancer-mediated osteolytic metastases [2,23,35]. In animal models, intracardiac injection of breast cancer cell lines (MDA-MB-231) engineered to overexpress PTHrP, resulted in a significant increase in the number of osteolytic metastases [35].

Several clinical studies have shown that metastatic breast cancer cells in bone express PTHrP more frequently than in other non-skeletal metastases or in the primary tumor [33,34,36]. However, it is not very clear whether PTHrP expression would promote the development of bone metastases in these patients, or that the bone matrix provides a favorable microenvironment, which is conducive to PTHrP production by breast cancer cells when these cells are already within bone matrix [36]. It may seem true that the ability of breast cancer cells to produce PTHrP in response to cross-talks within the

bone microenvironment is more important to the development of skeletal metastases than the expression of PTHrP by the primary breast cancer cells.

More recently, it has been shown that PTHrP may have roles in breast cancer bone metastasis independent of its roles in enhancement of osteoclastic function. PTHrP plays an important role in modulating the angiogenic and bone osteolytic actions of VEGF [37]. Furthermore, PTHrP up-regulates the expression of matrix metalloproteinase-13 in breast cancer cells, that can degrade bone matrix thus adding further invasive characters to the bone metastatic process [38]. These data strongly suggest that PTHrP represents a rational target to explore for the treatment of bone metastases in breast cancer. In a mouse model, neutralizing antibodies against PTHrP decreased both the size of osteolytic lesions and tumor area in bone [35]. A humanized monoclonal antibody targeting PTHrP, was under development in Japan but the research has been suspended [39]. At the present time and to the best of our knowledge no clinical study is under way.

Other factors like M-CSF, IL-11, VEGF contribute to osteolytic lesions via RANKL up-regulation by osteoblasts and stromal cells [13]. A unique exception of osteolytic cytokines expressed by breast cancer cells is IL-8, which seems to operate as a RANKL dependent as well as RANKL independent factor that can directly stimulate osteoclastic bone resorption [40]. Therefore, it has been proposed that IL-8 might be involved in the very early stage of osteoclastic bone resorption, which will be followed by the dominant action of PTHrP at a later phase of bone destruction [41].

The vicious cycle of bone destruction

The widely accepted soil and seed model of osteolytic bone metastasis in breast cancer is based on the hypothesis that the TGF- β (which is released from the bone matrix during osteoclastic resorption) induces tumor cell production of osteolytic factors including PTHrP and IL-11. This causes stromal cells to secrete RANKL, thus increasing osteoclast number and function with subsequent osteolysis with more TGF- β being released from bone [13,39].

TGF- β which is deposited in the bone matrix by osteoblasts and released and activated during osteoclastic resorption is not the most abundant growth factor in bone, but it plays the most significant role in the progression of osteolytic metastases [42]. In pre-clinical models, the role of bone-derived TGF- β to stimulate PTHrP production by breast cancer cells and enhance their growth in bone is well established [43,44]. TGF- β binds to its surface heterodimeric receptor, and mediates its functions through the intracellular mediators known as Smad protein family (cytoplasmic mediators of most TGF- β signals) and the Mitogen-activated Protein Kinase (MAP Kinase), which enhances PTHrP secretion by breast cancer cells [42,44]. In preclinical models, TGF- β signaling blockade inhibits PTHrP secretion by breast cancer cells and suppresses the development of bone metastases [45,46]. TGF- β also promotes osteolytic metastases by stimulating tumor expression of matrix metalloproteinases enzymes and increasing angiogenesis [47].

The fact that TGF- β is abundant in bone and can enhance PTHrP expression by cancer cells makes it an important target for the treatment of breast cancer bone metastases. However, although TGF- β acts as a tumor promoter in advanced cancer,

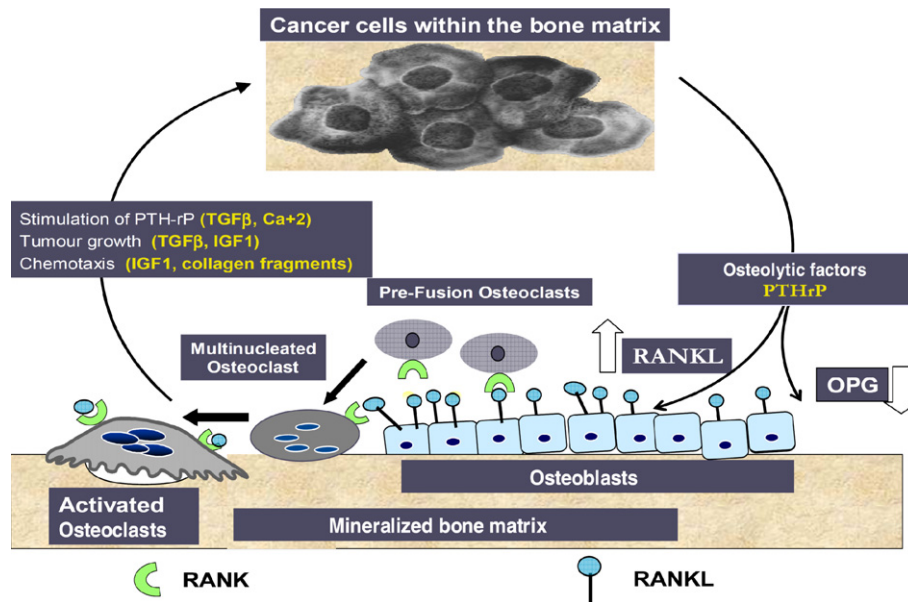
it actually functions as a tumor suppressor in early phases of cancer [48]. This dual role of TGF- β could pose a challenge when targeting the TGF- β signaling for cancer treatment [49]. Although TGF- β inhibitors have been investigated for other types of cancers, however, to date, there have been no clinical trials studying the effect of a TGF- β -related therapy for breast cancer with bone metastases.

IGF is another important molecule, which is also released during bone resorption and likely has significant effects on tumor cell growth [31]. Experimental evidence suggests that IGFs promote breast cancer cell proliferation within the bone matrix and neutralizing antibodies against IGF-I receptor markedly impaired the growth-stimulating effects of osteolysis on the tumor cells [2,13]. IGF inhibitors are in early phases of clinical trials in many solid tumors, but not as yet in the setting of bone metastases. Enhanced resorption of the mineralized bone matrix is also associated with excessive elevation of extracellular calcium. The levels of calcium in the vicinity of resorbing osteoclasts are many folds higher than the level of systemic calcium [50]. It has been shown that calcium-sensing receptors (CaR) are expressed on normal mammary epithelium and respond to low levels of ionized calcium by increasing the production of PTHrP [51,52]. Following the classic negative feedback, PTHrP stimulates osteoclasts to resorb bone, releasing calcium, and signaling back through the CaR to reduce PTHrP production. However, transformation into a malignant phenotype may involve reversal of the normal negative feedback that exists between CaR and PTHrP leading to stimulation rather than inhibition of PTHrP when extracellular calcium is elevated [53]. It has been shown that breast cancer cells express the CaR, which would then participate in stimulating the production of PTHrP by tumor cells within the bone matrix, thus adding more to the process of osteoclastic activation [53]. Interestingly, Mihai et al. have identified CaR predominantly expressed in patients who developed bone rather than visceral metastases [54]. These data may suggest CaR expression as a potential new predictive biomarker for bone metastases in breast cancer patients. Whether patients with CaR-positive tumors are more likely to develop bone metastases and whether they could benefit more from prophylactic treatment with bisphosphonates or RANKL inhibitors, are important questions that need to be addressed in prospective trials.

It seems true that many products of osteolysis join the concert to evoke further PTHrP release and worsening osteolysis in addition to supporting the growth of breast cancer cells within the bone [55,56]. This reciprocal feedback between tumor cells and the bone microenvironment has been referred to as the “vicious cycle” of bone destruction, in which osteoclast is the key cellular player, whereas PTHrP, RANKL and TGF β are the main molecular co-players in a master scene known as the ‘seed and soil’ hypothesis (Figure 1).

Treatment of bone metastases in breast cancer

Identifying the cellular and molecular components that promote the development of bone metastasis is an essential step to provide a rational treatment for skeletal metastases. The theme of therapy should be directed toward reduction of osteoclast differentiation and activation (with subsequent reduction in bone resorption and SRE).



PTHrP: parathyroid hormone related protein; TGFβ: transforming growth factor beta; Ca: calcium; IGF1: insulin growth factor 1; OPG: osteoprotegerin; RANKL: RANK ligand

Figure 1 Diagrammatic illustration of the interplay between breast cancer cells expressing PTHrP, osteoblasts, osteoclasts and bone matrix. Breast cancer cells secrete PTHrP, which stimulate osteoblast production of RANKL while OPG levels are reduced leading to enhanced osteoclastogenesis and increased bone resorption. Consequently the local milieu will be enriched by growth factors and other products of osteolysis (extracellular Ca + 2 and collagen fragments) which will induce: 1-stimulation of PTH-rP secretion (via TGF, Ca + 2), 2-stimulation of tumor growth (via TGF, IGF1) and 3-chemotaxis of circulating tumor cells to arrest in bone matrix (via IGF1, collagen fragments). This evokes further PTHrP release with worsening osteolysis, in addition to supporting the growth of breast cancer cells within the bone matrix. This reciprocal feedback between tumor cells and the bone microenvironment has been referred to as the “vicious cycle” of bone destruction.

Bisphosphonates

Bisphosphonates inhibit osteoclast formation and migration, increase production of OPG by osteoblasts and promote osteoclast apoptosis, resulting in suppression of physiological and pathological bone resorption [3,57]. They also reduce the release of bone-derived growth factors and cytokines associated with osteoclastic bone resorption, which potentially enhances tumor cell growth and proliferation in bone matrix [57,58]. Moreover, there are extensive data from preclinical studies, that these agents can also exert direct antitumor effects via inhibition of tumor cell adhesion, invasion, and proliferation, in addition to induction of apoptosis [59]. Furthermore, the Nitrogen containing bisphosphonates (e.g. zoledronic acid) may also act indirectly on tumor cells through antiangiogenic and immuno-modulatory mechanisms [59,60].

Zoledronic acid is known to be the most potent bisphosphonate to date [3,57]. Standard doses of zoledronic acid have been consistently reported to induce selective stimulation of $\gamma\delta$ T cells, which exert a beneficial anti-tumor function in vivo [60]. However, we would like to refer to the recent work by Fournier et al. [58] suggesting that the main anti-tumor effect of clinically relevant doses of bisphosphonates on breast cancer, is essentially mediated via the inhibition of osteoclast-mediated bone resorption rather than a direct cytotoxic effect. This supports the argument that targeting the bone microenvironment and not necessarily the primary tumor may be the corner stone for the beneficial anti-tumor effects

of bisphosphonates (if any) during the adjuvant phase of breast cancer.

In clinical practice, four bisphosphonates (clodronate, pamidronate, ibandronate, and zoledronic acid) have been widely used to treat breast cancer patients with bone metastases. In placebo controlled studies, these agents resulted in a significant decrease in the morbidity associated with bone metastases [4,5,7]. In a large double-blind phase 3 study comparing zoledronic acid with pamidronate, patients receiving the former had a significant 21% reduced risk of developing a SRE [61]. Subsequent follow-up, zoledronic acid could further reduce the risk of experiencing a second SRE by about one third compared with pamidronate, denoting the importance of maintaining these patients on a potent anti-resorption drug; even after the development of a SRE.

Targeting RANKL

As previously mentioned, RANKL is considered as the main molecular prerequisite in bone destruction. Therefore, targeting RANKL seems to be a very rational approach to treat bone metastases. Following the discovery of OPG, it was thought that increasing OPG levels would be an effective way to inhibit the bone resorbing effects of RANKL [62]. Administration of an Fc-OPG construct has shown promise as a potential therapy in animal models of bone metastasis [62,63]. Accordingly, a genetically engineered recombinant OPG-Fc construct (AMGN-0007) was developed as a potential therapeutic agent for patients with bone metastases. In a

double-blind trial, AMGN-0007 was at least as effective as pamidronate in reducing bone resorption marker levels in multiple myeloma and breast cancer patients [64]. However, there were some concerns that prevented further development of this drug in clinical trials. This agent had a short half-life, which raised some concerns on dose scheduling for clinical use. Furthermore, OPG is not specific to RANKL, as it can also block TRAIL [TNF related apoptosis inducing ligand] which is another ligand belonging to TNF family [65]. TRAIL is considered a very important component in natural immunity against cancer and is the principal mediator of tumor cell death induced by host immune cells [66]. Thus binding of pharmacological doses of OPG to TRAIL may protect breast cancer cells from undergoing TRAIL-induced apoptosis [67]. This may bear a potential risk of tumor growth with the long-term use of this drug. Therefore and as an alternative approach, an antibody specific to RANKL was developed which simulates the beneficial effects of OPG on bone health while avoiding any potential reaction with TRAIL [68].

Denosumab is a fully human anti-RANKL monoclonal antibody, but unlike OPG, it does not have any potential reaction with TRAIL [68]. In a recent large double-blind, randomized phase III study involving 2049 women with metastatic breast cancer, denosumab at a dose of 120 mg repeated every 4 weeks was compared to the standard 4-weekly zoledronic acid [69]. Denosumab was shown to significantly delay time to first SRE [HR: 0.82 (95% CI: 0.71–0.95; $p = 0.01$), time to first and subsequent SRE ($p = 0.001$) and malignant hypocalcaemia ($p = 0.007$).

The difference in efficacy between denosumab and zoledronic acid may be attributed to the difference in the mechanism of action between RANKL inhibition and bisphosphonate. Bisphosphonates act only when taken up by mature, actively resorbing osteoclasts, and thus residual osteoclasts can still be observed in bisphosphonate-treated bones [62,63]. On the other hand, RANKL inhibitors block the activation, survival, and differentiation of osteoclasts from their precursors resulting in complete absence of osteoclasts in the treated bones [63].

Currently, several phase III studies are ongoing to determine whether denosumab can also prevent the development of bone metastasis in the adjuvant phase of breast cancer.

RANKL beyond bone resorption

More interestingly, the RANK/RANKL pathway may also have a potential importance in breast cancer tumorigenesis. Two recent studies in animal models have demonstrated that RANKL mediates progestin-induced mammary breast cancer. In these studies, inhibition of RANKL function could result in a markedly decreased incidence and delayed onset of progestin-driven breast cancers. Blocking the RANKL not only reduced breast tumor formation but also decreased the spread of the cancer cells to the lungs. These data suggest that denosumab may be further considered as a novel approach to the prevention and/or treatment of hormone receptor positive breast cancer [70,71].

In summary, the development of established bone metastases involves complex, reciprocal interactions between cancer cells and the bone microenvironment, with a resultant vicious cycle of tumor cell growth and bone destruction. The affinity of breast cancer cells to bone is defined not only by a hosting

microenvironment favoring the survival of breast cancer cells, but also by the capacity of specific breast cancer cells to collaborate with stromal/osteoblastic cells in recruiting osteoclasts through the RANK/OPG/RANKL system. Interference with the micro environmental support of cancer cells via bisphosphonates has shown to be a valid approach to treat bone metastases. The introduction of denosumab will certainly provide an extra useful tool in the treatment and perhaps the prevention; of such a catastrophic complication of cancer.

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