

# Genomics and Proteomics of Bone Cancer

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**Abstract** Although the control of bone metastasis has been the focus of intensive investigation, relatively little is known about the molecular mechanisms that regulate or predict the process, even though widespread skeletal dissemination is an important step in the progression of many tumors. As a result, understanding the complex interactions contributing to the metastatic behavior of tumor cells is essential for the development of effective therapies. Using a state-of-the-art combination of gene expression profiling and functional annotation of human tumor cells, and surface-enhanced laser desorption/ionization time-of-flight mass spectrometry of patient serum, we have shown that changes in tumor biochemistry correlate with disease progression and help to define the aggressive tumor phenotype. Based on these approaches, it is apparent that the metastatic phenotype of tumor cells is extremely complex. The identification of the phenotype of tumor cells has benefited greatly from the application of gene expression profiling (microarray analysis). This technology has been used by many investigators to identify changes in gene expression and cytokine and growth factor elaboration (such as interleukin 8). The tumor phenotype(s) presumably also include changes in the cell surface carbohydrate profile (via altered glycosyltransferase expression) and heparan sulfate expression (via increased heparanase activity), to name but a few. These specific alterations in gene expression, identified by functional annotation of accumulated microarray data, have been validated using a variety of approaches. Collectively, the data described here suggest that each of these activities is associated with distinct aspects of the aggressive tumor cell phenotype. Collectively, the data suggest that multiple factors constitute the complex phenotype of metastatic tumor cells. In particular, the differences observed in gene expression profiles and serum protein biomarkers play a critical role in defining the mechanisms responsible for bone-specific colonization and growth of tumors in bone. Future studies will identify the mechanisms that participate in the formation of secondary tumor growths of cancers in bone.

Bone is a common site for cancer metastasis, and bone metastases are frequently associated with intractable bone pain, pathologic fractures, nerve compression, and hypercalcemia due to osteolysis (1). In addition, skeletal metastases and osteolysis denote a dramatic change in the prognosis for the patient and significantly increase the morbidity associated with the disease. For tumors to metastasize, they must proceed through multiple steps including primary tumor growth, the release of tumor cells into lymphatic and blood vessels, survival of the tumor cells within the circulation, arrest in the microvasculature of the target organ (leading to extravasation of tumor cells), invasion of target organs, and finally, growth of the tumor at the metastatic site (2, 3). Any step in the cascade is potentially a target for therapeutic intervention.

For bone metastasis, bone-colonizing tumor cells stimulate osteoclast-mediated bone resorption via the secretion of potent osteolytic agents (reviewed in refs. 1, 4) including parathyroid hormone-related protein (5, 6) and interleukin 8 (IL-8/CXCL-8; refs. 7, 8) among a variety of others. The increased bone resorption that follows releases bone-derived growth factors into the extracellular milieu and systemic circulation (1, 4, 9, 10), thereby further enhancing bone resorption, promoting tumor growth, and presumably altering the tumor microenvironment (and potentially the tumor cells themselves) to support tumor development (7). Although the control of bone metastasis has been the focus of intensive investigation, relatively little is known about the molecular mechanisms that control the process, even though widespread skeletal dissemination is an important step in the progression of many tumors (1, 3, 7, 9, 11–13). As a result, understanding the complex interactions contributing to the metastatic behavior of tumor cells is essential for developing biomarkers of disease progression, as well as for the development of more effective therapies.

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## Functional Annotation of Gene Expression Profiling

Gene expression profiling has proven useful in the subclassification and outcome assessment for a variety of human

tumors (8, 11, 12, 14, 15). Analysis of the biological significance of the hundreds or thousands of alterations in gene expression found using genomic profiling still remains a major challenge for the field. Moreover, it is increasingly evident that genes do not act as individual units but collaborate in a series of overlapping and interrelated networks, the deregulation of which is a classic hallmark of cancer. Thus, we and others have applied refined network knowledge to the analysis of key functions and pathways (so-called functional annotation) that are associated with tumor progression and/or development, using cDNA microarrays, inferential and descriptive statistics, and the dynamic mapping of gene expression data. These approaches have extended the existing knowledge about the organizational pattern of gene expression in human cancers and have begun to identify potential targets that hold promise for future therapeutic development (8, 15).

To focus our attention on groups of potentially important genes involved in the aggressive osteotropic phenotype of breast tumor cells, the functional annotation approach was initiated to determine the significance and relevance of genes differentially expressed in two genetically related, but functionally distinct breast cancer cell lines: MDA-MET (aggressive, osteotropic) versus MDA-231 (nonaggressive, nonosteotropic; ref. 8). The differentially expressed genes were assigned to seven nominally exclusive metastasis-associated categories: proliferation, transcription, oncogenes, motility and cytoskeleton, immune surveillance, adhesion, and angiogenesis (14). Gene assignment to individual categories was not mutually exclusive (8). The gene(s) represented in the most categories were considered to have a higher probability of involvement in the aggressive phenotype of MDA-MET cells and were selected for further analysis. On the basis of this osteotropism-related characterization, one gene (*IL-8*) was assigned to the most categories (present in five of seven categories) and was considered to be the most likely candidate to be associated with the aggressive behavior of MDA-MET compared with MDA-231 cells. Other genes present in multiple classifications that were validated by Northern blotting, immunohistochemistry, and real-time PCR include syndecan 1, heparanase-1, and the glycosyltransferases (16). This functional annotation approach was subsequently independently validated for stem cell differentiation (17, 18), as well as in numerous other biological systems (11, 12, 15, 19). Interestingly, many although clearly not all, of the genes identified by these overlapping approaches are present in multiple data sets.

It has been suggested that distinct tumor cell phenotypes are required for the establishment of metastatic lesions in different organs. However, the molecules and processes that allow breast cancer cells to take up residence and thrive in the bone marrow remain largely unknown. In fact, it has not been determined if the same molecules and processes are always involved in every metastasis of breast to bone. For the metastasis of breast cancer to bone, much of the work has been focused on the expression of parathyroid hormone-related protein (5) by the metastatic cells (1, 20) as the principal culprit in inducing tumor osteolysis. However, recent clinical evidence (21, 22) suggests that the role of parathyroid hormone-related protein is not in the early stages of tumor dissemination, but likely in the latter stages of the process, once the metastatic cells have colonized the bone marrow microenvironment. It is now becoming increasingly apparent that the metastatic phenotype of tumor

cells is extremely complex. The phenotype(s) presumably include (but are not limited to) changes in carbohydrate expression profiles, cell surface heparan sulfate proteoglycan expression, and cytokine and growth factor elaboration, all of which have been postulated to improve the survival of tumor cells in the circulation, allow adhesion of tumor cells to target organs, and facilitate positive interactions within the microenvironment of the target organ (1, 3).

Variations in carbohydrate sequences, which cell surfaces can present to lectins, adhesion molecules, and other receptors, create a refined pattern of potential sites for interactions between a tumor cell and its surrounding microenvironment. These observations suggest that the differences observed in the carbohydrate signatures of tumor cells play a role in the bone-specific colonization and growth of tumors in the marrow (23). Similarly, the tumor microenvironment is replete with heparan sulfate, an important component of the extracellular matrix and vascular basement membrane, where it serves as a physical barrier to tumor cell metastasis and interaction with normal tissue (16). In addition, heparan sulfates on the cell surface and within the extracellular matrix bind to, and regulate the activity of, numerous factors that control tumor growth and angiogenesis (23). Regulation of heparan sulfate content by the enhanced expression of human heparanase-1 *in vivo* dramatically up-regulates spontaneous metastasis of myeloma cells to bone (24). Heparanase-1 facilitates bone metastasis by promoting tumor cell invasion of the matrix through cleavage of heparan sulfate barriers to migration and by releasing bioactive fragments of heparan sulfate that promote tumor growth in bone (23).

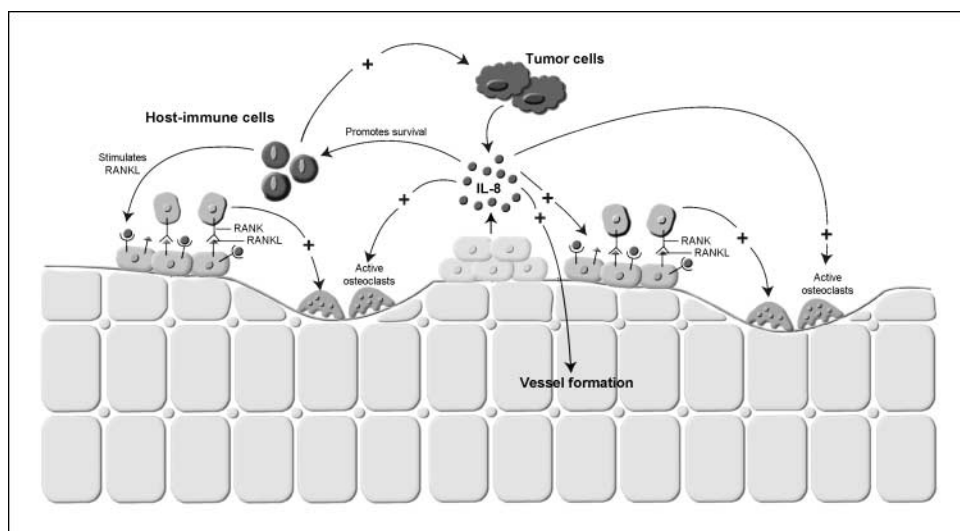
It is important to note that the action of many of the so-called osteolytic agents is not only to induce osteoclast activity but most likely multifactorial. Such is clearly the case with IL-8. Elevated levels of the chemokine IL-8 by breast cancer cells (8, 25) and prostate cancer (26) have been shown to correlate with increased bone metastasis, suggesting a prognostic biomarker role (see Discussion below). It is well recognized that IL-8 is a potent proangiogenic agent (27, 28). IL-8 was recently shown to be released following tumor cell-platelet aggregation (29), suggesting additional and important functions for platelets in the metastatic process. In addition, Sparmann and Bar-Sagi (30) characterized the functional significance of Ras-induced up-regulation of IL-8 in cervical and lung cancer. The up-regulation of IL-8 by oncogenic Ras seems to be required for the onset of tumor vascularization, further supporting a multifunctional role for the chemokine.

The release of IL-8 from metastatic tumor foci into the bone microenvironment provides an important and early direct source of neoangiogenic factors, osteoclast activators, and tumor mitogens. It is likely that IL-8 (and presumably many other factors) plays an important pathogenic role in several different aspects of the development and growth of bone lesions (Fig. 1). The dysregulation of IL-8 expression in human cancers suggests that this chemokine (and its receptors) are potentially attractive anticancer therapeutic targets.

### Identification of Biomarkers in Bone Cancer

Proteomics is more than the identification of proteins that are altered in expression as a consequence of pathophysiology, it also encompasses the search for novel biomarkers, a critical tool for the detection, treatment, and monitoring of disease

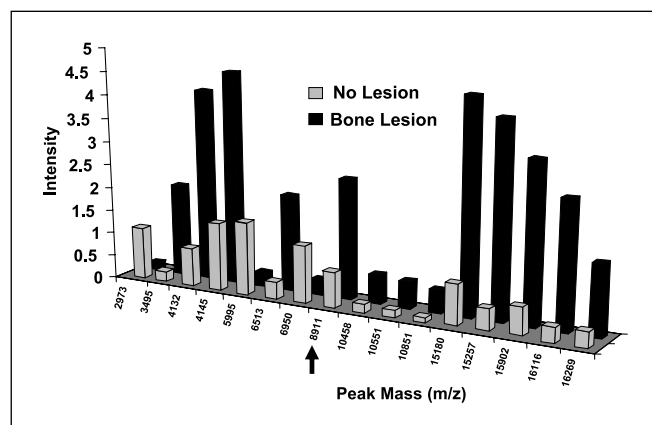
**Fig. 1.** IL-8 is an important regulator of tumor progression. IL-8-secreting tumor cells find their way to the bone marrow microenvironment. IL-8 directly activates existing osteoclasts and their precursors and increases RANKL expression by osteoblast precursors (and other cells in the marrow). In addition, IL-8 promotes the survival of and activates host-immune cells, to further enhance osteoclast formation, as well as the release of factors that support tumor proliferation and induce vascularization. Once bone resorption has been activated by IL-8, other growth factors such as transforming growth factor  $\beta$  and insulin-like growth factor II are released, stimulating parathyroid hormone-related proteins, IL-1 and IL-11 levels (to name but a few), in stromal cells and osteoblasts. The activation of cells on the bone surface also leads to the release of IL-8 from osteoblasts. Adapted from Martin (44).



(31). The necessity for new methods to identify and validate biomarkers is underscored by the increased survival of patients diagnosed at early stages of cancer. Serum proteomic pattern diagnostics is a new type of proteomic concept in which patterns of ion signatures generated from high dimensional mass spectrometry data are used as diagnostic classifiers. Intriguingly, it has been shown that this diagnostic information exists in a bound state, complexed with circulating highly abundant carrier proteins (32). Mass spectrometry-derived protein signatures have been identified, characterized, modeled, and are now moving into validation in extensive patient cohorts. Further characterization and sequencing of these key features should provide new insights into disease etiology, and presumably, intervention (33). Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) has emerged as the primary investigator-based modality for biomarker discovery of early stage cancers (31, 34), although the approach does have some limitations that have been identified of late (35, 36).

However, there is currently no way to reliably detect or predict which patients are at risk for metastatic cancer. Thus, the discovery of biomarkers that could distinguish patients with local disease from those with metastatic disease would be of great clinical value. Using SELDI protein chips, we tested the hypothesis that a unique biomarker pattern that distinguishes the skeletal involvement (or not) of patients with multiple myeloma (MM) was present in the serum proteome. Serum from MM patients with and without skeletal complications (University of Arkansas for Medical Sciences, Institutional Review Board-approved protocol) was profiled to identify protein patterns indicative of bone disease status. Using univariate statistical analysis as well as linear partial least squares discriminate analysis (37) and nonlinear random forest classification algorithms (38), a diagnostic fingerprint was generated that holds great promise as a potential serum biomarker profile for the diagnosis and treatment of MM progression (ref. 39; Fig. 2). Interestingly, one of the discriminatory peaks has a molecular weight (8.9 kDa) similar to that of intact human IL-8. Subsequent functional analysis suggests that this marker is IL-8, adding MM to the disease states associated with dysregulated IL-8 expression.

Clinically, prostate cancer progression from a hormone-sensitive to a hormone-refractory state is initially recognized by a biochemical failure in the form of rising levels of serum prostate-specific antigen (PSA). This biochemical change is followed by the appearance of new symptoms of progressive disease and/or radiographic evidence of relapsing disease, the traditional criteria for defining refractory prostate cancer. Therefore, the period of biochemical failure antedates clinical hormone refractoriness and lasts for a median of 6 months (40), during which time patients remain predominantly clinically asymptomatic. In such a setting, the serum proteome of patients with advanced prostate cancer on androgen ablation therapy who presented with an increasing PSA, were profiled and compared with the serum profile of patients with advanced prostate cancer on androgen ablation therapy who continued to exhibit a stable PSA response. We hypothesized that such prostate cancer pathologic states would be reflected as biomarker information within the low molecular weight region of



**Fig. 2.** Diagnostic fingerprint of MM patient samples (with and without bone disease). The proteomic profile generated by SELDI-TOF MS is able to discriminate between MM patients with bone disease (*black columns*) and MM patients without bone disease (*gray columns*). Not every peak in the profile has a significant individual contribution in differentiating the two groups. However, when combined, they generate a pattern with potential diagnostic value. Arrow, peak similar to the molecular weight of human IL-8.

the serum proteome and that such changes would be identifiable by SELDI-TOF MS. Low molecular weight protein profiles were generated from the serum of patients with prostate cancer (collected using a University of Arkansas for Medical Sciences, Institutional Review Board–approved protocol) and analyzed by multiple bioinformatics approaches, to determine if the low molecular weight component of the circulatory proteome contains potentially useful discriminatory information. Using this bioinformatics approach, specific low molecular weight molecules were identified that when combined with serum PSA levels in patients with advanced stages of prostate cancer, dramatically improved the prognostic value of an increasing serum PSA value. The addition of the obtained SELDI-TOF MS information to measured PSA status produces prognostically more informative subdivisions of the patient population. Specifically, patients with both risk factors (an increasing PSA and greater than median SELDI peak intensity) had the shortest survival, whereas patients with only one risk factor (rising PSA or greater than median SELDI peak intensity) had comparable and considerably longer survivals. Importantly, patients with neither risk factor had no deaths (after 33 months of follow-up). If confirmed in larger validation studies, efforts to identify the underlying diagnostic molecules by sequencing would be warranted. In the future, measurement of these biomarkers could be potentially used to improve the ability to identify patients with progressive advanced prostate cancer.

However, some questions remain regarding the reliability of SELDI-TOF MS to detect cancer and/or tumor-related biochemical changes associated with progressive disease (35, 36). These include reproducibility, contributory effects of nonspecific interference by circulatory molecules unrelated to tumor biology, and the lack of standardization of specimen collection, preparation, and techniques used in detecting differentiating *m/z* peaks, among others. These valid concerns are continually being addressed (34, 41). However, it is critical to note that the pipeline of new biomarkers for cancer diagnosis and/or for tracking cancer progression is drying up (42, 43). Currently, it seems that the proteomic profiling of patient serum remains the primary technology capable of rapid discovery of informative diagnostic and prognostic biomarkers.

### Summary and Future Directions

Collectively, these data suggest that multiple factors comprise the complex osteolytic phenotype of tumor cells. Differences observed in gene expression profile, which reflect changes in the carbohydrate signature, heparan sulfate proteoglycan expression, and cytokine expression (such as IL-8) play a critical role in the bone-specific colonization and growth of osteolytic tumors in bone. Further studies are required to identify the mechanisms that participate in the formation of secondary tumor growths of cancers in bone. The efficacy of novel preclinical treatments using specific modulators of platelet function (29) or perhaps even anti-IL-8 antibody therapy provides strong hope for the utility of such agents in the inhibition of tumor growth in bone. The utility of gene expression profiling and functional annotation supports the concept that novel therapeutic opportunities for the treatment of tumor growth and osteolysis remain to be identified.

Similarly, serum biomarker profiles, such as those described here, represent a critical first step in the discovery of new biomarkers for the treatment and monitoring of prostate cancer progression. Serum proteomic pattern diagnostics represent an important new technique in which proteomic signatures are used as a diagnostic classifier. The extent to which the identified protein peaks can serve as potential biomarkers of cancers other than prostate or MM must be investigated rigorously. Nevertheless, the data presented here support the existence of a highly accurate and distinct multiplex proteomic set that can accurately distinguish between MM patients with and without bone involvement (perhaps involving IL-8 dysregulation), and improve the prognostic value of the clinical prostate cancer marker, PSA.

It seems highly likely that this type of enabling technology (functional annotation and SELDI-TOF MS) has the potential to provide the foundation for the development of therapies to target tumor-specific pathways regulating key biological processes including proliferation, differentiation, apoptosis, immunity, and metastasis. In summary, these analyses suggest that further studies with additional patient cohorts, and continued investigation (both genomic and proteomic) of the role of the identified markers in the specific steps of tumor progression, should lead to a better understanding of the biology of tumor metastasis to bone and its susceptibilities to treatment.

### Open Discussion

**Dr. Roodman:** You commented that tumor cells can't bind to lining cells, and that to establish a bone metastasis, you have to move the lining cells out of the way. Do you think that the reason these *in vivo* models of bone metastasis work is because you are blocking the ability of the tumor to get at the surface because the osteoclast is not pushing the lining cells away?

**Dr. Suva:** I don't have any data, but that mechanism is plausible. In most bone metastases, resorption is critical.

**Dr. Berenson:** Have you looked at bortezomib in your model and whether it suppresses NF- $\kappa$ B dependence of IL-8?

**Dr. Suva:** We are currently doing that.

**Dr. Berenson:** We have now identified a novel protein that is highly produced by myeloma cells that we believe is an integral part of angiogenesis in not only myeloma but other tumors as well. It is able to take a fully matured monocyte and turn it into a blood vessel.

**Dr. Suva:** Is it regulated by IL-8 expression?

**Dr. Berenson:** No, and if you block it with antibody, you not only prevent angiogenesis, you greatly inhibit tumor growth.

**Dr. Suva:** Was it due to normal bone turnover?

**Dr. Berenson:** We know two things. Levels tend to be higher in osteoporotic patients, and in transgenics, over time, they develop very brittle bone, but it has not been well explored.

**Dr. Pearce:** Can you describe IL-8 receptor expression?

**Dr. Suva:** There are two receptors, CXCL-1 and CXCL-2. CXCL-1 binds IL-8 and a couple of other chemokine family members, and CXCL-2 is promiscuous and binds a bunch. CXCL-1 is expressed on the surface of osteoclasts and their precursors. CXCL-2 is not expressed on osteoclast lineage cells. CXCL-1 is also expressed on the surface of stromal osteoblasts.

**Dr. Pearce:** Are these chemokine receptors expressed on the surface of myeloma cells?

**Dr. Suva:** Yes.

**Dr. Pearce:** Is their expression by blood vessels in myeloma-infiltrated marrow different than their expression by blood vessels in breast cancer samples?

**Dr. Suva:** We have been ineffective at staining the vessels in the breast cancer cells *in vivo*, unless we have manipulated them with heparanase. In breast cancer, IL-8 is important for stimulating bone resorption, which is its primary function; other molecules probably mediate vessel formation. Therefore, the elaboration of IL-8 by the breast tumor cells doesn't necessarily have an impact on angiogenesis.

**Dr. Vessella:** Were your data from serum samples taken at the time the patients had inactive disease or had metastases? Were those same profiles predictive? Were they prognostic in patients who were going to develop bone metastasis down the line?

**Dr. Suva:** We have some samples but just not enough to determine that.

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