Identification of PTHrP(12-48) as a Plasma Biomarker Associated with Breast Cancer Bone Metastasis

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Abstract

Background: Breast cancer bone metastasis is a complication that significantly compromises patient survival, in part, to the lack of disease-specific biomarkers that allow early and accurate diagnosis.

Methods: Using mass spectrometry protein profiling, plasma samples were screened from three independent breast cancer patient cohorts with and without clinical evidence of bone metastasis.

Results: The results identified 13 biomarkers that classified all 110 patients with a sensitivity of 91% and specificity of 93% [receiver operating characteristics area under the curve (AUC = 1.00)]. The most discriminatory protein was subsequently identified as a unique 12-48aa peptide fragment of parathyroid hormone-related protein (PTHrP). PTHrP(12-48) was significantly increased in plasma of patients with bone metastasis compared with patients without bone metastasis (P < 0.0001). Logistic regression models were used to evaluate the diagnostic potential of PTHrP(12-48) as a single biomarker or in combination with the measurement of the clinical marker N-telopeptide of type I collagen (NTx). The PTHrP(12-48) and NTx logistic regression models were not significantly different and classified the patient groups with high accuracy (AUC = 0.85 and 0.95), respectively. Interestingly, in combination with serum NTx, the plasma concentration of PTHrP(12-48) increased diagnostic specificity and accuracy (AUC = 0.99).

Conclusions: These data show that PTHrP(12-48) circulates in plasma of patients with breast cancer and is a novel and predictive biomarker of breast cancer bone metastasis. Importantly, the clinical measurement of PTHrP(12-48) in combination with NTx improves the detection of breast cancer bone metastasis.

Impact: In summary, we present the first validated, plasma biomarker signature for diagnosis of breast cancer bone metastasis that may improve the early diagnosis of high-risk individuals. Cancer Epidemiol Biomarkers Prev; 22(5); 972–83. ©2013 AACR.

Introduction

Currently, there are approximately 2 million women in the United States living with breast cancer and the disease is the second leading cause of cancer-related death in women (1). Approximately, 80% of women with metastatic breast cancer will have tumors arise in bone during the course of their disease. Bone is a common site for cancer metastasis, and bone metastases are frequently associated with complications such as hypercalcemia due to osteolysis, nerve compression, intractable bone pain, and pathologic fractures (2). Clinically, metastasis to the skeleton can be characterized by the acceleration of bone remodeling, measured by increases in both bone formation and bone resorption markers (3, 4). The resulting bone loss raises serum calcium and significantly increases the morbidity associated with the disease.

Common bone biochemical markers measure increasing levels of blood calcium, alkaline phosphatase, or the by-products of bone resorption such as type I collagen cross-links (5). Surprisingly, relatively few cancer and bone turnover biomarkers have been shown to have clinical significance (6, 7). These proteins/peptides are often used in combination with other diagnostic tools such as bone densitometry (in the case of the skeleton; ref. 8) or in the monitoring of therapies and cancer recurrence (6). Although elevated marker values are somewhat associated with breast cancer progression in bone, they have not been widely used due to their lack of sensitivity and specificity (9, 10). As such, there is a critical need for new methods to reliably identify protein biomarker...
expression, given the increased survival of patients with cancer who are diagnosed early.

In this context, proteomic approaches are an important tool for the detection, treatment, and monitoring of cancer. We and others have used a variety of proteomic technologies to discover biomarkers for cancer (11), neurologic disorders, and postmenopausal osteoporosis (12), as well as diagnostic markers for prostate (13, 14), pancreatic (15–18), multiple myeloma (19, 20), breast (21–26), and ovarian cancer (27, 28). With the increased drive to independently validate proteomic profiles, proteomic technology and cancer biomarkers are closer to delivering on their clinical promise.

In this study, the plasma proteome of patients with breast cancer with and without clinical bone metastases was interrogated using surface-enhanced laser desorption time-of-flight mass spectrometry (SELDI-TOF MS) and other biochemical approaches. A series of 13 discriminating protein peaks were identified and blindly and repeatedly validated. The resulting biomarker profile discriminated breast cancer bone metastasis patients with high sensitivity and specificity. The most discriminatory protein component of the profile was identified as a specific and unique N-terminal 12-48aa fragment of parathyroid hormone-related protein (PTHrP). PTHrP is produced by many cancers including breast tumors (29, 30), and we were the first to show that PTHrP is directly associated with the humoral hypercalcemia of malignancy (31). However, the identity of the circulating form of PTHrP peptide fragment(s) remains elusive.

In the present study, we have for the first time identified and blindly validated a circulating plasma biomarker signature of breast cancer bone metastasis that discriminates patients with bone metastasis from patients without clinically detectable bone metastasis. The most discriminatory component was identified as PTHrP(12-48). PTHrP(12-48) can be measured in patient plasma, and in combination with the clinical measure of bone resorption, identifies at risk patients with high specificity and sensitivity. In the future, this could provide novel opportunities for the improved diagnosis of bone metastasis, as well as provide novel insight into the underlying and intricate disease biology.

Materials and Methods

Patient demographics, sample collection, and preparation

After informed consent, archival plasma samples from a total of 111 women with breast cancer from well-characterized clinical studies (32, 33) were analyzed. All the patients were postmenopausal and patient descriptions are outlined in Table 1. Because the analyzed samples were obtained from a study of second-line metastatic breast cancer, early breast cancer covariates including grade and histologic type were unavailable (34). The detailed covariates that are applicable to metastatic disease are shown in Table 2.

Cohort 1 consisted of 36 plasma specimens that were classified into 2 groups: 18 with and 18 without clinical evidence of bone metastasis. Cohort 2 consisted of 41 plasma samples with unknown class labels, and cohort 3 consisted of an additional 34 plasma samples with unknown class labels. All clinical characteristics were based on patient bone scan, X ray evidence of bone metastasis, and elevated blood N-telopeptide (NTx) levels, a clinical marker of bone resorption (35). The serum NTx levels of all patients that were used to help discern the presence or absence of bone metastasis are shown (Fig. 1A). The women ranged in age from 49 to 92 years with a median age of 70 years in the bone metastasis group and 67 years in the no bone metastasis group. Analysis of the

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<th>Table 2. Patient demographics</th>
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<td>Receptor status</td>
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<td>Disease-free interval, y</td>
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*All patients were postmenopausal by trials eligibility requirement.

*All patients had soft tissue as site of metastases in this group.

*There were no soft tissue metastases in this group.
archival plasma samples was approved by the University of Arkansas for Medical Sciences (UAMS; Little Rock, AR) and Penn State Institutional Review Boards. A power analysis was conducted to confirm that the size of the sample cohorts was sufficient to provide a statistical power of more than 80%. The initial experiment to identify the baseline diagnostic profile was carried out on cohort 1, whereas cohorts 2 and 3 were used as blinded and independent datasets for subsequent biomarker validation. Each archival sample contained at least 100 μL of plasma, and some samples underwent at least 2 freeze–thaw cycles. All archival plasma samples were stored in 50 μL aliquots at or below –80°C until processing and analysis.

**Protein chip SELDI-TOF MS analysis**

All plasma samples were analyzed using a weak cation exchange WCX2 (CM10) chip (Bio-Rad). The CM10 protein chip with low stringency buffer gives the least variation and most complex spectra of all chip surfaces (12). All reagents for sample preparation were analytic grade and obtained from Sigma-Aldrich unless otherwise stated. CM10 chips were prepared using the Biomek 2000 robotic system, as we have described previously (12). All protein chips were placed in the Protein Biological robotic system, as we have described previously (12). All protein chips were placed in the Protein Biological robotic system, as we have described previously (12).

Acquisition parameters were as described previously (12) and mass accuracy was calibrated using the All-in-one peptide and All-in-one protein molecular weight standards (Bio-Rad).

Acquisition and preprocessing of all spectral data were conducted using Bio-Rad ProteinChip software version 3.2.2. Peaks were baseline corrected, mass calibrated, and normalized with the total ion current of m/z between 1,500 and 10,000; 7,000 and 30,000; and 25,000 and 150,000 Da for the low, mid, and high molecular weight ranges, respectively. Only spectra with a normalization factor between 0.5 and 2 were used in the analysis. All peak intensity values were complied, log transformed (base 2), centered, and scaled to have a median of 0 and SD of 1 (12).

**Quality control and reproducibility**

To control and assess the reproducibility of the acquired SELDI spectra, human serum controls (Bio-Rad) were randomly applied to the chips and the correlation coefficient of variation (CV) calculated with SAS 9.2. To assess intrasample correlation, duplicate samples were applied to the protein chips in random locations. The greatest deviation between the 2 was 0.095. The median value of the Pearson/Spearman correlation for cohort 1 was 0.95 and 0.91, respectively, with an interquartile range of 0.61 to 0.99 and 0.55 to 0.97. The median value of the Pearson/Spearman correlation for the cohort 2 was

Figure 1. Cohort-specific bone resorption markers and workflow diagram for SELDI-TOF MS analysis and. A, serum NTx levels in all patients with breast cancer with bone metastasis (BM) (closed squares) and no bone metastasis (NBM) (open circles). Cohorts 1 and 2 show clear discrimination of NTx levels around the cutoff of 25 nmol/L NTx. Cohort 3 shows the overlap of serum NTx common to breast cancer patients. Dashed line indicates the 25 nmol/L cutoff that defines low versus elevated bone resorption. B, an initial test set of 36 samples with known class was obtained and spectra generated. The data were preprocessed, subjected to statistical analysis and a random forest (RF) training model for classification generated. A second set of 41 samples of unknown class were obtained 1 year later and spectra obtained and data analyzed in an identical fashion to the training set. The test set samples were then classified into respective groups based on the random forest training model derived from the training set (dashed arrow). Class identity was verified independently and sensitivity and specificity determined. All the samples were then combined (solid arrow) and analyzed to generate a new combined random forest model and overall diagnostic fingerprint.
0.96 and 0.93, respectively, with an interquartile range of 0.83 to 0.99 and 0.82 to 0.97. The median value of the Pearson/Spearman correlation for the cohort 3 was 0.98 and 0.96, respectively, with an interquartile range of 0.95 to 0.99 and 0.92 to 0.98. The high median correlation values indicate high spot-to-spot reproducibility, thus spectrum pairs were averaged together on a peak-by-peak basis for all subsequent analyses.

Data analyses

ANOVA. SELDI peaks were first selected on the basis of at least a 1.5-fold change in the median intensity between groups, and then analyzed for the statistical significance of the fold change. Statistical significance was assessed via ANOVA. Peak intensities with a $P \leq 0.05$ were considered significant. All statistical analyses were conducted using SAS version 9.1 statistical software, with ANOVA conducted with the Tukey and Student-Newman-Keuls multiple comparison test corrections.

Decision tree analysis: random forest training model.

The random forest ensemble decision tree algorithm (RandomForest v5.1.0.179, Salford Systems) was implemented to build a robust diagnostic classifier for the detection of breast cancer bone metastasis (12, 36). Tree building was repeated 1,000 times with 3 variables tested at each node to yield the best prediction success with the lowest error cost. The set of trees, a so-called random forest were then combined by an unweighted plurality-voting scheme and the important peaks selected on the basis of their median importance scores as described previously (12).

Plasma fractionation for identification

Plasma samples with the highest expression of the target $m/z$ 4,260 Da peak were selected and fractionated into 6 fractions containing proteins on the basis of their isoelectric point as we have described previously (12). Fractions containing the $m/z$ 4,260 Da peak were identified by SELDI-TOF MS analysis of all individual fractions using CM10 proteinchips as described earlier.

Tryptic peptide mapping

After fractionation, fraction 5 (F5) containing the peak of interest ($m/z$ 4,260 Da) was digested with trypsin. CM10 protein chips prepared as described earlier were loaded robotically with 5 µL F5 alone; 5 µL F5 + 20 ng trypsin; 2 ng PTHrP + 20 ng trypsin, all at pH 8.5 in Tris–HCl. The loaded SELDI CM10 chips were placed in a humid chamber and incubated for 4 hours at 37°C and air-dried. The SELDI protein chip with on-chip digestion of fraction 5 (F5) containing the peak of interest ($m/z$ 4,260 Da) was then used for matrix-assisted laser desorption/ionization (MALDI) identification. MALDI spectra were collected in the $m/z$ 1,000 to 10,000 Da molecular weight range, using a PerkinElmerSciex MALDI proTOF (PerkinElmer). The tryptic digested samples were analyzed by tandem mass spectrometry to identify PTHrP tryptic fragments. Comparison of tryptic peptides with PTHrP theoretical tryptic digestion using the PAWS proteomic analysis software (Genomics Solutions) was used to identify and confirm the specific peaks of interest.

Specific immunodepletion of PTHrP(12-48)

Immunodepletion of the discriminatory $m/z$ 4,260 Da peak was conducted using plasma from patients with breast cancer with bone metastasis showing the highest intensity value for the $m/z$ 4,260 Da peak. PTHrP antibodies A113 #6494 affinity purified immunoglobulin G (IgG; recognizes PTHrP(1-15); a kind gift of Dr. John Chirgwin, Indiana University, Bloomington, IN) and Ab#906 (recognizes PTHrP(21-40); a kind gift of Dr. T.J. Martin, St. Vincent’s Institute, Fitzroy, Australia) were prepared in PBS. Fifty microliter of plasma was incubated with 5 µL of PTHrP antibody for 2 hours at 4°C. Next, 1 µL of protein A/G agarose conjugate suspension was added and incubated at 4°C with rocking overnight. The sample was then centrifuged at 1,000 × g for 5 minutes at 4°C and the supernatant removed and added to the prepared SELDI protein chip (CM10).

Specific measurement of plasma PTHrP(12-48)

A novel mass spectrometry-based assay was developed to measure PTHrP(12-48) concentrations directly in plasma of patient with breast cancer. Quality control serum spiked with a working range of 0 to 1,000 ng/mL PTHrP (12-48) synthetic peptide (Biomatrik, Inc.) and plasma samples from cohort 3, 17 patients with breast cancer with and 17 without bone metastasis, as well as 4 samples from cohort 1 and 4 from cohort 2, 2 with and 2 without bone metastases, respectively, for a total of 21 with or without bone metastasis were analyzed in duplicate on CM10 proteinchip arrays. The PTHrP(12-48) 4,260 Da $m/z$ peak in each spectrum was manually identified and peak areas quantified with the Biomarker ProteinChip 3.2.2 software (Bio-Rad). Biologic and technical replicates were averaged and normalized to the unspiked control. Patient plasma concentrations were determined by comparison with the standard curve ($R^2 = 0.98$). Statistical significance between groups was assessed via the Wilcoxon two-sample test; $P \leq 0.05$ was considered significant and is reported as such.

Diagnostic potential

Receiver operating characteristic (ROC) curve analysis and logistic regression were used to evaluate the diagnostic potential of PTHrP(12-48) alone and in combination with NTx. Biomarker thresholds for subsequent clinical discrimination were selected via maximizing the Youden Index ($J$; ref. 37). To adjust for limitations in sample size and biases associated with data drive-cutoffs (38, 39). Bootstrap bias-corrected and accelerated 95% confidence intervals (1,000 replications) were calculated for each performance statistic using the approach developed by Efron (40). Binomial exact 95% confidence intervals are reported for the ROC area under the curve (AUC). All
logistic regression and ROC curve analyses were conducted with XLSTAT (v2012.5.01; Addinsoft) and MedCalc (v12.3.0; MedCalc Software).

Results

Classification of bone metastasis versus no bone metastasis patients

The initial patient cohort of 36 plasma specimens was classified into 2 groups; breast cancer without bone metastasis (18 samples) versus breast cancer with bone metastasis (18 samples) using molecular profiling by mass spectrometry, as we previously described (12, 19, 41). The entire biomarker discovery workflow including 2 independent validation steps were conducted as shown (Fig. 1B). Statistical, bioinformatics, and machine learning analyses were conducted to identify a panel of differentially expressed peaks that could distinguish the 2 breast cancer patient cohorts (bone or no bone metastasis) with high sensitivity and specificity. A total of 218 individual peaks were resolved in the low, mid, and high molecular weight ranges covering 1,500 to 150,000 Da. Thirteen of the discriminatory peaks identified in the low, mid, and high molecular weight range are shown in the representative SELDI spectra (Fig. 2A).

Refined biomarker signature for breast cancer bone metastasis diagnosis

After classifying the blinded patient plasma samples of cohort 2 using the initial random forest model with high sensitivity and specificity, all SELDI data were combined and used to generate a second random forest model (Fig. 1B). The refined random forest model classified all 3 cohorts of patients (n = 110) with an overall sensitivity and specificity of 91% and 93% and AUC of 1.00, respectively.

Multiple SELDI spectra from patients with and without bone metastasis showed the reproducibility of the spectra (Fig. 2A). The significant SELDI-TOF MS generated peaks that discriminate between patients with breast cancer with and without bone metastasis were confirmed and m/z 4,133.27, 4,260.92, 4,740.39, 11,386.2, 11,452.8, 11,525.9, 11,629.0, 11,679.7, 11,728.2, 11,894.2, and 39,096.5 Da were upregulated in patients with breast cancer with bone metastasis, whereas only peaks m/z 17,384.8 and 17,577.3 Da were downregulated (Fig. 2B).

Identification of PTHrP (12-48)

A search of the SWISS-PROT database using the TagIdent tool (ExPaSy. 2007 available from: http://ca.expasy.org/tools/tagident.html) and the m/z 4,260.92 Da significant peak (Fig. 2A), tentatively identified the peak as a fragment of PTHrP, (m/z 4,259.89 Da). PTHrP was originally identified as the cause of the humoral hypercalcemia of malignancy (31, 42, 43), and we and others have shown that PTHrP is expressed by the majority of breast cancer bone metastases (31, 42, 43). To confirm the tentative in silico identification of the m/z 4,260 Da peak as a PTHrP fragment, we next determined the accurate monoisotopic mass of the SELDI-derived m/z 4,260.92 Da peak.

The significant protein peak (m/z 4,260; Fig. 3C) from breast cancer bone metastasis patient plasma was analyzed by MALDI-TOF MS and found to have a m/z of 4,255.3 (Fig. 3A), the monoisotopic mass of which was subsequently identified as m/z 4,253.3 Da (Fig. 3B). After determination of the accurate and specific monoisotopic molecular weight of the discriminatory m/z 4,260 Da peak as m/z 4,253.3 Da (Fig. 3B), the PTHrP(1-173) protein sequence (31) was examined for any peptides with an
exact \( m/z \) 4,253.3 Da. PAWS proteomic analysis identified PTHrP(12-48) with a molecular weight of \( m/z \) 4,253.3 Da. Thus, we hypothesized that the \( m/z \) 4,260 Da peak in the SELDI profile was PTHrP(12-48). Subsequent analysis by SELDI-TOF MS and MS/MS was conducted to identify specific tryptic fragments. Breast cancer bone metastasis patient plasma was fractionated and digested with trypsin and compared with similarly trypsin-digested PTHrP(1-37; Fig. 3C–E). The SELDI proteinchip arrays were analyzed using an adapter that allows elucida-

tion by MALDI-based prOTOF mass spectrometry. Trypsin-digested breast cancer bone metastasis patient plasma generated peak maps with features identical to the theoretical tryptic map of PTHrP(12-48). The plasma sample tryptic peptide peaks that represent PTHrP(12-48) were \( m/z \) 1,947.06, 1,116.55, and 731.40 Da (Fig. 3D). The predicted tryptic peptide peak at \( m/z \) 731.40 Da is below the level of SELDI detection and was not observed. Tryptic mapping of PTHrP(1-37) as expected included the diagnostic \( m/z \) 1,947.06 tryptic peptide (Fig. 3E). The predicted pattern of overlapping and distinct tryptic peptides was observed and confirmed that the patient plasma sample peak (\( m/z \) 4,260 Da) contains a PTHrP(12-48) peptide fragment. Other peaks present in the tryptic mapping analysis are the result of trypsin digestion of other proteins in the partially purified patient plasma fraction 5 (Fig. 3C). Subsequent analysis by Thermo LTQ-XL ion trap mass spectrometry confirmed the identity of the PTHrP(12-48) tryptic peptide \( m/z \) 1,948.2 Da as PTHrP(12-48).

A biochemical approach was next used to confirm the identity of PTHrP(12-48) using selective antibody-based immunodepletion of the PTHrP(12-48) peak directly from breast cancer bone metastasis patient plasma. As a first step, the specificity and selectivity of the selected PTHrP antibodies for the detection of PTHrP(12-48) was determined using synthetic PTHrP(12-48) spiked into control plasma. A PTHrP monoclonal antibody raised against PTHrP(1-15) and a PTHrP polyclonal antibody raised against PTHrP(21-40; refs. 42, 43) were used to determine if either or both antibodies were able to recognize PTHrP(12-48; Fig. 4). As expected, the PTHrP(1-15) antibody was ineffective at immunodepleting PTHrP(12-48), as the antigenic region of this antibody has been suggested to be in the N-terminal 5 amino acids of PTHrP (42, 43). However, the PTHrP(21-40) antibody effectively depleted approximately 60% of the spiked PTHrP(12-48; Fig. 4), directly supporting our hypothesis that the antibody would be able to deplete PTHrP(12-48) directly from patient plasma.

To directly test this idea, patient plasma (50 \( \mu L \)) containing the \( m/z \) 4,260 Da peak was incubated with 5 \( \mu L \) of either the PTHrP(21-40) polyclonal antibody or the PTHrP PTHrP(1-15) monoclonal antibody. Addition of the PTHrP(21-40) antibody to patient plasma depleted the putative PTHrP(12-48) peak (\( m/z \) 4,260 Da; Fig. 5A) by approximately 70% (Fig. 5C–D) but had no effect on the intensity of unrelated peaks in the profile. Interestingly, in another bone metastasis patient plasma sample the addition of the monoclonal PTHrP(1-15) antibody did not diminish the PTHrP(12-48) peak at \( m/z \) 4,260 (Fig. 5B), or any other observable peak in the profile (Fig. 5B). Collectively, these data suggest that the immunodepleted \( m/z \) 4,260 Da peak is a PTHrP fragment that is not...
recognized by an N-terminal–specific monoclonal antibody and is selectively depleted by an antibody raised against PTHrP(21-40). Thus, the identified fragment does not contain the N-terminus of PTHrP and as suggested by our specific tryptic mapping and monoisotopic mass analysis is PTHrP(12-48).

Specific measurement of PTHrP(12-48) in patient plasma

To measure specific levels of PTHrP(12-48) in patient plasma a SELDI mass spectrometry-based assay to measure PTHrP(12-48) in the plasma of patients with breast cancer [with and without bone metastasis (Fig. 6)] was developed. Human control plasma was spiked with increasing concentrations of PTHrP(12-48) from 0 to 1,000 ng/mL (Fig. 6A). The correlation coefficient for the SELDI-based detection of PTHrP(12-48) peptide added to control plasma using this assay was 0.98 (Fig. 6B). This standard assay was used to measure PTHrP(12-48) in cohort 3 plasma samples as well as 4 samples from cohort 1 and 4 from cohort 2 (Fig. 6C). PTHrP(12-48) levels were significantly increased ($P < 0.0001$) in patients with bone metastasis (Fig. 6C). In fact, PTHrP(12-48) levels ranged between 45.1 and 205.7 ng/mL (mean 102.5 ng/mL) compared with no bone metastasis patient plasma 27.3 to 110.9 ng/mL (mean 53.2 ± 23.1 ng/mL; $P < 0.0001$; Fig. 6C).

Next, logistic regression models were used to evaluate the diagnostic potential of PTHrP(12-48) as a single biomarker or in combination with the measurement of the clinical marker of bone resorption NTx (9, 10) to discriminate patients in whom PTHrP(12-48) levels had been measured (Fig. 6C). The measurement of PTHrP(12-48) using a threshold of 51 ng/mL [12 mmol/L; $J = 0.57$ (0.29–0.67)] was not significantly different ($P = 0.07$) from serum NTx measured using a threshold of 20.2 mmol/L [BCE $I = 0.81$ (0.57–0.86)] and classified the 2 patient groups with similar accuracy [NTx: sensitivity (Sn) 81% (58%–95%); specificity (Sp): 100% (84%–100%); AUC = 0.95 (0.85–1.00)] and PTHrP(12-48) [Sn: 90% (70%–99%); Sp: 67% (43%–85%); AUC = 0.85 (0.70–0.94; Fig. 6D). However, class prediction by the combined PTHrP(12-48)/NTx logistic regression model significantly increased diagnostic specificity (Sn: 86%; Sp: 95%; AUC: 0.99 (0.90–1.00; Fig. 6D).

Discussion

Metastatic breast cancer is the second leading cause of cancer-related death in women and early detection is the key to patient survival (2, 44). However, little is known about the molecular mechanisms that regulate or even predict metastasis. Breast cancer bone metastasis has a poor prognosis mostly due to the advanced stage at the time of diagnosis (9, 44, 45). One way to improve prognosis would be to identify bone metastasis at an earlier stage, or even predict those patients with breast cancer at increased risk for developing a bone metastasis. However, current diagnostic tools are unable to conduct accordingly and no high-performing biomarker(s) for breast cancer bone metastasis are currently available.

We hypothesized that a proteomic pattern with the potential to distinguish patients with breast cancer with or without bone metastasis with high sensitivity and specificity could be identified in the circulation of patients with breast cancer. To test this hypothesis, a proteomic profile indicative of breast cancer bone metastasis was developed. This diagnostic profile was validated by analyzing plasma from patients with multiple breast cancer in a blinded fashion. The analysis uncovered a diagnostic profile containing 13 protein peaks that discriminated patients with breast cancer with bone metastasis from those without clinical evidence of bone metastasis with 97% sensitivity and a 82% specificity. The major discriminatory protein ($m/z$ 4,260 Da) in the profile was identified as a previously uncharacterized PTHrP(12-48) peptide fragment. PTHrP is a well-known component of human breast cancer (30, 31, 46), yet the identity of any specific circulating form has been lacking.

The mechanisms by which cancer cells cause osteolytic metastasis are beginning to be unraveled (2). In metastatic human breast cancer, a well-studied (though not the sole) mediator of osteolysis is tumor-derived PTHrP (2). Our initial cloning and identification of PTHrP from metastatic human lung cancer cells (31) and its demonstration as the causal agent of the humoral hypercalcemia of...
malignancy (47) identified PTHrP as a previously unrecognized hormone that acts generally upon the skeleton to increase bone resorption and on the kidney to reduce calcium excretion (48). PTHrP shares homology with the N-terminus of PTH(1–34; ref. 31) and binds and activates the PTH1 receptor, which is responsible for mediating the pleiotropic paracrine effects of PTHrP as well as the endocrine actions of PTH on calcium and skeletal homeostasis (49).

The human PTHrP gene locus is located at 12p12.1-p11.2 and the gene is alternatively spliced to yield an array of different length human PTHrP transcripts (1-139; 1-141; 1-173), whose specific function(s) remain unresolved (50). The genomic structure suggests the existence of tissue-specific alternative promoters and alternate 3' splicing mechanisms that explain the multiple PTHrP mRNA species observed (48). Interestingly, multiple fragments of human PTHrP that seem to have biologic activities have been described, but few have been shown to circulate or to serve any important physiologic function in vivo (51).

Confirmation of the identity of the discriminatory m/z 4,260 Da peak as a PTHrP fragment was obtained using a variety of biochemical approaches, namely immunodepletion, tryptic mapping, and MALDI mass spectrometry. The putative 4,260 Da PTHrP peak was significantly diminished by the addition of a specific PTHrP(21-40) antibody and was confirmed as PTHrP(12-48) by tryptic mapping MALDI. MALDI mass spectrometry has a higher mass resolving power than SELDI-TOF MS and resolves peaks into an isotopic fine structure with the precision of the mass measurement better than 0.01 Da compared with SELDI-TOF MS, which is accurate within 2 Da (12, 52). The monoisotopic mass of the m/z 4,260 peak determined by SELDI was found to be m/z 4,253.3 Da by MALDI. Detailed database mining in silico identified a unique PTHrP peptide of this specific molecular weight as PTHrP(12-48). In sum, these biochemical and in silico analyses support the identification of the highly discriminatory peak as PTHrP(12-48). Subsequent tandem mass spectrometry-sequencing efforts have confirmed the identity of the PTHrP(12-48) peptide.

Furthermore, a specific SELDI-based assay was developed with which to measure circulating PTHrP(12-48). In a third breast cancer patient cohort, levels of PTHrP(12-48) were measured and were significantly higher in patients with breast cancer with bone metastasis compared with

Figure 5. Specific immunodepletion of PTHrP(12-48) from breast cancer patient plasma. A, SELDI profile of unfractionated breast cancer bone metastasis (BM) patient plasma (U34). Top, arrow indicates the m/z 4,260 peak of interest. Bottom, the m/z 4,260 peak is completely abolished by the addition of PTHrP(21-40)AB. B, SELDI profile of unfractionated breast cancer bone metastasis patient plasma (A21). Position of the m/z 4,260 peak of interest is shown. The m/z 4,260 peak is unaffected by the addition of PTHrP(1-15)AB. C, gel view of SELDI spectra from patient U34. Duplicate immune depletion with PTHrP(21-40)AB or PTHrP(1-15)AB. Specificity of PTHrP(21-40)AB is seen. D, quantification of the immunodepletion from (C). Gray bar + (1-15)AB; Black bar + (21-40)AB. Bar labels identify percentage of PTHrP(12-48) remaining after immunodepletion and shows specific immunodepletion of PTHrP(12-48).
patients lacking clinical evidence of bone metastasis. Interestingly, there was no significant correlation between PTHrP(12-48) and NTx levels in the circulation of these patients and the diagnostic specificity and accuracy of either marker alone was not significantly different. However, the combination of plasma PTHrP(12-48) and serum NTx measurements increased the diagnostic specificity and accuracy compared with either biomarker alone (AUC = 0.99). Importantly, it should be noted that high NTx was one of the selection criteria used to identify patients with breast cancer with bone metastasis. As such, in these selected patients, it is perhaps not surprising that NTx is such a robust predictor of bone metastasis. Despite the selection bias for NTx, the measurement of plasma PTHrP(12-48) using the assay described here was equal to serum NTx in predicting bone metastasis in these patients. The use of the systemic measurement of PTHrP(12-48) to predict patients with breast cancer with bone metastasis, in combination with NTx and other markers, is currently the focus of intense investigation in our laboratory. This is an important point, as the eventual clinical use of the measurement of PTHrP(12-48) will likely require evidence that PTHrP(12-48) is identifiable before, or coincident with, the first symptoms of bone involvement. In the study described here, the presence of nonbone metastasis was not addressed, so we have no direct data to this end.
However, in patient cohort 3, there were 7 patients with clinical evidence of both bone and visceral metastasis. Interestingly, the PTHrP(12-48) threshold of 51 ng/mL was able to correctly identify these patients as having metastasis. Ongoing studies are focused on measuring PTHrP(12-48) levels in additional breast cancer patient populations, with and without bone and visceral metastasis.

The identification of a unique PTHrP (12-48) fragment in the plasma of patients with breast cancer with bone metastasis raises several important points. The data suggest that, although the demonstration of PTHrP in metastatic breast cancer is potentially useful for diagnosis, the circulating fragment, resulting from host- and/or tumor-specific proteolytic degradation, may be different. The specific plasma protein profile identified here, which includes PTHrP(12-48), is the reflection in the circulation of the presence and activity of active breast cancer bone metastases. The other proteins in the profile presumably reflect changes in the tumor milieu, altered osteoclast activity associated with elevated bone turnover and presumably tumor-induced osteolysis, as well as specific markers of other tumor activity. Uncovering the identity of these other discriminatory and diagnostic markers is required and is the focus of ongoing investigation.

The identification and independent and repeated validation of the unique PTHrP(12-48) fragment represents the first step in the discovery of biomarkers to improve the reliability of clinical measures of the presence of bone metastasis. As such, the role of PTHrP(12-48) in the discriminating plasma biomarker profile, as well as in the identification and management of patients with bone or other metastasis is intriguing.

As with any study, there were limitations that should be considered in the interpretation of our findings. First it is retrospective, with archival breast cancer patient plasma samples prospectively analyzed by mass spectrometry. Interestingly, despite the archival nature of the samples, multiple specific proteins were repeatedly and blindly identified, showing the robustness of our sample storage and assay capabilities. We recognize that the sample size was somewhat small; nonetheless, there were significant PTHrP(12-48) differences between patients with and without bone metastasis that can be further investigated in larger studies. This study may also suffer from selection bias. The analyses were conducted on selected patients with increased bone turnover and who had not yet received a bone-modifying agent, now an uncommon clinical scenario, as almost all patients with breast cancer with bone metastases are treated with bone modifying agent(s); (2). How the use of these therapeutics may impact circulating PTHrP(12-48) levels is unknown, but is an area of research in which we are actively involved.

In the end, it is likely that blood (or plasma)-based breast cancer bone metastasis biomarkers, such as PTHrP(12-48) can be used in conjunction with existing clinical measures, as we have shown here. Such use has the potential to improve the specificity of the detection of bone metastases by current modalities. This is a critically important application for biomarkers that have the potential to improve the diagnosis of breast cancer bone metastasis. The evaluation of such diagnostic paradigms awaits the measurement of PTHrP(12-48) and characterization of the plasma biomarker profile in expanded breast cancer patient cohorts.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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