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 (BM) is a complication that significantly compromises patient survival due, 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 3 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 BM patients plasma compared with patients without BM (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 demonstrate that PTHrP(12-48) circulates in breast cancer patient plasma and is a novel and predictive biomarker of breast cancer BM. Importantly, the clinical measurement of PTHrP(12-48) in combination with NTx improves the detection of breast cancer BM.
Impact: In summary, we present the first validated, plasma biomarker signature for diagnosis of breast cancer BM that may improve the early diagnosis of high-risk individuals.
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 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 pathological 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 crosslinks (5). Surprisingly, relatively few cancer and bone turnover biomarkers have been demonstrated 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) (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 cancer patients 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), neurological disorders, and post
menopausal 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 breast cancer patients 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 samples, 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. Since the analyzed samples were obtained from a study of 2nd-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 two 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 (Figure 1A). The women ranged in age from 49-92 years with a median age of 70 in the bone metastasis group and 67 in the no bone metastasis group. Analysis of the archival plasma samples was approved by the UAMS and Penn State Institutional IRBs. 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 data sets for subsequent biomarker validation. Each archival sample contained at least 100ul of plasma, and some samples underwent at least 2 freeze-thaw cycles. All archival plasma samples were stored in 50ul 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, Hercules, CA). 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 analytical grade and obtained from Sigma-Aldrich (St Louis, MO) unless otherwise stated. CM10 chips were prepared using the Biomek 2000 robotic system as we have described (12). All proteinchips were placed in the Protein Biological System II C mass spectrometer reader (Bio-Rad) and time-of-flight spectra collected in three mass ranges (1.5-10 kDa (low), 7-30 kDa (mid), and 25-150 kDa (high)). Acquisition parameters were as described (12) and mass accuracy was calibrated using the All-in-one peptide and All-in-one protein molecular weight standards (Bio-Rad).

Acquisition and pre-processing of all spectral data was performed 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, 25,000 and 150,000 Da for the low, mid, and high MW 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 standard deviation of 1 (12).

Quality Control and Reproducibility

In order 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 and CV calculated with SAS 9.2 (Carey, NC). In order to assess intra-sample correlation, duplicate samples were applied to the protein chips in random locations. The greatest deviation between the two was 0.095. The median value of the Pearson/Spearman Correlation for Cohort 1 was 0.95 and 0.91, respectively, with an
inter-quartile 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 0.96 and 0.93, respectively, with an inter-quartile 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 inter-quartile 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

Analysis of Variance (ANOVA)

SELDI peaks were first selected based on 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 analysis of variance (ANOVA). Peak intensities with a p-value \( \leq 0.05 \) were considered significant. All statistical analyses were performed using SAS version 9.1 statistical software, with ANOVA performed with the Tukey and SNK multiple comparison test corrections.

Decision Tree Analysis: Random Forest Training Model

The Random Forest (RF) ensemble decision tree algorithm (RandomForest v5.1.0.179, Salford Systems, San Diego, CA) 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 un-weighted plurality-voting scheme and the important peaks selected based on their median importance scores as described (12).

Plasma Fractionation for Identification
Plasma samples with the highest expression of the target m/z 4260 Da peak were selected and fractionated into six fractions containing proteins on the basis of their isoelectric point as we have described (12). Fractions containing the m/z 4260 Da peak were identified by SELDI TOF MS analysis of all individual fractions using CM10 protein chips as described above.

**Tryptic Peptide Mapping**

After fractionation, fraction 5 (F5) containing the peak of interest (m/z 4260 Da) was digested with trypsin. CM10 protein chips prepared as described above 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 4260 Da) was then used for MALDI identification. MALDI spectra were collected in the m/z 1,000 to 10,000 Da molecular weight range, using a PerkinElmerSciex MALDI prOTOF (Perkin Elmer, Waltham, MA). The tryptic digested samples were analyzed by MS/MS to identify PTHrP tryptic fragments. Comparison of tryptic peptides with PTHrP theoretical tryptic digestion using the PAWS proteomic analysis software (Genomics Solutions, Ann Arbor, MI) was used to identify and confirm the specific peaks of interest.

**Specific Immunodepletion of PTHrP(12-48)**

Immunodepletion of the discriminatory m/z 4260 Da peak was performed using plasma from breast cancer patients with bone metastasis demonstrating the highest intensity value for the m/z 4260 Da peak. PTHrP antibodies A113 #6496 affinity purified IgG (recognizes PTHrP(1-15); a kind gift of Dr. John Chirgwin, Indiana University) and Ab#906 (recognizes PTHrP(21-40); a kind gift of Dr. T.J. Martin, St Vincent’s Institute, Australia) were prepared in PBS. Fifty (50) µl of plasma was incubated with 5 µl of
PTHrP antibody for 2 hours at 4°C. Next, 1µl of protein A/G agarose conjugate suspension was added and incubated at 4°C with rocking overnight. The sample was then centrifuged at 1,000g 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 breast cancer patient plasma. Quality control serum spiked with a working range of 0–1,000ng/mL PTHrP(12-48) synthetic peptide (Biomatrik, Inc.) and plasma samples from Cohort 3, 17 breast cancer patients with and 17 without bone metastasis, as well as 4 samples from Cohort 1 and 4 from Cohort 2 two with and two 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) 4260Da m/z peak in each spectrum was manually identified and peak areas quantified with the Biomarker ProteinChip 3.2.2 software (Bio-Rad). Biological and technical replicates were averaged and normalized to the unspiked control. Patient plasma concentrations were determined by comparison to 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 ($J$) (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 AUC. All logistic regression and ROC curve analyses were performed with XLSTAT (v2012.5.01, Addinsoft, New York, NY) and MedCalc (v12.3.0, MedCalc Software, Mariakerke, Belgium).

Results

Classification of Bone Metastasis versus No Bone Metastasis Patients

The initial patient cohort of 36 plasma specimens was classified into two groups; breast cancer without bone metastasis (18 samples) vs. 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 performed as shown (Figure 1B). Statistical, bioinformatics, and machine learning analyses were performed to identify a panel of differentially expressed peaks that could distinguish the two 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 1.5-150 kDa. Thirteen of these were found via multiple bioinformatic tools to be statistically significant, with p-values <0.05. Several of the representative SELDI TOF MS generated peaks in the 1.5-10 kDa molecular weight range are shown in the representative SELDI spectra (Figure 2A).

Refined Biomarker Signature for Breast Cancer Bone Metastasis Diagnosis

After classifying the blinded patient plasma samples of Cohort 2 using the initial RF model with high sensitivity and specificity, all SELDI data were combined and used to generate a second RF model (Figure 1B). The refined RF 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 demonstrated the reproducibility of the spectra (Figure 2A). The significant SELDI TOF MS generated peaks that discriminate between breast cancer patients with and without bone metastasis were confirmed and m/z 4133.27, 4260.92, 4740.39, 11386.2, 11452.8, 11525.9, 11629.0, 11679.7, 11728.2, 11894.2, and 39096.5 Da were up-regulated in breast cancer patients with bone metastasis, whereas only peaks m/z 17384.8 and 17577.3 Da were down-regulated (Figure 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 4260.92 Da significant peak (Figure 2A), tentatively identified the peak as a fragment of parathyroid hormone-related protein (PTHrP), (m/z 4259.89 Da). PTHrP was originally identified as the cause of the humoral hypercalemia 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). In order to confirm the tentative in silico identification of the m/z 4260 Da peak as a PTHrP fragment, we next determined the accurate monoisotopic mass of the SELDI-derived m/z 4260.92 Da peak.

The significant protein peak (m/z 4260) (Figure 3C) from breast cancer bone metastasis patient plasma was analyzed by MALDI–TOF MS and found to have a m/z of 4255.3 (Figure 3A), the monoisotopic mass of which was subsequently identified as m/z 4253.3 Da (Figure 3B). After determination of the accurate and specific monoisotopic molecular weight of the discriminatory m/z 4260 Da peak as m/z 4253.3 Da (Figure 3B), the PTHrP(1-173) protein sequence (31) was examined for any peptides with an exact
m/z 4253.3 Da. PAWS proteomic analysis identified PThrP(12-48) with a molecular weight of m/z 4253.3 Da. Thus, we hypothesized that the m/z 4260 Da peak in the SELDI profile was PThrP(12-48). Subsequent analysis by SELDI-TOF MS and MS/MS was performed to identify specific tryptic fragments. Breast cancer bone metastasis patient plasma was fractionated and digested with trypsin and compared to similarly trypsin digested PThrP(1-37) (Figure 3C, D, E). The SELDI proteinchip arrays were analyzed using an adapter that allows elucidation by a MALDI-based prOTOF MS. 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 1947.06, 1116.55 and 731.40 Da (Figure 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 1947.06 tryptic peptide (Figure 3E). The predicted pattern of overlapping and distinct tryptic peptides was observed and confirmed that the patient plasma sample peak (m/z 4260 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 (Figure 3C). Subsequent analysis by Thermo LTQ-XL ion trap MS confirmed the identity of the PThrP tryptic peptide m/z 1948.2 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) (42, 43) were used to determine if either or both antibodies were able to recognize PTHrP(12-48) (Figure 4). As expected, the PTHrP(1-15) antibody was ineffective at immunodepleting PTHrP(12-48), since 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 ~60% of the spiked PTHrP(12-48) (Figure 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 µl) containing the m/z 4260 Da peak of plasma was incubated with 5 µl 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 4260 Da) (Figure 5A) by ~70% (Figure 5D) 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 4260 (Figure 5B), or any other observable peak in the profile (Figure 5D). Collectively, these data suggest that the immunodepleted m/z 4260 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**

In order 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 breast cancer patients (with and without bone metastasis (Figure 6) was developed. Human control plasma was spiked with increasing concentrations of PTHrP(12-48) from 0-1000 ng/ml
(Figure 6A). The correlation coefficient for the SELDI-based detection of PTHrP(12-48) peptide added to control plasma using this assay was 0.98 (Figure 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 (Figure 6C). PTHrP(12-48) levels were significantly increased (p<0.0001) in patients with bone metastasis (Figure 6C). In fact, PTHrP(12-48) levels ranged between 45.1 and 205.7 ng/mL (mean 102.5 ± 46.4 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) (Figure 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 (Figure 6C). The measurement of PTHrP(12-48) using a threshold of 51 ng/mL (12nM) ((J=0.57 (0.29 - 0.67)) was not significantly different (p=0.07) from serum NTx measured using a threshold of 20.2 nM/BCE ((J=0.81 (0.57 - 0.86)) and classified the two patient groups with similar accuracy (NTx: Sn 81% (58% - 95%); 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) (Figure 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) (Figure 6D).

Discussion

Metastatic breast cancer is the second leading cause of cancer 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 breast cancer patients at increased risk for developing a bone metastasis. However, current diagnostic tools are unable to perform 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 breast cancer patients with or without bone metastasis with high sensitivity and specificity could be identified in the circulation of breast cancer patients. In order to test this hypothesis, a proteomic profile indicative of breast cancer bone metastasis was developed. This diagnostic profile was validated by analyzing plasma from multiple breast cancer patients in a blinded fashion. The analysis uncovered a diagnostic profile containing 13 protein peaks that discriminated breast cancer patients with bone metastasis from those without clinical evidence of bone metastasis with 97% sensitivity and a 82% specificity. The major discriminatory protein (m/z 4260 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) (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 appear to have biologic activities have been described, but few have been shown to circulate or to serve any important physiological function in vivo (51).

Confirmation of the identity of the discriminatory m/z 4260 Da peak as a PTHrP fragment was obtained using a variety of biochemical approaches, namely immunodepletion, tryptic mapping and MALDI MS. The putative 4260 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. Third, MALDI MS 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 4260 peak determined by SELDI was found to be m/z 4253.3 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 MS-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 breast cancer patients with bone metastasis compared with 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 breast cancer patients 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 utility of the systemic measurement of PTHrP(12-48) to predict breast cancer patients 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, since the eventual clinical utility 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 non-bone 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 breast cancer patients with bone metastasis raises several important points. The data suggests 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, demonstrating 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 performed on selected patients with increased bone turnover and who had not yet received a bone modifying agent, now an uncommon clinical scenario, since almost all breast cancer patients with bone metastasis 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 demonstrated here. Such utility 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.
Acknowledgements

These studies are dedicated to the vision of the late Dr Carl L. Nelson who was a fierce advocate for discovery and innovation. This work was supported by a Graduate Studentship from the AR Breast Cancer Research Program (ABCRP) (CLW), NIH Fellowship NIH F32GM093614 (SDB), Pilot grant from ABCRP (LJS), the Carl L. Nelson Chair of Orthopaedic Creativity (LJS), the Fashion Footwear Association of New York (FFANY) (LJS), and the UAMS Translational Research Institute (TRI) (CTSA grant Award #1 UL1TR000039).
References


Table 1. Patient description

<table>
<thead>
<tr>
<th></th>
<th>Patients (N)</th>
<th>Bone Mets (BM)</th>
<th>No Bone Mets (NBM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>Age mean ± SD</td>
</tr>
<tr>
<td>Cohort 1</td>
<td>36</td>
<td>18</td>
<td>68±10.8</td>
</tr>
<tr>
<td>Cohort 2</td>
<td>41</td>
<td>21</td>
<td>70±9.8</td>
</tr>
<tr>
<td>Cohort 3</td>
<td>34</td>
<td>17</td>
<td>66±12.1</td>
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</tbody>
</table>
Table 2. Patient demographics

<table>
<thead>
<tr>
<th></th>
<th>Cohorts 1 and 2*</th>
<th>Cohort 3*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No bone (n=36)</td>
<td>Bone (n=41)</td>
</tr>
<tr>
<td>Receptor Status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>33 (92%)</td>
<td>34 (83%)</td>
</tr>
<tr>
<td>Negative / Unknown</td>
<td>3 (8%)</td>
<td>7 (17%)</td>
</tr>
<tr>
<td>Disease-free interval</td>
<td>2.47</td>
<td>2.49</td>
</tr>
<tr>
<td>(years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visceral metastasis</td>
<td>23 (64%)</td>
<td>9 (22%)</td>
</tr>
</tbody>
</table>

* 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.
Figure Legends

Figure 1. Cohort-specific bone resorption markers and workflow diagram for SELDI-TOF MS analysis and. (A) Serum NTx levels in all breast cancer patients with bone metastasis (open circles) and no bone metastasis (closed circles). Cohorts 1 and 2 show clear discrimination of NTx levels around the cut-off of 25 nM NTx. Cohort 3 shows the overlap of serum NTx common to breast cancer patients. Dashed line indicates the 25nM cut-off that defines low versus elevated bone resorption. (B) An initial test set of 36 samples with known class were obtained and spectra generated. The data was 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 one year later and spectra obtained and data analyzed in an identical fashion to the training set. The test set samples were then samples were classified into respective groups based on the RF 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 RF model and overall diagnostic fingerprint.

Figure 2. Biomarker signature of breast cancer bone metastasis. (A) Comparative low molecular weight SELDI TOF MS profiles of breast cancer patient plasma. Blue, no bone metastasis; Red with bone metastasis. Inset, expanded view showing the discriminatory 4260 Da peak present in patients with bone metastasis (red) and absent in patients with no bone metastasis (blue). (B) Diagnostic fingerprint showing intensity and molecular weight (m/z) of the 13 discriminatory peaks identified in breast cancer patient plasma. White bars, no bone metastasis; black bars, with bone metastasis.
Figure 3. Monoisotopic mass and tryptic mapping of PTHrP(12-48) in patient plasma. (A) Mass spectrometry profile of breast cancer patient plasma identifying the SELDI 4260 m/z peak as 4255.3 m/z using MALDI. (B) The isotopic profile of the 4255.3 peak identified the monoisotopic mass as 4253.3 m/z. (C) SELDI profile of fractionated bone metastasis patient plasma, fraction 5. Arrow indicates the m/z 4260 peak of interest. (D) Fraction 5 sample after trypsin digestion. Solid arrow m/z 1947.06; hashed arrow m/z 1116.55. Both tryptic peptides are as predicted for the trypsin cleavage of PTHrP(12-48). (E) Control PTHrP(1-37) tryptic peptide map identified the predicted m/z 1947.06 peptide (solid arrow).

Figure 4. Specificity of PTHrP antibodies. (A) Gel view of SELDI spectra. Quality control (QC) serum was spiked with PTHrP(12-48) (750 ng/ml) and then immunodepleted with either PTHrP(1-15)-AB or PTHrP(21-40)-AB. Addition of the (21-40)-AB significantly depletes spiked PTHrP(12-48). (B) Quantification of the immunodepletion from A. All bars QC serum spiked with PTHrP(12-48). Gray bar + (1-15)-AB; Black bar + (21-40)-AB. Bar labels identify percent of PTHrP(12-48) remaining after immunodepletion and demonstrate specificity of the (21-40)-AB for detecting PTHrP(12-48).

Figure 5. Specific immunodepletion of PTHrP(12-48) from breast cancer patient plasma. (A) SELDI profile of unfractionated breast cancer bone metastasis patient plasma (U34). (Top panel) Arrow indicates the m/z 4260 peak of interest. (Bottom panel) The m/z 4260 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 4260 peak of interest is shown. The m/z 4260 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 percent of PTHrP(12-48) remaining after immunodepletion and demonstrates specific immunodepletion of PTHrP(12-48).

Figure 6. Specific SELDI-based measurement of PTHrP(12-48) in patient plasma.
(A) Representative SELDI profiles of quality control (QC) plasma containing 0, 250, 500, 750, 1000 ng/ml PTHrP(12-48). Specific peak area measured is highlighted. (B) Standard curve for the measurement of PTHrP(12-48) in spiked QC plasma ($R^2 = 0.985$). (C) PTHrP(12-48) plasma concentrations measured in patients with (BM) and without bone metastasis (NBM). PTHrP(12-48) levels are significantly increased in patients with bone metastasis. *p<0.05 (D) ROC curves for the measurement of PTHrP(12-48) and NTx in patient plasma. The determined accuracy of PTHrP(12-48) and NTx is not significantly different. The combination of NTx and PTHrP(12-48) is the most accurate (AUC=0.99).
Figure 2: 

A. 

4260.92+H

Bone met

No bone met

B. 

No Bone Met

Bone Met

m/z

Intensity

Low MW range (1.5 – 10 kDa)

2500 5000 7500 10000
Figure 4

A

B

% PTH P

0%

25%

50%

75%

100%

100.0% 100.0%

99.7% 99.2%

+ (1-15) AB + (21-40) AB

+ (1-15) AB + (21-40) AB

+PTH P (12-48) + (1-15) AB + (21-40) AB

QC
Figure 5
Figure 6

- **A**: Mass spectrometry analysis showing peak intensity at different concentrations of PTHrP(1-48)
  - 1000 ng/mL
  - 750 ng/mL
  - 500 ng/mL
  - 250 ng/mL
  - 0 ng/mL

- **B**: Graph showing the relationship between peak area (AUC) and PTHrP(1-48) concentration (ng/mL)

- **C**: Scatter plot of PTHrP(1-48) concentration (pg/ul) for BM and NBM samples

- **D**: Receiver Operating Characteristic (ROC) curve for PTHrP(1-48), NTx, and PTHrP(1-48) + NTx:
  - PTHrP(1-48): (AUC=0.85)
  - NTx: (AUC=0.96)
  - PTHrP(1-48) + NTx: (AUC=0.99)
Identification of PTHrP(12-48) as a plasma biomarker associated with breast cancer bone metastasis

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