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Breast Cancer

Reverse-Phase Protein Array for Prediction of Patients at Low Risk of Developing Bone Metastasis From Breast Cancer

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Disclosures of potential conflicts of interest may be found at the end of this article.

Key Words. Breast neoplasm • Bone metastasis • Reverse-phase protein array • Prediction model • Phosphorylated protein

Abstract

Background. A biomarker that predicts bone metastasis based on a protein laboratory assay has not been demonstrated. Reverse-phase protein array (RPPA) enables quantification of total and phosphorylated proteins, providing information about their functional status. The aim of this study was to identify bone-metastasis-related markers in patients with primary breast cancer using RPPA analysis.

Patients and Methods. Tumor samples were obtained from 169 patients with primary invasive breast carcinoma who underwent surgery. The patients were categorized by whether they developed breast cancer bone metastasis (BCBM) during follow-up. Clinical characteristics and protein expression by RPPA were compared and verified by leave-one-out cross-validation.

Results. Lymph node status ($p = .023$) and expression level of 22 proteins by RPPA were significantly correlated with BCBM in logistic regression analysis. These variables were used to build a logistic regression model. After filtering the variables through a stepwise algorithm, the final model, consisting of 8 proteins and lymph node status, had sensitivity of 30.0%, specificity of 90.5%, positive predictive value of 30.0%, and negative predictive value of 90.5% in the cross-validation. Most of the identified proteins were associated with cell cycle or signal transduction (CDK2, CDKN1A, Rb1, Src, phosphorylated-ribosomal S6 kinase, HER2, BCL11A, and MYH11).

Conclusion. Our validated model, in which the primary tumor is tested with RPPA, can predict patients who are at low risk of developing BCBM and thus who likely would not benefit from receiving a bisphosphonate in the adjuvant setting. Clinical trials excluding these patients have the potential to clarify the benefit of bisphosphonates in the adjuvant setting. ***The Oncologist*** 2014;19:909–914

Implications for Practice: We present a validated model involving testing of the primary tumor with reverse-phase protein array to predict patients who are at low risk of developing bone metastasis from breast cancer. The model, which consists of eight proteins and lymph node status, showed novel predictive potential. Patients with low assessed risk are unlikely to benefit from receiving a bisphosphonate in the adjuvant setting. Further clinical trials excluding these patients will clarify the benefit of bisphosphonates.

Introduction

Bone is a very common site of metastasis from breast cancer. Breast cancer bone metastasis (BCBM) presents in 30%–85% of patients during the course of disease. In 1889, Dr. Stephen Paget first described the "seed and soil" theory, in which some characterized cancer cells (the "seeds") metastasize to certain favorable organs (the "soil") [1]. Bone, which has rich blood flow, may be an optimal organ for development of metastasis.

The mechanism of BCBM is not sufficiently known. Cancer cells do not destroy bone directly, but cytokines and hormones secreted by the cancer cells cause bone destruction and absorption [2]. Furthermore, these cytokines and proteins cause unbalanced activity of osteoblasts and osteoclasts, which then induce osteoblastic and osteolytic changes. These complicated biological processes involved in BCBM may make it difficult to predict. Patients who could be predicted to have a greater risk of BCBM might benefit from adjuvant therapy with an agent such as a bisphosphonate, which inhibits osteoclastic function.

It is very difficult to predict the occurrence of metastasis using only clinical characteristics. Recent advanced technologies have begun to reveal the roles of genes and cell signaling pathways related to metastasis [2]. Gene analysis may predict

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which patients in a population may develop metastasis, and microarray-based prediction models, such as Oncotype DX [3] and the MammaPrint assay [4], are currently commercially available. However, these prediction models still cannot predict specific metastatic sites.

The reverse-phase protein array (RPPA) is a new type of antibody-based assay. This method has great advantages over immunohistochemistry (IHC) and microarray analysis in terms of quantifying protein expression and providing functional information by revealing the expression of total and modified (phosphorylated) proteins. The aim of this study was to use RPPA analysis to identify BCBM-related markers in patients with primary breast cancer.

Materials and Methods

Human Tumor Samples

Tumor samples were obtained from biopsies in 169 patients with primary invasive ductal or invasive lobular breast carcinoma who underwent surgery between June 1992 and March 2007 at the University of Texas MD Anderson Cancer Center. The samples were obtained from MD Anderson's breast tissue frozen tumor bank. Patients with ductal carcinoma in situ, metaplastic carcinoma, or sarcoma were excluded.

Clinical data were collected from MD Anderson's Breast Cancer Management System database. All specimens were collected and analyzed under institutional review board approval. The presence of metastasis was diagnosed by biopsy or radiological findings. Estrogen receptor (ER), progesterone receptor, and HER2 levels were compared for correlation with bone metastasis; these hormone receptor levels were evaluated not as positive or negative by IHC and/or fluorescence in situ hybridization but rather as continuous variables by RPPA analysis.

Reverse-Phase Protein Array

RPPA analysis was performed in our laboratory, as described previously [5–7]. Briefly, frozen tumors were lysed by homogenization with lysis buffer. Tumor lysates were normalized to 1-μg/μL concentration using a bicinchoninic acid assay. The lysates were then boiled with 1% sodium dodecyl sulfate, and the supernatants were manually diluted in six or eight twofold serial dilutions with lysis buffer. An Aushon Biosystems 2470 arrayer was used to create sample arrays from the serial dilutions on nitrocellulose-coated FAST slides (Schleicher and Schuell, Keene, NH, www.schleicher-schuell.com/bioscience). The slides were analyzed and protein expression quantitated with the use of Microvigene software (VigeneTech Inc., Carlisle, MA, http://www.vigenetech.com). The RPPA data set consists of relative expression levels for 108 full proteins as well as 46 phosphoproteins (supplemental online Table 1). The RPPA data were normalized using SuperCurve [8].

Gene Expression Data

For microarray analysis, tumor samples were obtained from fine-needle biopsy before any systemic therapy. Samples were placed in RNAlater (Ambion, Austin, TX, http://www.ambion.com) storage reagent and stored at −80°C until gene expression analysis. All gene expression data were generated using Affymetrix U133A gene chips (Affymetrix, Inc, Santa Clara, CA, http://www.affymetrix.com) [9]. The Robust Multi-array Analysis algorithm was used to quantify the Affymetrix arrays [10].

Statistical Methods

Baseline patient characteristics were summarized with medians and ranges for age and follow-up time and with frequencies and percentages for all other characteristics.

Each model contained terms for age at diagnosis, menopausal status, the presence of lymph node metastasis at diagnosis, stage, hormone receptor status, and receipt of pre- or postoperative chemotherapy. *P* values $<.05$ were considered statistically significant. All statistical tests were two-sided. All statistical analyses were done using SPSS version 17 (IBM Corp., Armonk, NY, http://www-01.ibm.com/software/analytics/spss/) and R software version 2.10.1.

We performed univariate analysis to identify clinical parameters and proteins (from the RPPA data) associated with BCBM. The χ^2 test was used to identify significant clinical parameters associated with bone metastasis for categorical values. The Wilcoxon rank-sum test was used to identify proteins that were differentially expressed between patients who did and did not have BCBM. The variables identified as significant in univariate analysis were used to perform logistic regression analyses comparing the two sample groups. Leave-one-out cross-validation was done to verify the results obtained from the regression model. The correlation of expression levels between proteins (determined by RPPA) and their respective genes (determined by microarray analysis) was assessed by Spearman rank correlation.

Results

Patient Characteristics

Clinicopathological characteristics of the derivation cohort of 169 patients are listed in Table 1. We categorized the samples into two groups. The BCBM group consisted of patients who had metastasis to bone or to bone and other sites during the follow-up period. The "Other" group consisted of patients who did not have metastasis to bone during the follow-up period. The median ages of the BCBM and Other groups were 52 years (range: 30–66 years) and 53 years (range: 27–83 years), respectively. Thirty-seven of the 169 patients had died at the time of our analysis. Among these 169 patients, 61 were diagnosed with metastasis during the study period. The first metastatic lesion was exclusively in bone in 5 patients, in bone and in other sites in 10 patients, and only in nonbone sites in 46 patients. Among these 46 patients, the second metastatic site was bone for 6 patients. Consequently, a total of 21 patients were placed in the BCBM group, and 148 patients were placed in the Other group.

We compared the BCBM and Other groups to identify clinical parameters associated with BCBM (Table 2). Only lymph node status was significantly correlated with BCBM ($p = .023$) in univariate analysis. Age at diagnosis, menopausal status, pathological stage, tumor grade, and receipt of chemotherapy for the primary tumor were not significantly associated with occurrence of BCBM.

Table 1. Patient characteristics

Characteristic	All patients ($N = 169$)	BCBM group ($n = 21$)	Other group ($n = 148$)
Age, years, median (range)	53 (27–83)	52 (30–66)	53 (27–83)
Follow-up time, months, median (range)	47.3 (2.5–236.7)	49.3 (12–170.8)	47.3 (2.5–236.7)
Menopausal status, *n* (%)			
Premenopausal	105 (62.1)	10 (47.6)	95 (64.2)
Postmenopausal	64 (37.9)	11 (52.4)	53 (35.8)
ER and/or PR status (by IHC) , *n* (%)			
Positive	76 (45.0)	12 (57.1)	64 (43.2)
Negative	93 (55.0)	9 (42.9)	84 (56.8)
HER-2/neu status (by IHC/FISH) , *n* (%)			
Positive (3+, 2+/FISH+)	33 (19.5)	14 (66.7)	19 (12.8)
Negative (0, 1+, 2+/FISH–)	136 (80.5)	7 (33.3)	129 (87.2)
Tumor grade, *n* (%)			
1	6 (3.6)	0 (0)	6 (4.1)
2	45 (26.6)	4 (19.0)	41 (27.7)
3	114 (67.4)	15 (71.4)	99 (66.9)
NA	4 (2.4)	2 (9.5)	2 (1.3)
Lymph node metastasis status, *n* (%)			
Positive	90 (53.2)	16 (76.2)	74 (50.0)
Negative	77 (45.6)	4 (19.0)	73 (49.3)
NA	2 (1.2)	1 (4.8)	1 (0.7)
Pathologic stage, *n* (%)			
0	2 (1.2)	0 (0)	2 (1.4)
I	27 (16.0)	1 (4.8)	26 (17.6)
II	95 (56.2)	9 (42.9)	86 (58.1)
III	43 (25.4)	9 (42.9)	34 (22.9)
NA	2 (1.2)	2 (9.5)	0 (0)
Receiving chemotherapy, *n* (%)			
Yes	136 (80.5)	18 (85.7)	118 (79.7)
No	33 (19.5)	3 (14.3)	30 (20.3)
Survival status at last follow-up, *n* (%)			
Alive	132 (78.1)	7 (33.3)	125 (84.5)
Dead	37 (21.9)	14 (66.7)	23 (15.5)

Abbreviations: BCBM, breast cancer bone metastasis; ER, estrogen receptor; FISH, fluorescence in situ hybridization; HER2, human epidermal growth factor receptor type 2; IHC, immunohistochemistry; NA, not available; PR, progesterone receptor.

Identification of Predictive Markers for Bone Metastasis

To analyze the RPPA data, we treated the assayed protein expression levels as continuous variables and the occurrence of BCBM as a categorical variable using the Wilcoxon rank-sum test (Table 3). In this analysis, the expression levels of 22 proteins were significantly correlated with BCBM in univariate analysis (Table 3). None of these proteins were correlated with metastasis in the Other group.

The 22 significant proteins from the RPPA data and lymph node status from the clinical data were used to build a logistic regression model. Of the 22 proteins, only 8 (CDK2, CDKN1A, Rb1, Src, phosphorylated-ribosomal S6 kinase [*p*-RSK], HER2, BCL11A, and MYH11) and lymph node status remained in the final model after filtering the variables using a stepwise algorithm. The final model with the 9 variables had sensitivity of 55%, specificity of 97.9%, positive predictive value of 78.5%, and negative predictive value of 94.1%. Using leave-one-out cross-validation, the model had sensitivity of 30%, specificity of 90.5%, positive predictive value of 30.0%, and negative predictive value of 90.5%. These performance metrics give more realistic estimates for practical use of the model.

In the cross-validation, because the samples differed in each iteration, the significant proteins identified and the actual proteins identified in the final filtered model varied in every iteration. EGFR, although not selected in the original model, was identified as significant in all 167 iterations and was present in 161 of the 167 models in cross-validation. The protein JAZF1 was also identified as significant in all 167 iterations and was present in 155 of the 167 models in cross-validation. Most of the proteins (CDK2, CDKN1A, Rb1, *p*-RSK, and HER2) that remained in the final model after validation are associated with cell cycle or signal transduction pathways.

Table 2. Clinical and tumor characteristics of patients

Characteristic	*p* value[a]
Age at diagnosis (<50 or ≥50 years)	.146
Menopausal status (pre- or postmenopausal)	.220
Tumor grade (1, 2, or 3)	.468
Lymph node metastasis status (positive or negative)	.023
Pathologic stage (I, II, III, IV)	.108
Chemotherapy (received or not)	.723

[a]The χ^2 test was used to perform univariate analysis between the breast cancer bone metastasis (BCBM) group and the Other group using the listed clinical parameters. The lymph node metastasis status was significantly ($p = .023$) different between the BCBM group and the Other group.

Table 3. Significant proteins in the breast cancer bone metastasis group compared with the Other group

Protein name	*p* value
BAD P1	.0009
BCL2	.04715
CDK2	.01014
EGFR	.02653
EGFR P2	.00351
STK11 P1	.0087
CDKN1A	.0126
PDK1 P2	.04769
RAB25	.0461
RB1	.02888
SRC	.02718
KDR	.01056
CD4	.0025
RSK P1	.0262
HER2	.00004
HER2 P1	.00035
BCL11A	.02588
FGFR2	.02121
JAZF1	.01227
KIT	.01729
MYH11	.02374
SGK1	.01685

Correlation Between RPPA and Microarray Analysis

It has not been clarified whether RPPA provides information independent of that from microarray analysis. Consequently, we examined the correlations between microarray data and RPPA data prior to validating our results using microarray gene expression data related to the 22 RPPA-identified proteins that were significantly correlated with BCBM in univariate analysis.

Microarray data were available for 57 of the patient tumors. There were 31 probe sets on the microarray corresponding to the proteins in the RPPA signature. The correlations were poor between the proteins identified by RPPA and the genes identified by the microarray probe sets (supplemental online Table 2).

Discussion

In this study, we showed that our model for predicting BCBM with nine variables—the expression levels of eight proteins and lymph node status—can predict with very high accuracy which patients are unlikely to develop BCBM. Our results suggested that RPPA analysis and microarray analysis provide different information. Although it has not been sufficiently proven that bisphosphonates provide a benefit in terms of extending BCBM-free survival, our results indicate that a subset of patients is unlikely to develop bone metastasis and thus would not benefit from receiving a bisphosphonate in the adjuvant setting. Conducting clinical trials that exclude such patients has the potential to clarify the benefit of receiving a bisphosphonate.

We could not predict which patients would develop BCBM using our RPPA model. However, our prediction rate for patients who would not develop BCBM was much higher than that of the traditional predictive factor, ER status, which has sensitivity of 74% and specificity of 63% with microarray analysis, according to Smid et al. [11]. Because a bisphosphonate is not currently prescribed routinely for patients in the adjuvant setting, the >90% of breast cancer patients who are predicted not to develop BCBM are justified in not receiving it. The rest of the patients, who have a possibility of developing bone metastasis, are good candidates for a clinical trial to determine the survival benefit of bisphosphonate use in the adjuvant setting.

This RPPA method has the potential for detecting a predictive marker or identifying a target for novel therapy and for revealing the need for prophylactic treatment. The advantage of RPPA is that it permits, with just a small amount of material, quantification of the expression level and modification of proteins as a continuous value for a large number of patients compared with IHC and microarray analysis. Reproducibility of RPPA has been confirmed in a previous study [12]. From previous studies of microarray analysis, some predictive genes associated with BCBM have been reported; however, only a few of these genes overlapped among the studies [11, 13, 14]. The different backgrounds of samples in each study may have caused these different results. In our analysis, the proteins identified by RPPA were not correlated with the genes identified by microarray probe sets. These results suggest that RPPA provides different information from microarray analysis, including the functional state of proteins, and the RPPA findings could not be validated using microarray data for expression of genes related to the proteins. For these reasons, our method using RPPA is novel and has great potential to predict bone metastasis.

In our final prediction model, BCL11A and MYH11—the function of which in breast cancer was unknown—remained, in addition to proteins associated with cell cycle or signal transduction pathways, including CDK2, CDKN1A, Rb1, *p*-RSK, and HER2. BCL11A is essential for pre-B-cell development, thymocyte maturation, and globin switching. The *MYH11* gene encodes the smooth-muscle myosin heavy chain, which has a key role in smooth muscle contraction. Myosin functions in the use of the energy of ATP hydrolysis to move actin filaments and produce muscle force, and also is implicated in a variety of other cellular functions that are relevant for cancer formation. We still need to evaluate the actual function of these proteins

in developing BCBM. The function of current therapeutic agents such as bisphosphonates is mainly to block bone absorption. Such agents may improve bone health but cannot be expected to serve as fundamental therapy with survival improvement at this time. Novel targeting agents, including a receptor activator of nuclear factor κ-B ligand inhibitor (denosumab) and an Src inhibitor, have shown relevance in clinical trials. A new strategy of therapy for BCBM is development of novel targeting agents that block signaling molecules such as chemokine receptor 4. Given the currently known mechanism of BCBM and the microenvironment of bone, combination therapies may have better efficacy for patients with BCBM. Measuring the functional state of proteins or cell signaling pathways through RPPA may reveal a more reasonable therapeutic strategy.

Bisphosphonates have shown clinical benefit in large randomized controlled trials for breast cancer with bone metastasis. Bisphosphonates in the metastatic setting improve patients' quality of life by reduction of skeletal-related events [15–18]. However, the survival benefit of bisphosphonates has been controversial in each clinical trial [17–26]. Three adjuvant clodronate trials had conflicting results in terms of prevention of bone or other metastases [20, 21, 25, 26]. A meta-analysis of these trials also did not show any survival benefit with clodronate [23]. Recently, potential antitumor effects of zoledronic acid have been revealed in large clinical studies. In the randomized Austrian Breast and Colorectal Cancer Study Group (ABCSG-12) trial ($n = 1{,}803$), at a median follow-up of 62 months, addition of zoledronic acid to adjuvant endocrine therapy improved disease-free survival by 32% in premenopausal women with hormone-receptor-positive early breast cancer ($p = .009$), and there was no significant difference between the tamoxifen and anastrozole arms [27]. A similar result was also observed in the Zometa-Femara Adjuvant Synergy Trial (ZO-FAST) at a median follow-up of 48 months. The trial showed a significant improvement in median disease-free survival, including bone-metastasis-free survival, by 41% ($p = .0175$) in postmenopausal women [28]. However, the Adjuvant Zoledronic Acid to Reduce Recurrence (AZURE) trial ($n = 3{,}360$) showed that addition of zoledronic acid to adjuvant chemotherapy did not have an impact in patients with stage II/III breast cancer; in subset analysis, improvement of disease-free survival and overall survival was seen only in postmenopausal patients [29]. The investigators concluded that the results did not support the routine use of zoledronic acid in the adjuvant setting. Recently, the meta-analysis showed that adjuvant use of bisphosphonates significantly reduced the risk of bone metastasis recurrence by 34% and improved breast cancer survival by 17% in postmenopausal women with early breast cancer [30]. However, no similar effects were observed in premenopausal women.

Another consideration is the cost versus the benefit of bisphosphonate treatment. Although low incidence of osteonecrosis of the jaw (ONJ) and other serious adverse events was reported in the AZURE and ABCSG-12 trials [18, 20, 26], patients who develop ONJ will suffer severely from its symptoms. Consequently, it is reasonable for patients who are not likely to develop BCBM to be excluded from receiving a bisphosphonate in the adjuvant setting, even among postmenopausal women. Because of the unavailability of other RPPA data in a large clinical trial using adjuvant bisphosphonate, further prospectively collected samples would be useful to address the issue of how bisphosphonates affect the markers that we found in our study.

There are some limitations of this exploratory research for prediction of BCBM. Only 57 patients had both RPPA and microarray data for determination of the correlations between them. Further analysis using a larger data sample with the same patient background is needed to confirm the difference between these two methods. Because this study had a short median follow-up time and a small number of events, we determined a predictive model for early onset of BCBM. We further need to determine a predictive model for late-onset BCBM with long-term follow-up. We also need to validate this model in a large prospective cohort with recent external data.

Conclusion

We demonstrated that a combination of expression levels of specific proteins and modified proteins and lymph node status predicted patients who are unlikely to develop bone metastasis from breast cancer. We need a definitive prospective study to assess the survival benefit of bisphosphonate in an adjuvant setting in a population that excludes such patients.

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Disclosures

Naoto T. Ueno: Amgen (RF). The other authors indicated no financial relationships.

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