Bone Metastasis in Advanced Breast Cancer: Analysis of Gene Expression Microarray

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Abstract

In this study we analyzed gene expression profiles in advanced breast cancer patients to elucidate genes that can be used to predict bone metastasis. A combination of the identified 3 genes in bone metastasis and other organs and 8 genes in only bone metastasis showed better prediction, and can be used as a training set.

Background: Approximately 30% to 40% of breast cancer recurrences involve bone metastasis (BM). Certain genes have been linked to BM; however, none have been able to predict bone involvement. In this study, we analyzed gene expression profiles in advanced breast cancer patients to elucidate genes that can be used to predict BM. **Patients and Methods:** A total of 92 advanced breast cancer patients, including 46 patients with BM and 46 patients without BM, were identified for this study. Immunohistochemistry and gene expression analysis was performed on 81 formalin-fixed paraffin-embedded samples. Data were collected through medical records, and gene expression of 200 selected genes compiled from 6 previous studies was performed using NanoString nCounter. **Results:** Genetic expression profiles showed that 22 genes were significantly differentially expressed between breast cancer patients with metastasis in bone and other organs (BM+) and non-BM, whereas subjects with only BM showed 17 significantly differentially expressed genes. The following genes were associated with an increasing incidence of BM in the BM+ group: *estrogen receptor 1 (ESR1)*, GATA binding protein 3 *(GATA3)*, and *melanophilin* with an area under the curve (AUC) of 0.804. In the BM group, the following genes were associated with an increasing incidence of BM: *ESR1*, *progesterone receptor*, *B-cell lymphoma 2*, *Rab escort protein*, *N-acetyltransferase 1*, *GATA3*, *annexin A9*, and *chromosome 9 open reading frame 116*. *ESR1* and *GATA3* showed an increased strength of association with an AUC of 0.928. **Conclusion:** A combination of the identified 3 genes in BM+ and 8 genes in BM showed better prediction than did each individual gene, and this combination can be used as a training set.

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Keywords: Advanced breast cancer, Bone metastasis, ESR1, Gene expression profile, NanoString microarray

Introduction

Breast cancer is the second leading cause of cancer-related death in women, and it is the top-ranked cancer among women in developed countries. In the past few years, breast cancer has become the leading cancer type globally.[1-4] Breast cancer mortality is mostly related to distant metastasis to other vital organs. Approximately 30% to 40% of breast cancer recurrence cases involve bone metastasis (BM), although autopsy studies have reported bone involvement in almost 70% of breast cancer-related mortality cases.[5] A previous study conducted at Dharmais Cancer Hospital Indonesia showed a 24.4% BM incidence in advanced stage breast cancer. St Gallen consensus reported 14.9% and 40.8% cumulative incidence of BM in node-positive breast cancer at 2 and 10 years, respectively, after diagnosis.[6,7]

Despite multiple clinicopathological and molecular studies, the etiology of BM in breast cancer patients remains unknown, and it is still impossible to accurately predict when BM will occur. Cancer metastasis to various organs, including bone, is not a random event, and we believe it proceeds in a systematic sequence that involves the acquisition of different mutations, microenvironmental cues, inflammatory responses, and other factors.[8] Recently, several groups have published multigene expression profiles that are predictive for BM in breast cancer.[9-11]

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Certain subtypes, such as intrinsic luminal, are well known to relapse only 20 years after diagnosis and treatment, which differs from the HER2 and triple-negative breast cancer (TNBC) subtypes that have the tendency to relapse within the first 5 years after treatment.[12,13] Various immunohistochemistry studies have identified several genes as possible early BM markers, but these findings have yet to be substantiated with proper evidence.[9,10,14,15]

In patients with early-stage breast cancer, several clinical trials have suggested that the adjuvant use of bisphosphonates (BP) reduced rates of recurrence and death.[16-18] However, these findings do not support the routine use of zoledronic acid in the adjuvant management of breast cancer.[19,20] A meta-analysis study indicated that a positive effect for adjuvant BP on survival was only in postmenopausal patients.[21] The microRNA expression profile can help to identify novel targets for zoledronic acid in breast cancer.[22] However, some clinical trials have not had specific variables that can be used to either predict the incidence of BM or decide on which cases that benefit from the use of BP.

The identification of factors that can predict BM events using clinicopathologic characteristics and gene expression profiles in advanced breast cancer has yet to be done. We will used gene expression profiles from a previous study to identify the genes that can predict BM in advanced breast cancer patients.[9,10,15,23-25]

Patients and Methods

Patient Recruitment

This study first started in July 2012 and lasted until February 2015, using 92 subjects with advanced breast cancer on the basis of study criteria. The subjects were categorized on the basis of their metastasis status: patients with BM (n = 46) and patients without BM/non-BM (NBM) (n = 46). Patients with BM were further categorized on the basis of whether they had only BM or BM and metastasis to other organs (BM+). All experiments were performed in accordance with the Declaration of Helsinki and other relevant guidelines. This study was also approved by the ethics committee of the Medical Faculty, Universitas Indonesia. The clinicopathological characteristics are summarized in Table 1.

RNA Extraction

Total RNA was extracted from formalin-fixed paraffin-embedded (FFPE) samples. Each paraffin block was tested on the basis of

Table 1 Association Immunohistochemistry With Bone and Non-Bone Metastases in Advanced Breast Cancer Patients

Clinicopathology	Metastasis		Crude OR (95% confidence interval [CI])	*P*
	Bone, n (%)	Non-Bone, n (%)		
Age				
>64 Years	3 (33.3)	6 (66.7)	0.500 (0.105-2.379)	.384
55-64 Years	8 (47.1)	9 (52.9)	0.889 (0.270-2.925)	.846
45-54 Years	20 (55.6)	16 (44.4)	1.250 (0.473-3.303)	.653
35-44 Years	15 (50.0)	15 (50.0)	Reff	
Histopathology				
Ductal invasive/NST	37 (50.7)	36 (49.3)	1.142 (0.415-3.137)	1.000
Lobular invasive	9 (47.4)	10 (52.6)	Reff	
Malignancy Degree				
Low	13 (43.3)	17 (56.7)	0.328 (0.070-1.518)	.154
Intermediate	26 (50.0)	26 (50.0)	0.429 (0.099-1.841)	.255
High	7 (70.0)	3 (30.0)	Reff	
Primary/Relapse				
Primary	25 (43.9)	32 (56.1)	0.521 (0.222-1.225)	.198
Relapse	21 (60.0)	14 (40.0)	Reff	
Estrogen Receptors				
Positive	39 (66.1)	20 (33.9)	7.243 (2.682-19.561)	<.001
Negative	7 (21.2)	26 (78.8)	Reff	
Progesterone Receptors				
Positive	33 (58.9)	23 (41.1)	2.538 (1.070-6.021)	.033
Negative	13 (36,1)	23 (63.9)	Reff	
HER2				
High expression	15 (40.5)	22 (59.5)	0.528 (0.227-1.229)	.202
Low expression	31 (56.4)	24 (43.6)	Reff	
Molecular subtype				
Luminal	39 (58.2)	28 (41.8)	3.582 (1.319-9.726)	.019
TNBC	0 (0.0)	8 (100.0)	–	.006
HER2	7 (41.2)	10 (58.8)	0.646 (0.222-1.878)	.591

Abbreviations: NST = no specific type; Reff = represents the group with the lowest risk; TNBC = triple negative breast cancer.

National Cancer Center Singapore standard of RNA amount ≥ 50% to reach 250 base pairs. After quality control of the FFPE samples and RNA extraction, 81 samples were analyzed.

NanoString Quantification

A NanoString (Nanostring Technologies Inc, Seattle, WA) panel was designed, which comprised 200 genes (including 8 housekeeping genes) compiled from 6 previous studies.[9,10,15,23-25] The probes and RNA were hybridized according to the manufacturer's protocol. The raw expression data were normalized and $\log_2$-transformed for further analyses.

Statistical Analysis

The normalized and $\log_2$-transformed data were analyzed using unsupervised hierarchical clustering, and heat maps were generated to show the grouping of samples on the basis of their gene expression patterns. Supervised hierarchical clustering was also performed on the basis of the clinical status (BM+, BM, and NBM) of the samples.

Univariate analysis was used to evaluate data distribution. We used χ^2 or Fisher exact tests to evaluate the correlations between gene expression patterns and clinicopathological characteristics. Multivariate analysis using variables with $P < .25$ in the bivariate analysis was performed using logistic regression test.

Results

Association of Clinicopathological Characteristics With BM Incidence

The following clinicopathological characteristics were used in our analysis: age, histopathology, degree of malignancy, cancer status, molecular subtype, presence of estrogen receptors (ERs), progesterone receptors (PRs) and epidermal growth factor receptor HER2. Tumor size and node status were not analyzed in this study because tumor size was unmeasurable in most of the samples obtained (subcutaneous lesion, bone lytic lesion, etc), and the node status was only on the basis of clinical impression.

Most of these factors were not associated with a BM event ($P > .05$), but age older than 64 years, ductal invasive, low malignancy degree, and relapse exhibited a slightly higher risk for BM. Samples that were ER-positive (crude odds ratio [OR], 7.243; $P < .001$), luminal subtype (crude OR, 3.582; $P = .019$), and PR-positive (crude OR, 2.538; $P = .033$) have a higher risk rate for BM. However, PR-positive and luminal subtype were not statistically significant ($P = .055$) in multiregression analysis because of their interaction with ER-positive (Table 1).

Gene Expression Analysis of the BM+ Group

Gene expression profiling has yielded 22 genes with significant changes between the BM+ and NBM groups. Of these 22 genes, 13 genes were upregulated in the BM+ group and ranked in descending order as follows: estrogen receptor 1 *(ESR1)*, progesterone receptor *(PGR)*, signal peptide, CUB domain and EGF like domain containing 23 *(SCUBE2)*, N-acetyltransferase 1 *(NAT1)*, GATA binding protein 3 *(GATA3)*, annexin A9 *(ANXA9)*, kinesin family member 5C *(KIF5C)*, Rab escort protein *(REPS2)*, insulin like growth factor binding protein 5 *(IGFBP5)*, dynein axonemal light intermediate chain 1 *(DNALI1)*, B-cell lymphoma 2 *(BCL2)*, melanophilin *(MLPH)*, and *reticulon 4 receptor like 1*. The following 9 genes were downregulated: *origin recognition complex subunit 6*, *cell division cycle associated 7*, *G protein-coupled receptor 1*, *tweety family member 1*, cathepsin V *(CTSV)*, Erb-B2 receptor tyrosine kinase 2 *(ERBB2)*, *MMP1*, growth factor receptor bound protein 7 *(GRB7)*, and chloride channel accessory *(CLCA2)*.

Using this gene list, unsupervised hierarchical clustering was done and 2 relatively distinct subgroups were identified, herein termed as group 1 and group 2 as shown in Figure 1, which had significantly different proportions of BM+ and NBM samples ($P < .001$). Group 1 had a higher number of NBM samples, whereas group 2 showed a higher number of BM+ samples. However, the distinct groupings were lost when the samples were clustered on the basis of their clinical status (BM+ and NBM).

To identify the genes that are associated with BM incidence, we generated receiver operating characteristic curves of the upregulated genes in the BM+ group. The following genes were identified: *ESR1*, *GATA3*, and *MLPH* with an area under the curve (AUC) of 0.702, 0.489, and 0.651, respectively. Combining these 3 genes increased the AUC to 0.804.

Gene Expression Analysis of the BM Group

Similarly, we discovered 17 genes with significant changes between the BM and NBM groups. The 13 genes upregulated in BM are as follows: *ESR1*, *NAT1*, *PGR*, *SCUBE2*, *GATA3*, *ANXA9*, *IGFBP5*, *REPS2*, *KIF5C*, *DNALI1*, *BCL2*, *C9orfl16*, and *transforming growth factor beta 3*. Four genes, *CTSV*, *Kallikrein related peptidase*, *GRB7*, and *CLCA2* were downregulated in BM.

Unsupervised hierarchical clustering was done and 2 relatively distinct groups were identified, herein termed group 1 and group 2 as shown in Figure 2, which had significantly different proportions of BM and NBM samples ($P < .001$). Group 1 consisted of 1 BM and 25 NBM, whereas group 2 had 16 BM and 14 NBM.

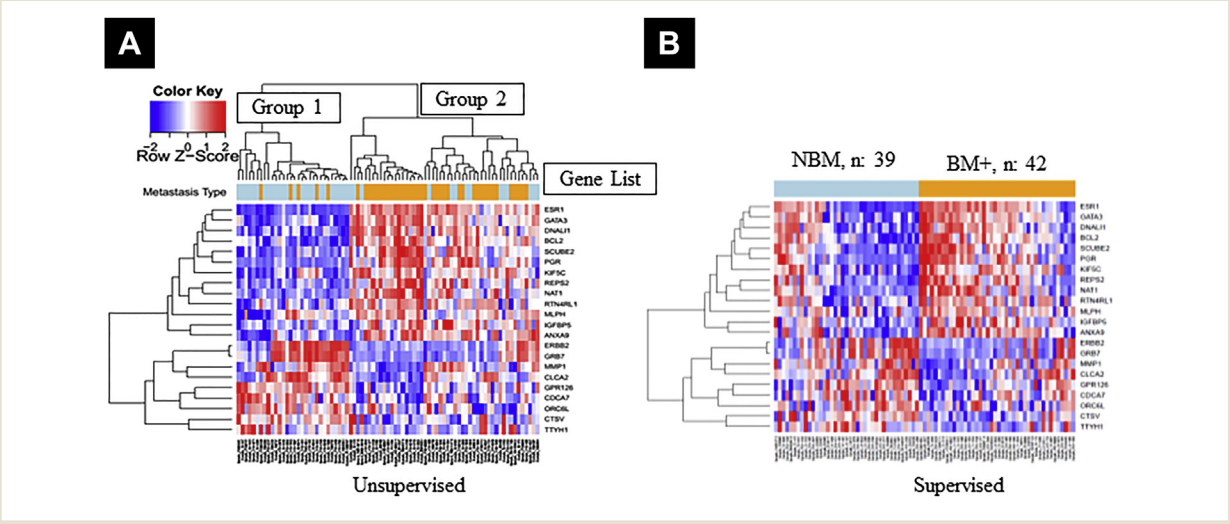
Supervised hierarchical clustering was then performed to identify genes that were associated with an increased BM incidence. Although the distinct groupings were lost, a group of 8 genes exhibited an upward trend in the 17 BM samples. These 8 genes were *ESR1*, *PGR*, *BCL2*, *REPS2*, *NAT1*, *GATA3*, *ANXA9* and *chromosome 9 open reading frame 116. ESR1* and *GATA3* were also associated with an increased BM incidence in the BM+ group. Combining these 8 genes showed an increased strength of association with an AUC of 0.928.

Discussion

The main complication of breast cancer is the metastasis of cancer cells to surrounding tissues and other organs. It has been estimated that approximately 40% to 75% of breast cancer cases include BM.[3,7,26,27] The mechanism of metastasis to the bone is a complex process.

On the basis of our clinicopathology data, approximately 72% of breast cancer patients were younger than 54 years, with the bulk of the patients between 45 and 54 years. In contrast to the belief that cancer incidence usually increases with age, this result shows a tendency for breast cancer to afflict women at a younger age, and similar trends have also been observed in Pakistan, India, and Sri Lanka.[3,28-30] The difference in age distribution suggests that ethnicity, nutrition, and genetic factors could play key contributing roles.

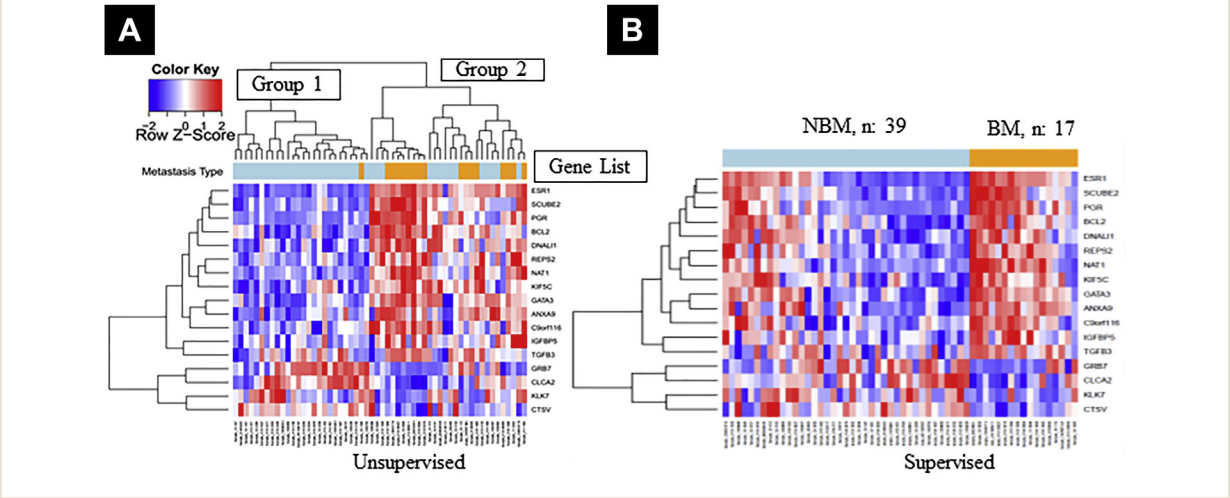
Figure 1 Gene Expression (mRNA) Heat Map of Bone Metastasis With Other Organs (BM+) and Non-Bone Metastasis Breast Cancer (NBM); (A) Unsupervised; (B) Supervised



Our data also show there is a higher tendency for BM to occur in recurrent breast cancer patients. Despite treatment, the return of the cancer indicates that the primary cancer cells might have acquired the certain genetic mutations that allow them to metastasize to a secondary location.[31,32]

Our study has shown that compared with advanced breast cancers without BM, those with BM were associated with the luminal subtype with ER-positive and PR-positive tissues, although no significant differences were seen with HER2 expression between these 2 groups. Similar results were also observed in studies by Wei B et al[33] and Wei S et al,[34] therefore showing the significance of increased ER expression in breast cancer patients with BM. Smid et al[35] have also shown that approximately two-thirds of patients with BM were ER-positive luminal cases, whereas only 7% were of the basal subtype. Our previous study showed that a combination of ER-positive, mouse monoclonal antibody *(MLF1P)*, and interleukin 11 receptor subunit alpha *(IL11-RA)* yielded an AUC of almost 80% compared with individual *IL11-RA* and *MLF1P* with an AUC of 0.652 and 0.670, respectively. This indicates that the combination of those factors would be a better predictor of BM.[36] In this study, we have shown that ER-positive and *ESR1* show the strongest association with BM.

Figure 2 Gene Expression (mRNA) Heat Map of Only Bone Metastasis (BM) and Non-Bone Metastasis Breast Cancer (NBM); (A) Unsupervised; (B) Supervised



Our gene expression analyses of the BM+ and NBM groups have shown that *ESR1*, *PGR*, *NAT1*, *SCUBE2*, and *GATA3* have significantly different expression patterns between the 2 groups. Of these 5 genes, *ESR1*, *PGR*, and *NAT1* were shown to be upregulated in the BM group. *ESR1* showed the highest level of upregulation in the BM as well as BM+ groups, which complements other studies that have shown the influence of ERs in the malignancy of breast cancers.

Because 73% of our cohort was diagnosed with the luminal subtype, the crude OR has shown that the luminal subtype has 3.6 times increased risk of developing BM, suggesting that *ESR1*, *PGR*, and *NAT1* have specific roles in BM. Taken together with the increased risk associated with ER-positive tissues, it would suggest that a positive correlation can be drawn between *ESR1*, *PGR*, and *NAT1* upregulation with BM.

On the basis of our data, the highly upregulated genes identified in the BM+ and BM groups could be probable candidates for the next training set. We have also identified 6 genes that are present in the BM group but absent in the BM+ group, which could be explained by the following. First, the BM genotype was covered by other genotypes with more aggressive characteristics, such as TNBC or HER2, thus they tend to metastasize to other organs, such as lung, liver, and brain.[37-39] Second, downregulated genes (eg, *ERBB2* in BM+), could be used as a predictor of disease aggression and poor prognosis.[40-42]

Compared with previous studies, this study yielded no osteolytic prospective gene that was significantly expressed in the BM process in the 22-gene or 17-gene panel, which differentiated the BM group from the NBM group ($P < .000$). Compared with other studies, our study showed a different gene expression pattern. This is influenced by several factors: different origins of samples, FFPE versus fresh samples, patient type (advanced stage with no metastasis), and results of immunohistochemistry staining intensities that were affected by FFPE samples and reagent optimization.

A plausible explanation for increased risk of BM is the interaction *ESR1* has with other variables.[43] In addition, Zhang et al[38] have shown that 90% of ER-positive tumors contain the proto-oncogene c-*Src* (Src) response signature (SRS), where Src activation elicits further downstream signaling that promotes cancer cell survival and the acquisition of a metastatic profile. In addition, ER in the cytoplasm interacts with Src, thereby activating SRS to stimulate the proliferation and survival of tumor cells. Increased activity of Src is also a cell survival response to cytokines such as tumor necrosis factor-related apoptosis-inducing ligand, *C-X-C motif chemokine ligand 12*, and *insulin-like growth factor 1*, which are expressed in the BM microenvironment, hence activating the Akt/PKB signaling pathway (protein kinase B) signaling pathway and avoiding apoptosis. This suggests that Src has a role in the antiapoptosis mechanism. Furthermore, Src increases the activity of the phosphatidylinositol 3-kinase and Akt pathways to enrich primary tumor clones that are able to metastasize to the bone,[26,38] therefore solidifying the role of Src as a possible key player in BM. Therefore, a further study with the addition of Src as a prospective gene is required.

Conclusion

Compared with the AUC of a single gene or the AUC of 13 genes in the unsupervised group, the combined AUC of the 3 or 8 genes discussed previously showed higher performance. Despite the random grouping in both sets of clustering, either unsupervised or supervised, the results produced were consistent and significantly different. A combination of the identified 3 genes in BM+ and 8 genes in BM showed better prediction and can be used as a training set.

Clinical Practice Points

- Cancer metastasis to various organs,including bone, is not a random event, and we believe it proceeds in asystematic sequence that involves the acquisition of different mutations, microenvironmental cues, inflammatory responses, and other factors.
- Certain genes have been linked to BM; however, none have been able to predict bone involvement.
- Several clinical trials have suggested that the adjuvant use of BP reduced rates of recurrence and death. However, some clinical trials have not had specific variables that can be used to either predict the incidence of BM or decide on which patients might benefit from the use of BP.
- Our genetic expression profiles showed that 22 genes were significantly differentially expressed in breast cancer patients with BM+ and non-BM, whereas subjects with only BM showed 17 significantly differentially expressed genes.
- The following genes were associated with an increasing incidence of BM in the BM+ group: *ESR1*, *GATA3*, and *MLPH*. In the BM group, and the following genes were associated with an increasing incidence of BM: *ESR1*, *PGR*, *BCL2*, *REPS2*, *NAT1*, *GATA3*, *ANXA9*, and *C9orf116*. *ESR1* and *GATA3* showed an increased strength of association.
- A combination of the identified 3 genes in the BM+ group and 8 genes in BM group showed better prediction than did each individual gene, and this combination can be used as a training set.
- The microRNA expression profile can help to identify novel targets for BP in breast cancer.

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