

# Exploring the molecular mechanism associated with breast cancer bone metastasis using bioinformatic analysis and microarray genetic interaction network

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## Abstract

**Background:** Bone metastases are common in advanced breast cancer patients and frequently leading to skeletal-related morbidity and deterioration in the quality of life. Although chemotherapy and hormone therapy are able to control the symptoms caused by bone destruction, the underlying molecular mechanisms for the affinity of breast cancer cells towards skeletal bones are still not completely understood.

**Methods:** In this study, bioinformatic analysis was performed on patients' microarray gene expression data to explore the molecular mechanism associated with breast cancer bone metastasis. Microarray gene expression profile regarding patients with breast cancer and disseminated tumor cells was downloaded from Gene Expression Omnibus (GEO) database (NCBI, NIH). Raw data were normalized and differently expressed genes were identified by using Significance Analysis of Microarrays (SAM) methods. Protein interaction networks were expanded using String. Moreover, molecular functions, biological processes and signaling pathway enrichment analysis were performed using Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG).

**Results:** We identified 66 differentially expressed genes. After submitting the set of genes to String, genetic interaction network was expanded, which consisted of 110 nodes and 869 edges. Pathway enrichment analysis suggested that adhesion kinase, ECM-receptor interaction, calcium signaling, Wnt pathways, and PI3K/AKT signaling pathway are highly associated with breast cancer bone metastasis.

**Conclusion:** In this study, we established a microarray genetic interaction network associated with breast cancer bone metastasis. This information provides some potential molecular therapeutic targets for breast cancer initiation and progression.

**Abbreviations:** BC = breast cancer, cc = clustering coefficient, GEO = gene expression omnibus, GO = gene ontology, KEGG = Kyoto encyclopedia of genes and genomes, SAM = significance analysis of microarrays.

**Keywords:** bone metastasis, breast cancer, enrichment analysis, interaction network, microarray

## 1. Introduction

Globally, breast cancer is the most frequently diagnosed cancer and the leading cause of cancer-related death in women.<sup>[1]</sup> Metastatic diseases occur in most women with advanced breast cancer and bone is one of the most preferential distant organs for

metastasis of breast cancer. Evidence from clinical and postmortem studies suggests that 47% to 85% of breast cancer patients will have bone metastasis.<sup>[2]</sup> It has also been reported that the breast cancer tumor subtypes affect the metastases sites and rates. The lowest rate of bone metastases are patients with estrogen (ER)-negative/human epidermal growth factor receptor 2 (HER2)-negative tumors, which is 55.2%; meanwhile, this rate was significantly increased to 69.8% (HER2-positive tumors), 87.8% (ER-positive/HER2-negative/Ki67high tumors), and 73.1% (ER-positive/HER2-negative/Ki67low tumors). The most common sites of bone metastases are the spine, ribs, pelvis, proximal femur, and skull. The destruction of these bones frequently leads to excessive skeletal-related complication such as bone pain, pathological fractures, life-threatening hypercalcemia, spinal cord compression, and other nerve compression syndromes. Some of them can be fatal and significantly reduce the quality of life.

Bone metastasis is a complex, multistage process that requires breast cancer cells to detach from the primary tumor, travel through the blood or lymphatic system, survive in bone microenvironment, and then proliferate in bone tissue.<sup>[3]</sup> To date, genomic studies have suggested that each step of metastasis was associated with a series of molecular events. However, the interaction network of molecular mechanism associated bone metastases from breast cancer is still not completely understood.

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Motivated by this, we established a comprehensive protein interaction network by building a microarray gene expression profile originating from breast cancer patients with bone metastases, hoping to reveal the molecular mechanisms in breast cancer bone metastasis. In our analysis, 66 genes with significant expression changes were identified to confer bone metastasis. Pathway enrichment analysis highlighted that adhesion kinase, extracellular matrix (ECM)–receptor interaction, calcium signaling pathway, and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway are potential key regulators, which may involve in breast cancer bone metastasis. These results advanced our understanding of molecular information of bone metastasis from breast cancer and provided potential targets for clinical interventions.

## 2. Material and methods

### 2.1. Microarray dataset resources

After searching in Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>), a public functional genomics data repository, a microarray dataset was downloaded with the accession number GSE14776. In this study, Cawthorn et al.<sup>[4]</sup> explored the analyzable yield of genetic material from human biopsy samples in order to describe differences in gene expression between disseminated tumor cells and bone metastatic tumor

cells. Total RNA was extracted from disseminated tumor cells and bone metastatic tumor cells and mRNA array was performed on Illumina HumanRef-8 v3.0 platform. Other involved online databases were listed in the String website.

### 2.2. Aberrant expressed genes identification

To standardize the microarray data set, comparison of the gene expression profiles of metastatic tumor cells versus disseminated tumor cells was normalized using log<sub>2</sub> transformation, a method previously developed by Fan et al.<sup>[5]</sup> Subsequently, Significance Analysis of Microarrays (SAM, <http://statweb.stanford.edu/~tibs/SAM/>) was applied to produce a cluster of up- or downregulated variant genes according to previous publications.<sup>[6,7]</sup>

### 2.3. Functional protein association network construction

Protein–protein/Gene–protein interaction networks were expanded on the basis of the result from 2.2 using String consortium (<http://string-db.org/>).<sup>[8]</sup> Gene Ontology consortium (GO, <http://www.geneontology.org/>) and Kyoto Encyclopedia of Genes and Genomes (KEGG, <http://www.genome.jp/kegg/>) functional enrichment were also applied via Database for Annotation, Visualization and Integrated Discovery (DAVID, <https://david.ncifcrf.gov/>).<sup>[9,10]</sup>

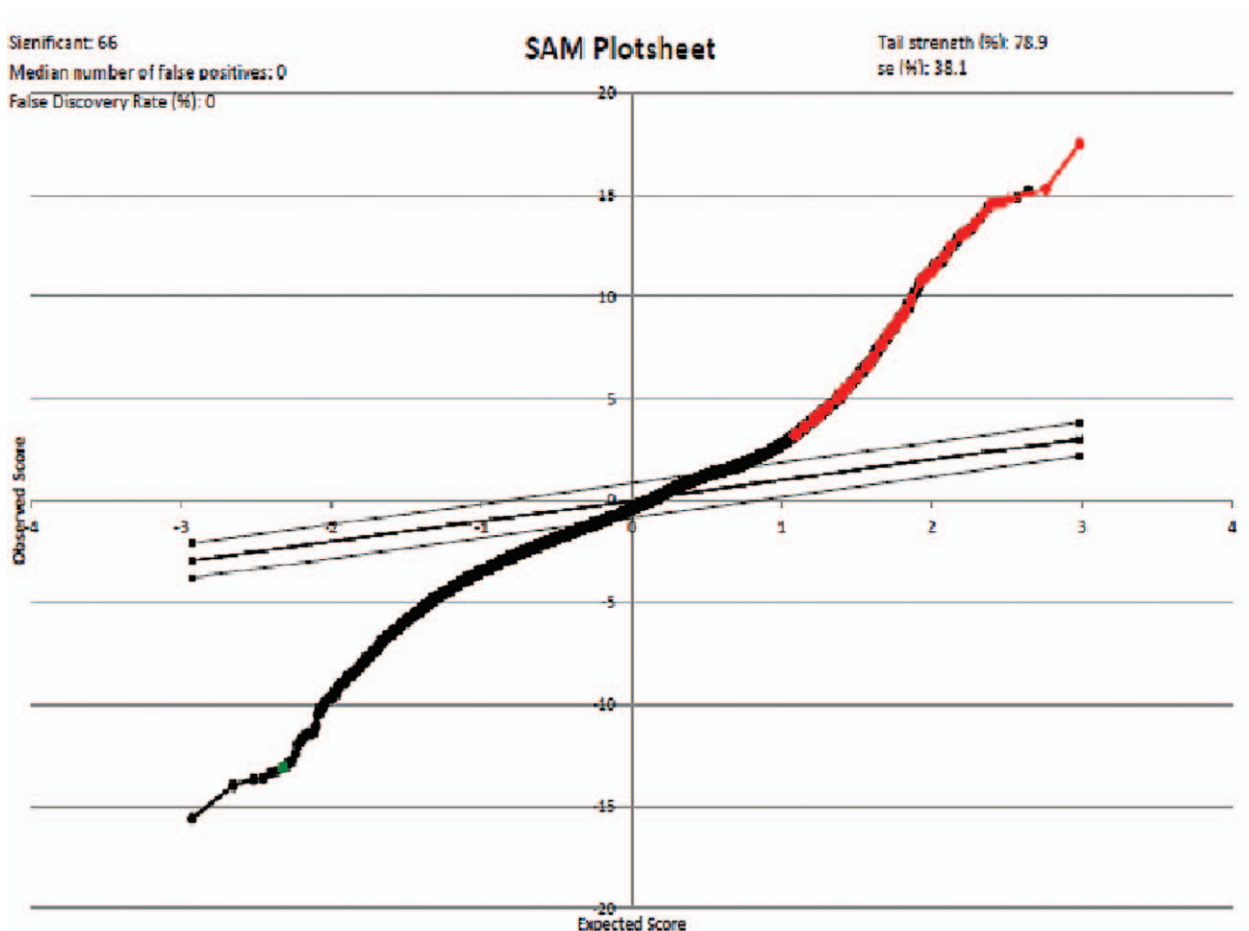


Figure 1. Significance analysis of microarrays (SAM) plot result generated by SAM plugin in Excel platform.

## 2.4. Statistical analysis

Gene expression was considered to be significant if the threshold of false discovery rate (FDR)  $\leq 5\%$  and fold change  $\geq 5$ . For GO and KEGG enrichment analysis, biological process, molecular function, and signaling pathways were identified as different if the  $P$  value was  $\leq 5\%$ .

## 2.5. Ethical Experimentation

The study does not involve any patient consent, so ethical approval is not necessary.

## 3. Results

### 3.1. Sixty-six genes were found to be significantly expressed in bone-specific metastatic breast tumor cells

A total of 14 breast tumor samples were profiled in this study, consisting of 8 disseminated tumor cell samples and 6 metastatic tumor cell samples. After performing SAM, 66 genes were found to be differently expressed in metastatic tumor cells comparing to disseminated tumor cells as shown in Fig. 1 and Table 1. Totally, 65 genes increased and 1 gene decreased dramatically with the threshold of FDR  $\leq 5\%$  and fold change  $\geq 5$ .

### 3.2. Gene-gene interaction network construction associated with breast cancer bone metastasis

To better identify how these genes regulated breast cancer bone metastasis in a system biology perspective, all these significant genes were applied to String platform for further analysis. As shown in Fig. 2, the interaction network involved in bone metastasis consists of 110 nodes and 869 edges with the average node degree of 15.8. Network analysis also indicated that the clustering coefficient (cc) was 0.58, which means that the network has a reliable robustness Figure 3.

### 3.3. GO analysis in terms of molecular function and biological processes

To explore the genetic interaction network involved in bone metastasis in the context of GO, all the nodes were submitted to DAVID for functional annotation. As summarized in Table 2, molecular function analysis indicated that most of these genes regulated protein binding and activities. We also elevated the biological processes involved in this bone metastasis network (Table 3). Table 3 summarized all the potential biological processes for bone metastasis. In particular, all these genes seemed to be involved in skeletal muscle development and differentiation, and cell development Table 4.

### 3.4. Signaling pathway enrichment analysis

To assess the relationship between the significantly expressed genes and bone metastasis, we also elevated the potential signaling pathways involved in this pathogenesis (Table 3). Notably, focal adhesion kinase (FAK), ECM-receptor interaction, calcium signaling pathway, and PI3K/AKT signaling pathways seem to confer bone metastasis in metastatic tumor cells.

## 4. Discussion

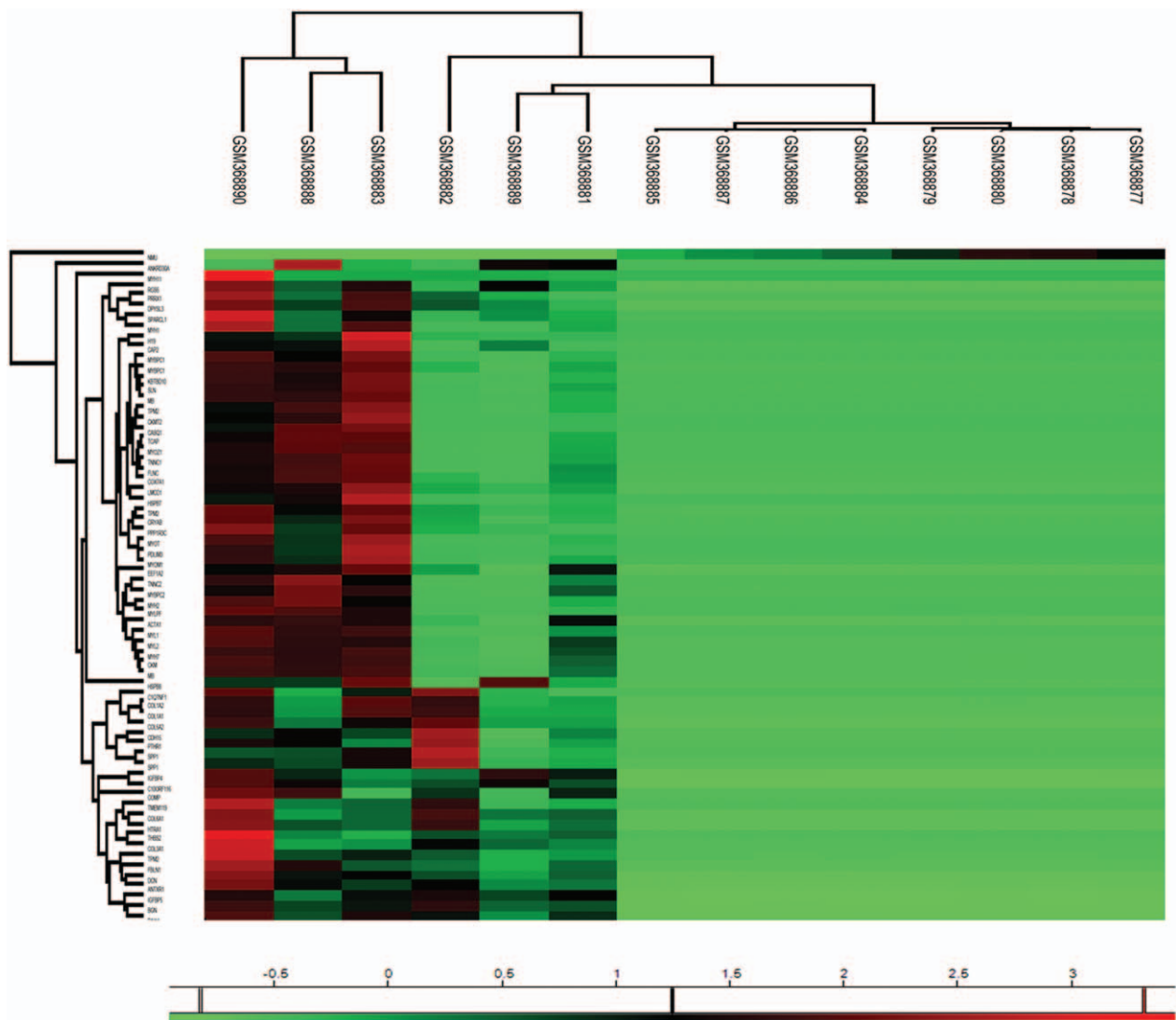
As we described previously, breast cancer bone metastasis is a complex process that includes tumor cells dissemination into

**Table 1**

**Significant genes identified by significant analysis of microarray (SAM) in metastatic tumor cells versus disseminated tumor cells.**

Gene ID	Gene name	Fold change	Gene regulation
ILMN_1682139	RAI14	5.171365018	Up
ILMN_1773079	COL3A1	6.982849802	Up
ILMN_1676563	HTRA1	6.186672925	Up
ILMN_1680110	C10ORF116	5.62413739	Up
ILMN_1651354	SPP1	8.120311169	Up
ILMN_2206746	BGN	6.788616982	Up
ILMN_1732151	COL6A1	5.19206243	Up
ILMN_1729117	COL5A2	6.02889456	Up
ILMN_1701308	COL1A1	8.685506406	Up
ILMN_1679262	DPYSL3	5.858419075	Up
ILMN_1665865	IGFBP4	5.371994388	Up
ILMN_2132982	IGFBP5	6.756401053	Up
ILMN_1678842	THBS2	5.19456082	Up
ILMN_2082273	RGS5	5.147027272	Up
ILMN_2104356	COL1A2	7.473049934	Up
ILMN_2374449	SPP1	6.811305803	Up
ILMN_1738116	TMEM119	6.420385133	Up
ILMN_1670379	ANTXR1	5.353581087	Up
ILMN_2347145	DCN	6.871239303	Up
ILMN_1672536	FBLN1	5.42833538	Up
ILMN_1754969	LMCD1	5.181945655	Up
ILMN_2218208	SPARCL1	5.847127838	Up
ILMN_1677636	COMP	5.612414957	Up
ILMN_1795166	PTHR1	6.334048145	Up
ILMN_1660086	MYH11	5.767671444	Up
ILMN_1662419	COX7A1	6.825572263	Up
ILMN_1739496	PRRX1	5.21056501	Up
ILMN_2148527	H19	5.758324154	Up
ILMN_1736670	PPP1R3C	6.254000814	Up
ILMN_1729216	CRYAB	7.982347327	Up
ILMN_1789196	TPM2	5.446569426	Up
ILMN_1757604	TPM2	5.964153134	Up
ILMN_2125869	ACTA1	7.954883327	Up
ILMN_1666109	MB	6.14745385	Up
ILMN_2108735	EEF1A2	6.732135912	Up
ILMN_2380237	C1QTNF1	5.408489082	Up
ILMN_1757521	CKM	7.606526359	Up
ILMN_1784036	CDH15	5.203647347	Up
ILMN_2113807	MYL2	7.868579764	Up
ILMN_2364864	MB	6.899035766	Up
ILMN_1752075	MYBPC1	8.003827301	Up
ILMN_1691237	CAP2	5.258749008	Up
ILMN_1669714	MYH7	7.21233735	Up
ILMN_1715748	FLNC	6.519045099	Up
ILMN_1778595	SLN	7.411203576	Up
ILMN_2330170	MYBPC1	7.089137326	Up
ILMN_1791280	HSPB8	5.236337651	Up
ILMN_1680344	MYOM1	5.146825247	Up
ILMN_2230025	PDLIM3	5.869918788	Up
ILMN_1796059	ANKRD30A	5.632681051	Up
ILMN_2218604	KBTBD10	6.19605488	Up
ILMN_1700860	MYL1	7.441270503	Up
ILMN_1656395	MYOT	6.565788174	Up
ILMN_1731157	MYOZ1	6.323047458	Up
ILMN_1809842	TNNC1	6.362462097	Up
ILMN_2200836	HSPB7	5.2389758	Up
ILMN_2051113	MYH1	6.184257861	Up
ILMN_1799743	MYBPC2	5.492847488	Up
ILMN_1813949	CASQ1	5.015400744	Up
ILMN_1789624	TPM2	6.300902539	Up
ILMN_1764266	CKMT2	5.389847298	Up
ILMN_1804316	TCAP	5.020894462	Up
ILMN_1693428	TNNC2	6.335950114	Up
ILMN_1759962	MYLPF	5.44701728	Up
ILMN_1692638	MYH2	5.709780098	Up
ILMN_2162253	NMU	0.17518273	Down

SAM=Significance Analysis Microarray.



**Figure 2.** Heatmap visualization of the significant genes identified by SAM. All the detailed information can be seen in Table 1.

circulation, homing to bone, and proliferation in bone tissue. Underlying these complicated, multistep scenarios, it has been known that a sophisticated network of molecular events is crucial in the development of metastasis to bone, which was not fully understood. In this literature, the authors identified a microarray gene expression profile and established a comprehensive genetic interaction network to reveal the molecular mechanisms in breast cancer bone metastasis. The results suggested that ECM–receptor interaction, FAK, calcium signaling pathway, and PI3K/AKT signaling pathway were highly associated with breast cancer bone metastasis.

Previous publications have already confirmed the role of ECM components in breast cancer dissemination and metastases.<sup>[11,12]</sup> As polysaccharides and fibrous proteins, ECM which induced by either cancer cells or stromal components is a crucial component of cancer microenvironment, initiating downstream signaling events that lead to the aggressive behavior of breast cancer.<sup>[13,14]</sup> The interaction of cancer cells and ECM components is profoundly altered at all steps of cancer metastasis, which include detachment from the primary tumor, migration through

adjacent tissue, invasion into and extravasation from the vasculature.<sup>[15–17]</sup> Studies on interaction of tumor cells with ECM components showed increased extracellular protease activity mediated by the family of matrix metalloproteinases (MMPs).<sup>[18,19]</sup>

Several previously studies indicated that FAK mediated cancer metastasis in various cancers.<sup>[20–22]</sup> (FAK) is a nonreceptor protein tyrosine kinase that resides at the sites of at focal adhesions, which plays an essential role in cancer cells survival, proliferation, migration, and invasion.<sup>[23,24]</sup> FAK coordinates a signaling network that orchestrates these processes through both kinase-dependent and independent mechanisms.<sup>[25]</sup> FAK cooperates with SRC and leads to SRC phosphorylation and then FAK/SRC phosphorylation at multiple sites, relaying the external signal into cells associated with various genes and multiple signaling pathways, such as PI3K/AKT and MAPK.<sup>[26]</sup>

A previous study suggested the regulation of the metastasis formation either directly through mutations in the involved adhesion molecules or indirectly through impaired calcium signaling pathway.<sup>[27]</sup> The ubiquitous second messenger calcium



**Table 3****Biological process analysis of the genetic interaction network associated with metastatic tumor cells in terms of Gene Ontology (GO).**

GO ID	Biological process	Observed gene count	FDR
GO.0003012	Muscle system process	37	1.65E-40
GO.0030049	Muscle filament sliding	23	1.65E-40
GO.0006936	Muscle contraction	35	2.82E-40
GO.0030048	Actin filament based movement	24	1.13E-35
GO.0030029	Actin filament based process	31	2.19E-24
GO.0006941	Striated muscle contraction	18	7.16E-22
GO.0006928	Movement of cell or subcellular component	43	1.86E-21
GO.0003008	System process	46	2.52E-20
GO.0061061	Muscle structure development	28	1.28E-19
GO.0007517	Muscle organ development	23	2.34E-18
GO.0060537	Muscle tissue development	21	1.01E-15
GO.0009887	Organ morphogenesis	31	2.02E-15
GO.0009653	Anatomical structure morphogenesis	44	5.22E-15
GO.0048729	Tissue morphogenesis	26	7.18E-15
GO.0009888	Tissue development	38	1.60E-14
GO.0051146	Striated muscle cell differentiation	17	3.90E-14
GO.0006937	Regulation of muscle contraction	16	1.08E-13
GO.0014706	Striated muscle tissue development	19	1.09E-13
GO.0048468	Cell development	37	3.10E-13
GO.0060048	Cardiac muscle contraction	11	3.55E-13

FDR = false discovery rate, GO = Gene Ontology, MAPK = mitogen-activated protein kinase.

bone metastasis.<sup>[38]</sup> Through the PI3K/AKT pathway, mPR $\alpha$  promoted the expression of MMP-9 during breast cancer cells invasion to local lymph nodes.<sup>[39]</sup> The angiogenesis and metastasis of breast cancer cells were inhibited by down-regulating PI3K/AKT/ERK signaling pathway mediated by

**Table 4****Signaling pathway analysis of the genetic interaction network associated with metastatic bone cells in terms of Gene Ontology (GO).**

Pathway ID	Signaling pathway	Observed gene count	FDR
4510	Focal adhesion	21	6.69E-19
5410	Hypertrophic cardiomyopathy (HCM)	13	7.12E-14
5414	Dilated cardiomyopathy	13	1.24E-13
4530	Tight junction	12	2.91E-10
4512	ECM-receptor interaction	10	1.71E-09
4260	Cardiac muscle contraction	9	1.20E-08
4810	Regulation of actin cytoskeleton	12	5.35E-08
5205	Proteoglycans in cancer	12	7.99E-08
4611	Platelet activation	9	8.39E-07
4151	PI3K-Akt signaling pathway	12	7.58E-06
4261	Adrenergic signaling in cardiomyocytes	8	2.47E-05
4670	Leukocyte transendothelial migration	6	0.000798
4974	Protein digestion and absorption	5	0.00199
5144	Malaria	4	0.00222
4921	Oxytocin signaling pathway	6	0.00331
4022	cGMP-PKG signaling pathway	6	0.0037
5146	Amoebiasis	5	0.00417
4270	Vascular smooth muscle contraction	5	0.00628
4360	Axon guidance	5	0.00809
5132	Salmonella infection	4	0.0137
5200	Pathways in cancer	7	0.0236
4020	Calcium signaling pathway	5	0.0327
5416	Viral myocarditis	3	0.0404
330	Arginine and proline metabolism	3	0.0429

FDR = false discovery rate, GO = Gene Ontology, phosphatidylinositol 3-kinase (PI3K)-protein kinase B (AKT).

connective tissue growth factor.<sup>[40]</sup> Several drugs against PI3K, Mammalian target of rapamycin (mTOR), and AKT had already been invented and tested in clinical trials.

Besides the signaling pathways mentioned above, we also discovered many pathways, including tight junction, regulation of actin cytoskeleton, leukocyte transendothelial migration, etc, were involved in breast cancer bone metastasis. However, detailed information regarding the association between these pathways and bone metastasis has not been fully investigated.

In conclusion, using the integrated microarray gene expression profile and genetic interaction network, we characterized some molecular signaling pathways (ECM-receptor interaction, FAK, calcium signaling pathway, and PI3K/AKT signaling pathway), which may mediate the aggressive behavior of breast cancer in terms of bone polarization.

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### Author contributions

All authors contributed toward data analysis, drafting and revising the paper, and agree to be accountable for all aspects of the work.

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