



Immune response pathways enriched in breast cancer samples with brain metastasis

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Background: Breast cancer (BC) is the most common form of cancer in women. BC brain metastasis (BM) is associated with poor prognosis, especially for Triple negative breast cancer (TNBC). However, the driver genes of this clinical characteristic are poorly understood.

Methods: This study conducted a transcriptome-wide analysis of gene expression levels in BCBM samples from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) datasets. Clinical data and gene expression matrix of TNBC samples were collected. Differential analysis and functional enrichment of metastasis *vs.* non metastasis data samples were conducted. Genes associated with overall survival and BM event was scanned.

Results: Up-regulation in 120 genes and down-regulation in 56 genes were found in TNBC metastasis data. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) clustering using expression alternated genes showed unique immune-gene enrichment in BM samples. Immune response category GO:000695 was found as the most significant term associated with metastasis event. KEGG pathways including cytokine pathways and Primary immunodeficiency were significantly changed in metastasis samples. ESR1 and FYB2 genes expression changes were found to be linked to survival or BM events.

Conclusions: Our results suggest that data-mining on the immune microenvironment of BM might be useful in future study.

Keywords: Breast cancer (BC); brain metastasis (BM); transcriptome; differential expression analysis; immune profile

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Introduction

Breast cancer (BC) is the most common form of cancer in women, accounting for almost one-third of cancer cases among US women (1). With considerable increases in diagnosis rates, mostly due to better imaging and screening, the rate of cancer deaths has steadily declined since 1975. BC brain metastasis (BM), which occurs 34 months (on average)

after diagnosis of BC, is a sign of poor prognosis, with a mean survival time of 15 months. A random sampling of BC patients shows that approximately 15% have diagnosed BM. Owing to better detection methods, there has been a trend of increasing BM rates over time, with BM being detected in up to 30% of all BC patients in autopsies. Human epidermal growth factor receptor 2 positive (HER2⁺) BC patients have

an up to 50% chance of being diagnosed with BM, while triple-negative patients have a 25–46% chance (2,3).

The risk of BCBM is correlated with certain mutations. A gene panel from 2015 with a sample size of 60 has shown that mutations in *TP53* (59.5%), *MLH1* (14.3%), and *PIK3CA* (14.3%) were relatively common, and that there were no significant genetic differences between BM and non-BM BC cells (4). A DNA panel of over 10,000 cancer patients from 2017 demonstrated similar mutation rates for *TP53* and *PIK3CA* in unrelated cancer cells (5). More recent analyses have shown considerable genetic differences between primary BC cells and BM cells, with BM cells having more mutations and copy-number-alterations (6). A 2007 study of 44 BC cases with 23 BMs showed that while only 15–20% of BC in general are estrogen receptor negative (ER⁻) and progesterone receptor negative (PR⁻), more than 64% of BCBMs are steroid hormone receptor negative (7). Triple negative breast cancer (TNBC) is defined as BC that is ER⁻, PR⁻, and HER2⁻, and has a survival rate between those of steroid receptor-positive and HER2⁺ BC; although, it is the most likely to have recurrent metastases (8). The *HER2* gene is closely related to *HER1* (also known as epidermal growth factor receptor, *EGFR*) and is targeted for treatment in roughly 30% of BC patients (9). Both are known oncogenes and activate mechanisms for cell survival, proliferation, migration, and angiogenesis, all of which are factors that contribute to oncogenesis and metastasis (10). An analysis of 20 BCBM patient genomes has shown gain-of-function in *FGFR4* (30%) and loss of function in *ESR1* (45%). In addition, the expression of *ERBB2/HER2* were found increased greater than two-fold in 35% of patients (11).

Overall, many effective treatments for BC are less effective for BCBM (12). A monoclonal-antibody-based treatment, trastuzumab, targets the HER2 receptor and is considered one of the most effective treatments for HER2⁺ BC. However, it is less effective in BCBM compared to combination chemotherapy (13). Despite brain tumors secreting angiogenic factors, which result in a blood-tumor barrier (BTB) that is more porous than the blood-brain barrier (BBB), antibodies are still too large to effectively cross the BTB (14). In addition, the exact molecular mechanisms for BCBM are uncertain, although signal proteins like C-X-C Motif chemokine receptor 4 (*CXCR4*) and Vascular Endothelial Growth Factor (*VEGF*) are known to play a role (12). However, the use of trastuzumab and other antibody therapies prior to BM have been shown to decrease the probability of BM in cases of metastatic

HER2⁺ BC, from a 1-year BCBM probability of 87.7% for the control group to 66.9% for the test group (15). Interestingly, the resistance of HER2⁺ BCBM to HER2⁺ targeted therapy seems to extend to some small-molecule treatments, although combination therapies have shown a significant effect (16,17). A 2010 study reported considerable differences in BCBM patient survival times by type and treatment. For example, the mean survival time of TNBC BM patients was 4 months, HER2⁺ patients was 9 months, and luminal HER2⁺ patients was 15 months. HER2⁺ BCBM had an untreated mean survival time of 3 months, which increased to 8 months with chemotherapy, and 11 months with chemotherapy + radiation (18). Recently, cell proliferation, immune response and cell migration as critical pathways in a prognostic signature for HER2⁺:ERα⁻ breast cancer using Gene Set Enrichment Analysis (GSEA) (19). Cancer immunologic pathways were found related to clinical outcomes in basal-like breast cancer (20). Immune response pathways were speculated as potential novel drivers in brain metastatic samples of TNBC samples with homologous recombination (HR) signature conserved (21). Whether immune pathway affects TNBC brain metastases needs to be checked in transcriptome data. To test this assumption, this study conducted a transcriptome-wide analysis of TNBC BCBM. Our findings can help to improve the outcomes of TNBC BCBM patients. We present the following article in accordance with the MDAR reporting checklist (available at <https://dx.doi.org/10.21037/gs-21-745>).

Methods

Data collection

Messenger RNA (mRNA) expression data for 204 samples from the GSE12276 dataset (22) was downloaded from the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>) using R package GEOquery (23). This was the total BCBM expression available from the GEO database. The probe with the highest expression was used as the gene expression value for each gene. Probes failing to map and outlier samples were discarded. In total 15,222 genes in 198 samples were used in following analysis. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Bioinformatics analysis

Differential analysis of gene expression between primary samples with/without metastasis was conducted using

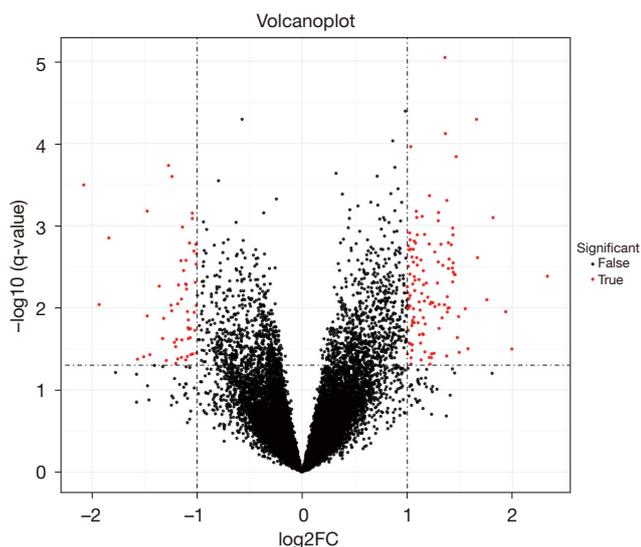


Figure 1 Volcano plot of differential gene expression. The vertical axis represents statistical significance while the horizontal axis represents the size of expression difference. The red dots represent genes that meet the significance thresholds for both.

R-package DESeq2 (24). Samples without metastasis were used as control group. Significant genes were selected using an adjusted P value cutoff of 0.05 and absolute value of $\log_2FC > 1$. Functional enrichment was investigated using the R ClusterProfiler package (25). R-package ggplot2 (26) was used to visualize the top enriched terms of the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, using $P < 0.05$ as the significance threshold. The TNBC-related gene list and external verification gene list were extracted from The Cancer Genome Atlas Breast Invasive Carcinoma (TCGA-BRCA) using R-package GEOquery. The code for this paper is available upon request.

Survival analysis

In addition, R package survminer (27) was used to conduct 3-year survival and BM analyses, using P value < 0.05 as the significance threshold.

Statistical analysis

All statistical analysis were conducted in R 3.8.

Results

The GSE12276 dataset were used to conduct BCBM

analysis. The expression levels were normalized and transformed through \log_2+1 . The differential expression (DE) analysis results showed up-regulation in 120 genes and down-regulation in 56 genes (Figure 1) for BM in TNBC, as opposed to non-metastatic TNBC.

GO enrichment

GO enrichment analysis of the differentially-expressed genes showed that 97 DE genes were classified as biological processes (BP), two as cellular components (CC), and 20 as molecular functions (MF) (Figure 2A). The most statistically significant and differentially expressed GO group was GO:000695, which was the group for immune response. A more detailed analysis of GO enrichment indicated that the enriched immune response contained categories with a humoral immune response, immune signaling, immune chemotaxis, immune chemokine, and calcium ion hemostasis (Figure 2B).

KEGG enrichment

KEGG enrichment results showed enrichment in multiple immune pathways (Figure 3). Notably, cytokines are involved with immune-cell-induced apoptosis, and dysfunction in cytokine pathways are associated with oncogenesis and proliferation (28). Primary immunodeficiency is similarly associated with cancer, and the interleukin-17 (IL-17) signaling pathway has been shown to directly cause BC through granulocyte colony-stimulating factor (29). Overall, KEGG analysis results showed a systematic enrichment of genes related to oncogenesis and cancer progression, with anti-apoptotic and immune system pathways increasing the survival of mutated cells and inflammation pathways, resulting in increased cancer proliferation and metastasis.

Survival analysis

Our survival analysis of gene expression identified estrogen receptor alpha (*ESR1*) as the only statistically significant gene (Table 1). *ESR1* mutations are frequently found in metastatic BC, and are often acquired after aromatase inhibitor treatments for metastatic BC (30).

Metastasis analysis identified *FYB2* as the only statistically significant gene, which is part of T-cell receptor-mediated signaling pathway activation (Table 2). Validation results showed similar trends, with no gene DE levels related to BM events. Furthermore, there was no

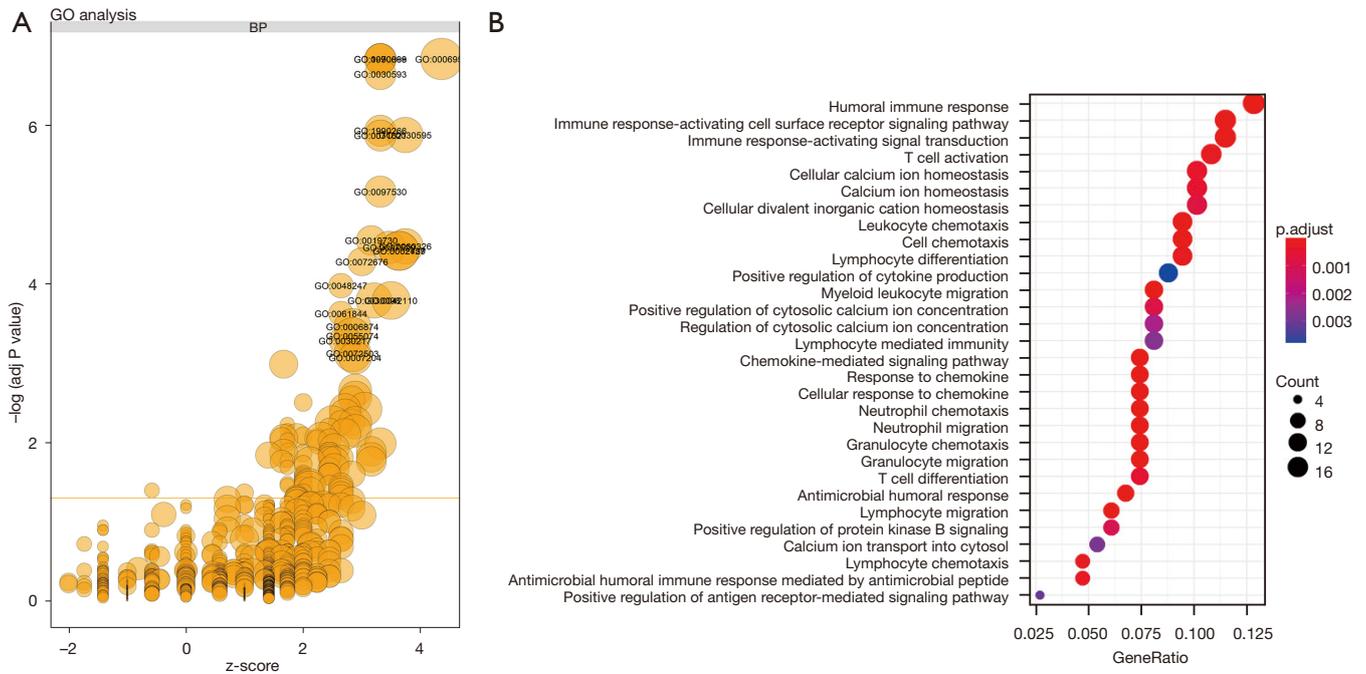


Figure 2 Bubble plot of Gene Ontology (GO) enrichment analysis (A) and detailed inspection of immune-related processes (B). (A) The horizontal axis represents size of effect and the vertical axis represents statistical significance. The circles represent GO groups, with size representing the number of differentially-expressed genes within a GO group. (B) The horizontal axis represents gene ratio. The dot size shows gene count, and the red color indicates smaller adjusted P values.

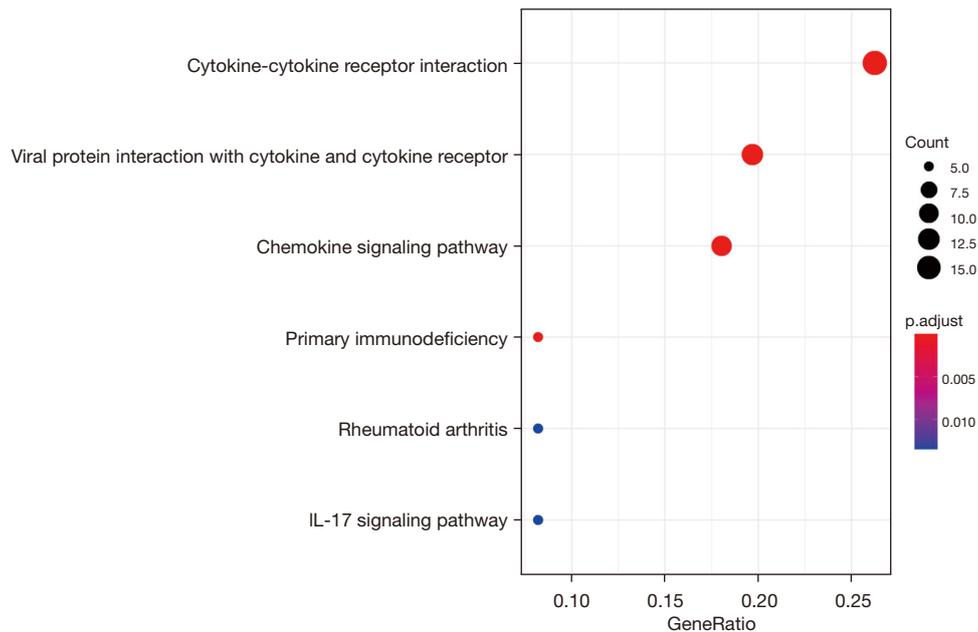


Figure 3 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of differential expression (DE) genes. The horizontal axis represents gene ratios of DE genes in pathways. The dot size shows gene count, and the red color shows the smaller adjusted P value.

Table 1 Differentially expressed genes in 3-year survival

Term	HR (95% CI)	Statistics	P value
<i>ESR1</i>	-0.21 (-0.32 to -0.09)	-3.65	<0.01
<i>PRR15</i>	-0.13 (-0.27-0.02)	-1.73	0.08
<i>ERBB4</i>	0.09 (-0.01-0.2)	1.72	0.09
<i>BCL11A</i>	-0.08 (-0.22-0.05)	-1.27	0.2
<i>VGLL1</i>	-0.04 (-0.11-0.03)	-1.11	0.26
<i>MMP1</i>	0.04 (-0.03-0.1)	1.09	0.27
<i>HORMAD1</i>	-0.03 (-0.11-0.04)	-0.85	0.4
<i>FYB2</i>	-0.04 (-0.14-0.05)	-0.84	0.4
<i>LINC00993</i>	-0.03 (-0.11-0.06)	-0.61	0.54
<i>CA12</i>	-0.04 (-0.18-0.11)	-0.52	0.6
<i>TSPAN1</i>	-0.02 (-0.12-0.09)	-0.35	0.73
<i>ANXA9</i>	-0.01 (-0.13-0.12)	-0.11	0.91

Table 2 Differentially expressed genes in brain metastasis

Term	Estimate	SE	Statistics
Intercept	-8.48	6.09	-1.39
<i>FYB2</i>	-0.53	0.19	-2.73
<i>ERBB4</i>	-0.15	0.18	-0.86
<i>CA12</i>	-0.04	0.26	-0.16
<i>VGLL1</i>	0	0.12	-0.03
<i>ESR1</i>	0.01	0.19	0.06
<i>TSPAN1</i>	0.11	0.19	0.58
<i>LINC00993</i>	0.09	0.16	0.59
<i>BCL11A</i>	0.18	0.25	0.71
<i>PRR15</i>	0.26	0.26	1
<i>MMP1</i>	0.21	0.15	1.44
<i>HORMAD1</i>	0.19	0.13	1.49

SE, standard error.

difference in primary and meta-samples in our dataset for TNBC-related genes.

Discussion

In this study, we attempted to identify the differential gene expression differences between TNBC and non-TNBC. Multiple genes were found to be associated with BCBM,

with 120 up-regulated and 56 down-regulated genes. These genes were enriched in the following categories: humoral immune response, immune signaling, immune chemotaxis, immune chemokine, and calcium ion hemostasis. The humoral immune response directly attacks and helps to induce apoptosis in cancer cells, and has been shown to have predictive value for BC survival (31). Immune signaling is a vital aspect of inflammation pathways and contributes

directly to cancer proliferation (32). Immune chemotaxis and immune chemokine are both functions that help guide immune cells to cancerous and pre-cancerous cells for destruction, and disruption helps cancer cells evade the immune system (33). High levels of cellular calcium are known to play a role in the calcification of breast tissue, which is associated with BC progression (34). Primary immunodeficiency is similarly associated with cancer, and the IL-17 signaling pathway has been shown to directly cause BC through the granulocyte colony-stimulating factor (29). Through multivariate analysis, we also found that *ESR1* and *FYB2* were independently linked to survival and metastasis. *ESR1* is a well-known metastatic and drug-resistance gene in BC (35,36). Recent real-world data and cell line studies have found infiltrating immune cell status reveals therapeutic vulnerabilities of *ESR1*-mutant breast cancer (37,38). *FYB2* is part of the T-cell receptor-mediated activation of signaling pathways and T-cell activation (39); however, its relationship to cancer has not been specifically investigated.

Compared to other forms of BC, TNBC has different survival and metastasis patterns, and is generally associated with lower survival and a higher likelihood of metastasis (8). There have been few reports regarding gene expression characteristics specific to metastatic TNBC (40). Much of this has been due to a limited number of samples available for comparative analysis (41). Our re-analysis found that *FYB2* may act as a key differentially-expressed gene in TNBC BM. T-cell activation plays a vital role in regulating cancer proliferation and metastasis (42). *FYB2* might provide a novel target of drug discovery.

In conclusion, this study confirmed that metastasis is closely correlated to the expression of inflammation- and immune-related genes. Notably, immunotherapy, which affects both, is known to decrease both the likelihood of further BMs and increase survival in patients with metastatic melanoma (43).

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <https://dx.doi.org/10.21037/gs-21-745>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/gs-21-745>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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