



Focal pyroptosis-related genes *AIM2* and *ZBP1* are prognostic markers for triple-negative breast cancer with brain metastases

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Background: Extensive research has shown the role of pyroptosis in the occurrence, progression, and prognosis of breast cancer. The study sought to screen important pyroptosis-related genes and their role in the prognosis of triple-negative breast cancer (TNBC) patients with brain metastasis (BM).

Methods: The Gene Expression Omnibus database was used to obtain transcriptome data from primary TNBC and from TNBC BM patients. Differentially expressed genes (DEGs) between the primary tumors and BMs were analyzed, and the expression, prognostic significance, immune infiltration, function, and drug sensitivity of the pyroptosis genes in the DEGs were analyzed.

Results: In both data sets, 456 genes differed between primary TNBC and TNBC BM. *Absent in melanoma 2 (AIM2)* and *Z-deoxyribonucleic acid-binding protein 1 (ZBP1)* were found to be important pyroptosis genes in DEGs, and significant differences in their expression in primary lesions and BMs were observed. Patients with a high expression of *AIM2* had a worse prognosis than low expression, while patients with a high expression of *ZBP1* had a better prognosis than low expression. *AIM2* and *ZBP1* were positively correlated with the infiltration of most immune cells; however, *AIM2* was negatively correlated with the infiltration of neural cell adhesion molecule 1 (CD56) bright natural killer cells and central memory cluster of differentiation 8 (CD8) T cells. Increased expression of *ZBP1* is negatively correlated with high infiltration levels of central memory CD8 T cells and memory B cells.

Conclusions: Our findings suggest that *AIM2* and *ZBP1* increase immune cell infiltration and may be potential targets for predicting and treating TNBC BM.

Keywords: Triple-negative breast cancer (TNBC); pyroptosis; bioinformatics

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Introduction

According to the latest data on the global cancer burden, breast cancer has surpassed lung cancer as the most common cancer in the world, and its mortality rate ranks first among female malignancies (1). Triple-negative breast cancer

(TNBC) refers to estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) negative subtypes of breast cancer, and the incidence of invasive breast cancer is about 12–17% (2). Studies have shown that compared with other subtypes of breast cancer, TNBC has a higher risk of recurrence after

surgery and is prone to brain metastasis (BM) (3). When TNBC patients are diagnosed, they often have high tumor grades and are in the late stages of the disease. Due to the lack of ER, PR, and HER-2 receptors in TNBC patients, conventional endocrine therapy and targeted therapy are not very effective, and there is a lack of effective treatment methods. TNBC patients have worse disease-free survival and overall survival compared with luminal and HER-2 positive subtype breast cancers (4). The mechanism of brain metastases of TNBC has not been clear, but there are several ways be accepted by the researchers. For example, grant breast circulating tumor cells (CTCs) extravasation into de brain parenchyma (5), organ-specific colonization dependent on the interaction between cancer cells and their microenvironment (6) or undergo genetic and epigenetic changes to gain brain metastatic competence and so on (7). As the number of TNBC patients increases, so does the number of patients with BM. Thus, the challenging search for effective therapeutic targets and prognostic biomarkers has become the focus of TNBC research.

Pyroptosis is a type of programmed cell death characterized by an innate immune response that relies on caspase and classical inflammasome (8). Studies have shown that pyroptosis can inhibit the occurrence and development of tumors, promote inflammatory death, and form a microenvironment suitable for the growth of tumor cells that promotes tumor growth (9). Several studies have pointed out that apoptosis-related molecules are closely related to the growth, invasion, and metastasis of breast cancer. Storr *et al.* found that the expression level of Gasdermin B (GSDMB) in breast cancer cells was higher than that in normal breast tissue, and was associated with a high metastasis rate and a low survival rate in patients. Further, cell experiments have shown that interleukin 1 beta (IL-1 β) produced by macrophages promotes the migration and lymphatic adhesion of breast cancer cells (10). Notably, the activation process of pyrogen death involves the release of a large number of inflammatory mediators, such as IL-1 β and IL-18, which results in a cascade of inflammatory responses that affect the tumor microenvironment (TME). In addition, the low expression of Gasdermin E (GSDME) in breast cancer cells is associated with a significantly increased risk of lymph node metastasis (11). Thus, the expression level of GSDME may induce the pyroptosis of tumor cells. Immunology-related studies have shown that human umbilical cord mesenchymal stem cells can induce cell apoptosis and Michigan Cancer Foundation-7 cell death by secreting IL-1 β (12). In terms of immunity, Homo

sapiens antiviral innate immune response receptor (RIG-I) mediated innate immune response leads to breast cancer cell death and is associated with the increased expression of inflammatory cytokines induced by pyroptosis (13). These studies suggest that cell pyroptosis plays an important role in the occurrence, development, and prognosis of breast cancer, and may be involved in the regulation of the TME.

Changes in the TME are widely considered an important target that can affect the prognosis of TNBC patients. The TME is composed of cellular and non-cellular compartments. Cancer cells, immune cells, blood vessels, and lymphatics, and fibroblasts comprise the cellular portion of the TME. The non-cellular portion encompasses cytokines, chemokines, mediators, and growth factors. The TME is necessary for in-vasion, metastasis, and settling in a distant location mediates the process by which tumors involve the central nervous system (14). Due to the high infiltration levels of immune cells in the TNBC tumor microenvironment, immunotherapy has quickly become an emerging treatment method for TNBC patients. However, we found that there is currently a lack of research on the TME and pyroptosis genes of TNBC BM. In this study, bioinformatics method was used to evaluate the expression and prognostic role of pyroptosis genes in BM TNBC, and to explore the mechanism of focal death related to brain metastasis in TNBC patients. Additionally, the infiltration of immune cells in the TME was analyzed to provide a prediction and therapeutic target for the clinical treatment of TNBC BM. We present the following article in accordance with the STREGA reporting checklist (available at <https://dx.doi.org/10.21037/tcr-21-2182>).

Methods

Datasets

Ribonucleic acid-sequencing (RNA-seq) transcriptome data and the related clinical data of patients with primary TNBC and TNBC BM were obtained from the Gene Expression Omnibus (GEO) database, and standardization was performed on RNA-seq gene expression data for the subsequent analysis. The GSE12276 data set (which comprised 169 patients with TNBC without BM and 35 patients with TNBC with BM, and included their survival data) and the GSE52604 data set (which comprised 20 patients with TNBC without BM and 35 patients with TNBC with BM, but no survival data) were included. The study was conducted in accordance with the Declaration of

Helsinki (as revised in 2013).

Differential expressed genes screening based on pyroptosis genes

First, the GSE12276 and GSE52604 data sets were analyzed for differential genes with or without BM. Pyroptosis genes are derived from the Gene Set Enrichment Analysis (GSEA) database. And then, the intersection genes of DEGs in GSE12276 data set, DEGs in GSE52604 data set and pyroptosis-related genes were seen as TNBC BM related pyroptosis genes.

Survival analysis

The expression and survival of the DEGs in the tumors were analyzed to evaluate the prognostic effect of DEGs. The Kaplan-Meier method was used to plot survival curves. A log-rank P value <0.05 was considered statistically significant.

Immune infiltration and correlation analysis

The single sample gene set enrichment analysis (ssGSEA) algorithm was used to calculate the level of immune infiltration of primary TNBC and TNBC BM. Additionally, the correlations between DEGs and immune cells were analyzed. Finally, the content of immune cells and the prognosis of patients were evaluated.

Drug sensitivity analysis

The TNBC gene expression matrix of the GEO database was used to calculate the sensitivity of the samples to drugs based on the gene expression amount of the samples, and the sensitivity of the drugs was determined using the rank-sum test.

Statistical analysis

All data were analyzed using the R language software package (<https://www.r-project.org/>). Wilcoxon rank and inspection comparisons of primary TNBC and TNBC BM were conducted to determine differences in gene expression using the Kaplan-Meier survival curve drawing method. Genetic variations with the logarithmic ratio change of $|\log FC| > 0.3$ were considered significant. A P value <0.05 was considered statistically significant.

Results

Differential gene screening based on pyroptosis

We first performed a differential gene analysis on the genes of TNBC and TNBC BM in the GSE12276 and GSE52604 data sets, and found that 456 genes were differentially expressed. To further understand the relationship between the pyroptosis genes and the 456 different genes, we conducted an intersection analysis, and found that in DEGs, absent in melanoma 2 (*AIM2*) and *Z-deoxyribonucleic acid-binding protein 1* (*ZBP1*) were pyroptosis genes (see *Figure 1*). We hypothesized that these 2 pyroptosis genes might be related to the metastasis and prognosis of TNBC, and thus conducted further investigations.

Survival analysis

To understand the prognostic value of *AIM2* and *ZBP1* in TNBC primary tumor and TNBC BMs, we first performed an expression analysis. The results showed that *AIM2* and *ZBP1* were significantly overexpressed in TNBC BM in the GSE12276 and GSE52604 data sets (see *Figure 2A-2D*). A survival analysis showed that patients with high *ZBP1* expression had a better prognosis than low expression patients ($P=0.036$), and patients with high *AIM2* expression had a worse prognosis ($P=0.075$; see *Figure 2E,2F*). This suggests that *AIM2* and *ZBP1* play important roles in the occurrence, development and prognosis of TNBC, but the roles of *AIM2* and *ZBP1* may be opposite to one another.

Immune infiltration and correlation analysis

The ssGSEA algorithm was used to evaluate the level of immune infiltration in TNBC primary tumors and TNBC BMs. The results showed that TNBC had a rich infiltration of immune cells, including activated cluster of differentiation 8 (CD8) T cells, effector memory CD8 T cells, activated CD4 T cells, central memory CD4 T cells, T follicular helper cells, gamma delta T cells, type 1 T helper cells, type 2 T helper cells, regulatory T cells, activated B cells, immature B cells, natural killer cells, myeloid-derived suppressor cells, natural killer T cells, plasmacytoid dendritic cells, Immature B cell, and Immature dendritic cell. The infiltration levels of dendritic cells, macrophages, mast cells, and monocytes in primary TNBC tumors were significantly lower than those in TNBC BMs. However, the expressions of central memory CD8 T cells and type 17 T helper cells in primary TNBC

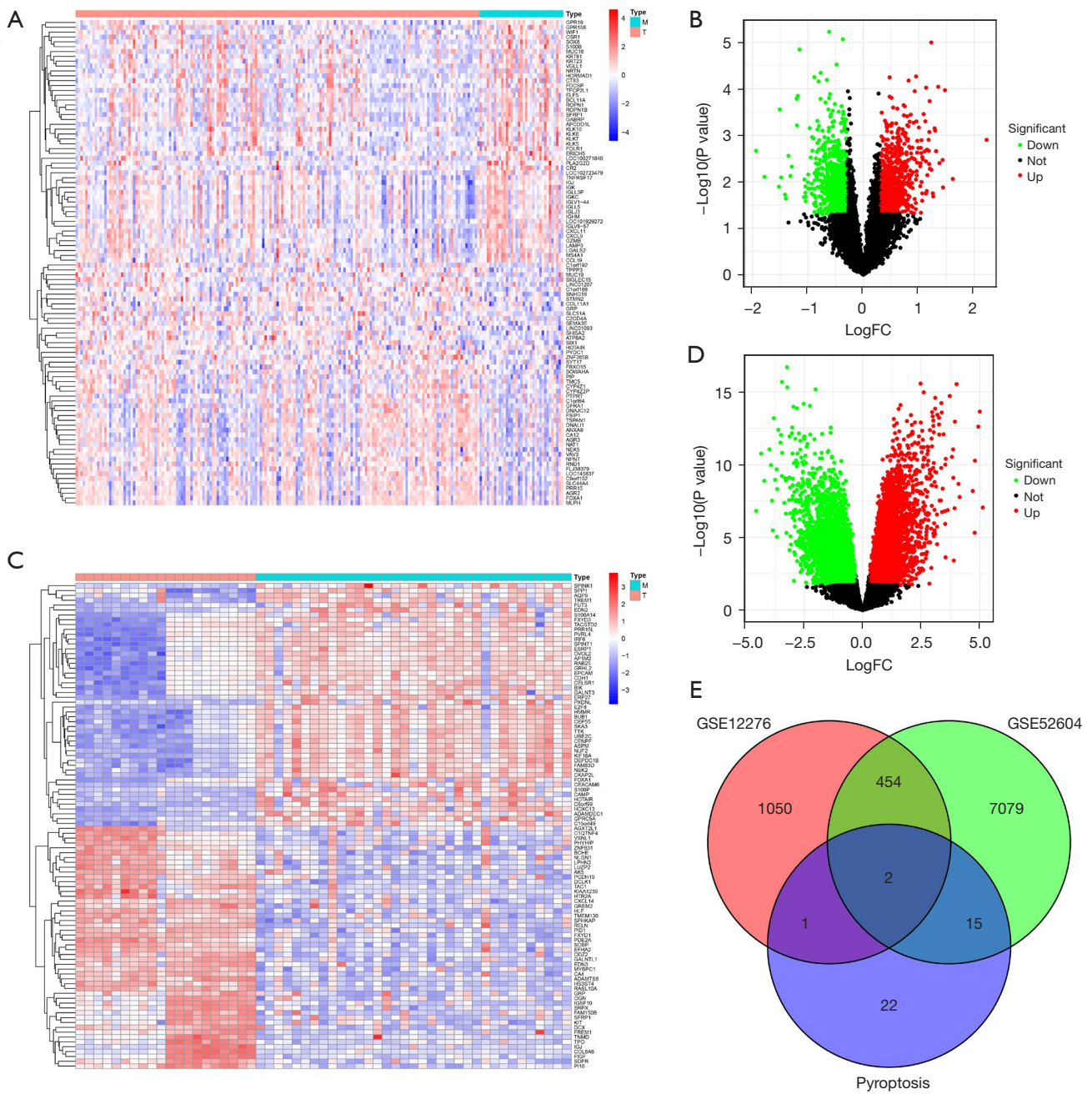


Figure 1 Differential gene screening based on GSE12276, GSE52604 and pyroptosis genes. The identification of DEGs in heat maps for the (A) GSE12276 and (C) GSE52604 data sets. The identification of DEGs in volcanic maps for the (B) GSE12276 and (D) GSE52604 data sets. Compared with primary TNBC tumor tissues, the red or green dots represent upregulated or downregulated DEG in BM tissues, and the black dots represent genes that are not significantly differentially expressed. (E) Venn diagrams of DEGs and pyroptosis genes. DEGs, differentially expressed genes; T, primary TNBC; M, brain metastasis; TNBC, triple-negative breast cancer.

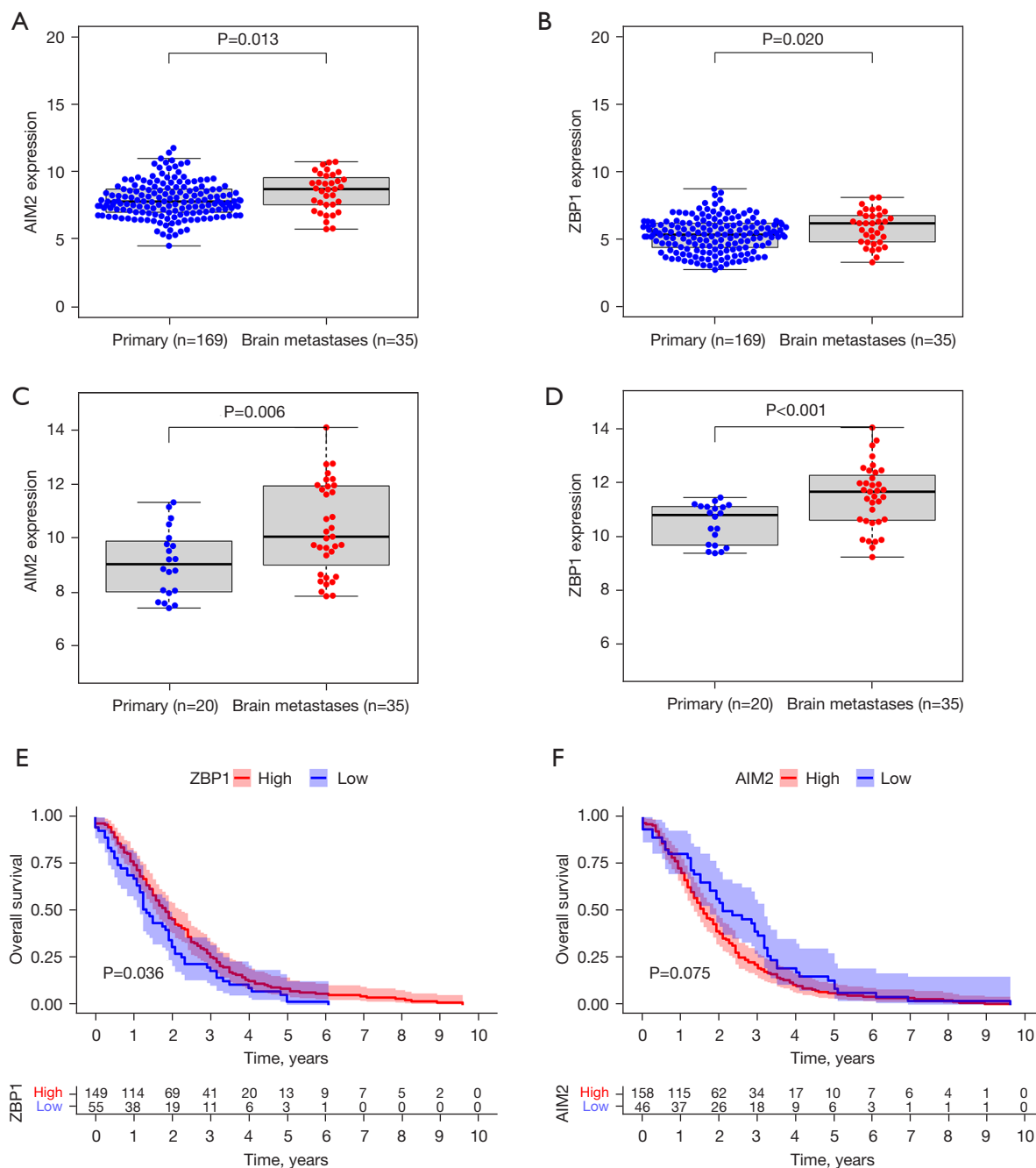


Figure 2 Expression and survival analysis of DEG pyroptosis genes in primary TNBC tumor and brain metastasis. (A,B) Expression of *AIM2* and *ZBP1* in the GSE12276 data set. (C,D) *AIM2*, and *ZBP1* expressions in the GSE52604 data set. (E,F) Survival analysis of *ZBP1* and *AIM2* in the GSE12276 data set. *AIM2*, Absent In Melanoma 2; *ZBP1*, Z-DNA Binding Protein 1; DEGs, differentially expressed genes; TNBC, triple-negative breast cancer.

tumor was significantly higher than those in TNBC BMs. Second, TNBC was positively correlated with the level of most immune cell infiltrates, monocytes, effector

memory CD8 T cells, gamma delta T cells, natural killer T cells, immature dendritic cells, and natural killer cells (see *Figure 3*). The correlation analysis between *AIM2* and

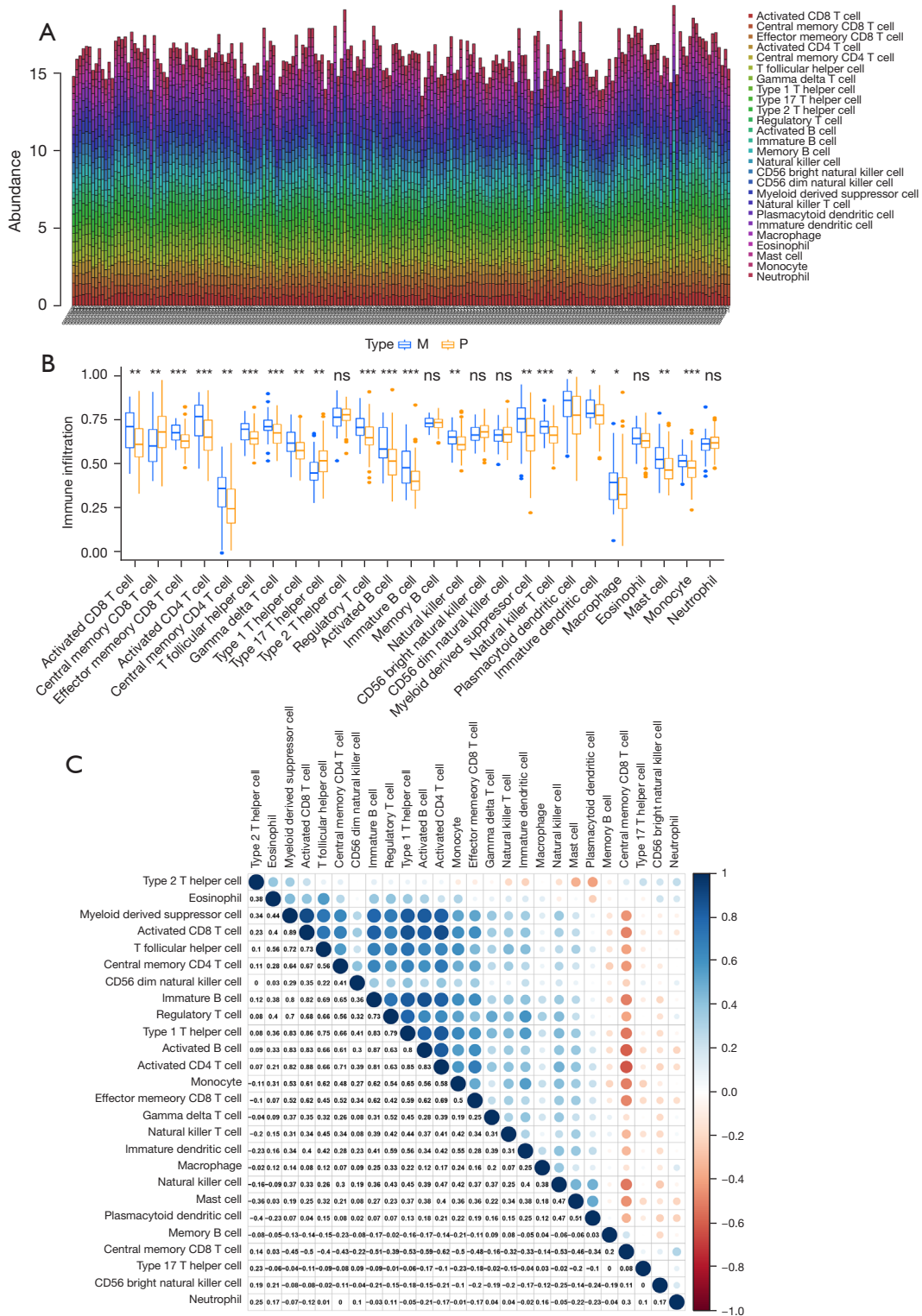


Figure 3 Immune infiltration analysis. (A) Relative levels of 26 immune cells in a single breast cancer sample. (B) Analysis of 26 types of immune cells in primary TNBC tumor and brain metastasis, NS, *, **, *** represent $P > 0.05$, $P < 0.05$ and $P < 0.01$ and $P < 0.001$ respectively. (C) Correlation analysis of 26 immune cell types between primary TNBC tumor and brain metastasis. P, original TNBC. M, brain metastases. Blue, positive correlation. Red, negative correlation. TNBC, triple-negative breast cancer.

ZBP1 and immune cells showed that *AIM2* and *ZBP1* were significantly correlated with the levels of most immune cells (see *Figures 4,5*). The analysis of immune cell survival showed that high infiltrating of effector memory CD8 T cells ($P=0.014$), Immature dendritic cells ($P=0.010$), mast cells ($P=0.019$), and natural killer cells ($P=0.039$) were lead to poor prognosis in TNBC patients without BM. Moreover, high infiltrating of immature dendritic cells ($P=0.014$) also lead to poor prognosis in TNBC BM patients (see *Figure 6*).

Drug sensitivity analysis

The sensitivity of the drugs in primary TNBC and TNBC BM was analyzed, and the data sets were analyzed. The results showed that Bosutinib ($P=0.0035$), Methotrexate ($P=0.0092$), Paclitaxel ($P=0.0081$), Parthenolide ($P=0.033$), Salubrinal ($P=0.02$), and Sunitinib ($P=0.0027$) were more sensitive to primary TNBC, while Bicalutamide ($P=0.024$) and Imatinib ($P=0.038$) were more sensitive to TNBC BM (see *Figure 7*). These findings have significant implications for treatment decisions for patients with TNBC.

Discussion

Cranial computer tomography, magnetic resonance imaging, positron emission computed tomography and lumbar puncture examination were the diagnostic methods of breast cancer patients with brain metastasis. Compared with computer tomography and positron emission computed tomography, magnetic resonance imaging is more effective in the diagnosis of small intracranial lesions. Nevertheless, these methods are all traditional diagnostic methods. At present, we urgently need new biomarkers to diagnose patients with brain metastasis. In this study, we conducted a bioinformatics analysis of TNBC and TNBC BM, and identified *AIM2* and *ZBP1* as important genes related to the death of TNBC BM patients. There were significant expression differences of *AIM2* and *ZBP1* in primary lesions and BMs. Patients with a high expression of *AIM2* had a worse prognosis than low expression, while patients with a high expression of *ZBP1* had a better prognosis than low expression. These results suggest that *AIM2* and *ZBP1* may be effective biomarkers in patients with TNBC BM.

AIM2, which is a member of the IFI202x /IFI16 family, plays a putative role in tumorigenic reversion and may control cell proliferation. *AIM2* is a cytoplasmic innate immune receptor that assembles inflammasome

complexes corresponding to cytoplasmic double-stranded deoxyribonucleic acid (DNA), driving the secretion of IL-1 β and IL-18, which in turn induces cell pyroptosis (15). In 2 independent studies of colorectal cancer, *AIM2* was found to significantly inhibit colon stem cell proliferation and promote cell death by inhibiting the phosphatidylinositol-3-kinase and protein kinase B (PI3K/Akt) pathway, and is believed to play a regulatory role primarily through the NOD-like receptor family CARD domain containing 3 (NLRC3) (16,17).

AIM2 delays the development of hepatocellular carcinoma. The silencing or overexpression of *AIM2* in hepatocellular carcinoma was found to have an anti-cancer effect by forming inflammatory corpuscles and inducing cell pyroptosis, mainly by inhibiting the tumorigenicity of immune-impaired nude mice by inhibiting the mechanistic target of rapamycin (mTOR-S6K1) pathway (18,19). In addition, the disruption of mitochondrial iron metabolism in the absence of phosphatase and tensin homolog induced kinase 1 (PINK1) and parkin ubiquitin protein ligase 2 (PARK2) induces the activation of *AIM2* inflammasomes in pancreatic ductal carcinoma and promotes the upregulation of programmed cell death ligand 1 (PD-L1) (20). These studies suggest that *AIM2* plays an important role in tumorigenesis and disease progression. Studies of breast cancer have shown that *AIM2* inhibits human breast cancer cell proliferation *in vitro* and breast tumor growth in mouse models, and that *AIM2* is associated with tumor suppressor activity (21). Currently, very few studies have explored the relationship between *AIM2* and TNBC, and its function in tumors has not yet been fully elucidated.

ZBP1 encodes Z-DNA binding protein, which plays a role in the innate immune response by binding with exogenous DNA and inducing the production of type I interferon. Once *ZBP1* is activated by binding with Z-RNA, *ZBP1* interacts with RIPK3 to induce cell pyroptosis (22). *ZBP1* family proteins are usually silenced or suppressed in normal adult tissues, but studies have shown that the reactivation or increased expression of this gene can be detected in breast cancer, colon cancer, and non-small cell lung cancer, and it is considered to be a characteristic marker of these cancers (23-25). Subsequent studies have shown that *ZBP1* activation regulates beta-actin messenger RNA (mRNA) localization, maintains cell polarity and directional movement, and inhibits the chemotaxis and metastasis of breast cancer cells (26-28). *In-vitro* experiments have demonstrated that blocking the binding of β -catenin to the *ZBP1* promoter inhibits the expression of *ZBP1*, leading to the increased proliferation and migration

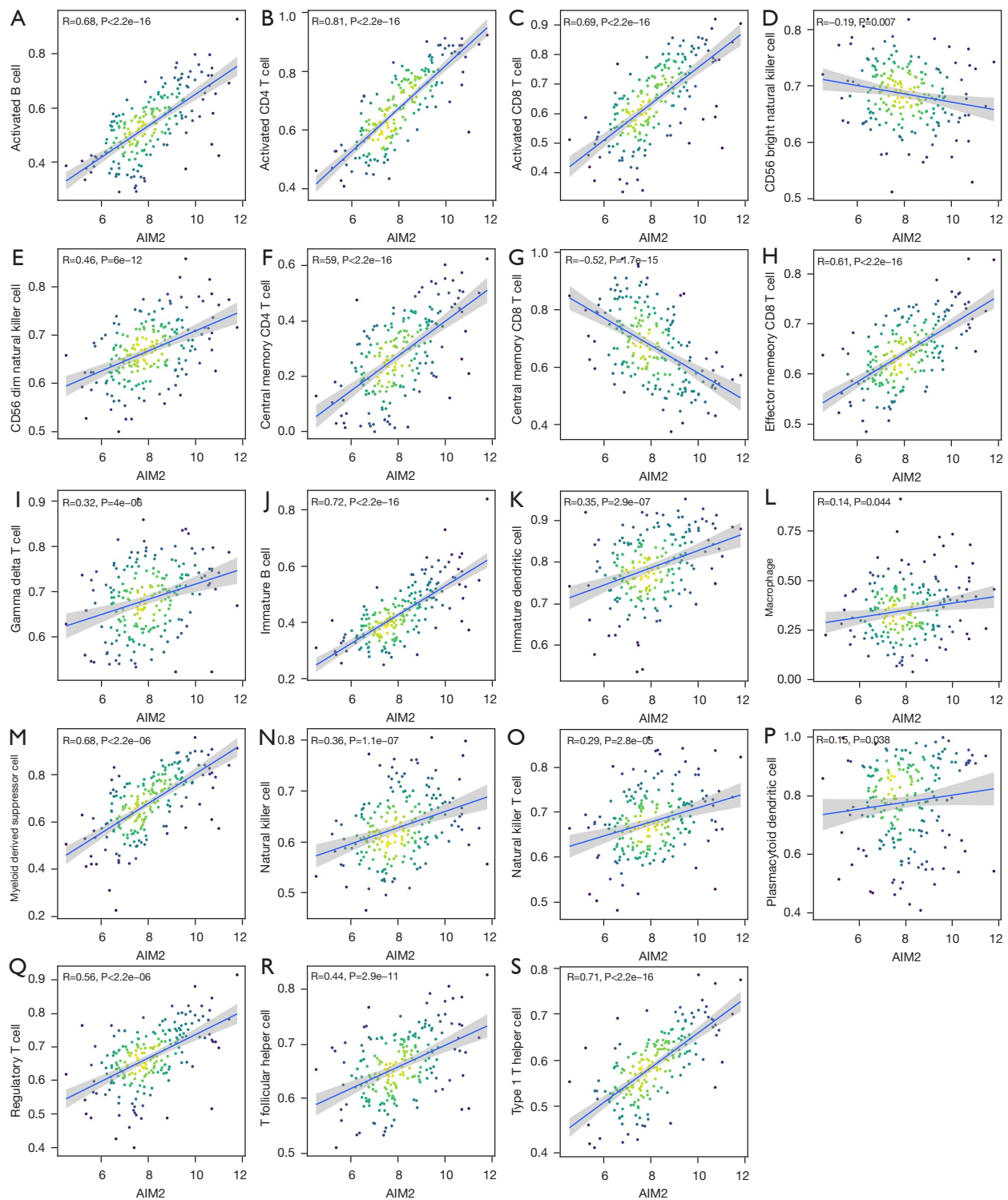


Figure 4 Correlation between *AIM2* and immune cells. (A) Activated B cell; (B) activated CD4 T cell; (C) activated CD8 T cell; (D) CD56bright natural killer cell; (E) CD56dim natural killer cell; (F) central memory CD4 T cell; (G) central memory CD8 T cell; (H) effector memory CD8 T cell; (I) gamma delta T cell; (J) immature B cell; (K) immature dendritic cell; (L) macrophage (M) myeloid-derived suppressor cell; (N) natural killer cell; (O) natural killer T cell; (P) plasmacytoid dendritic cell; (Q) regulatory T cell; (R) T follicular helper cell; and (S) type 1 T helper cell. *AIM2*, Absent In Melanoma 2.

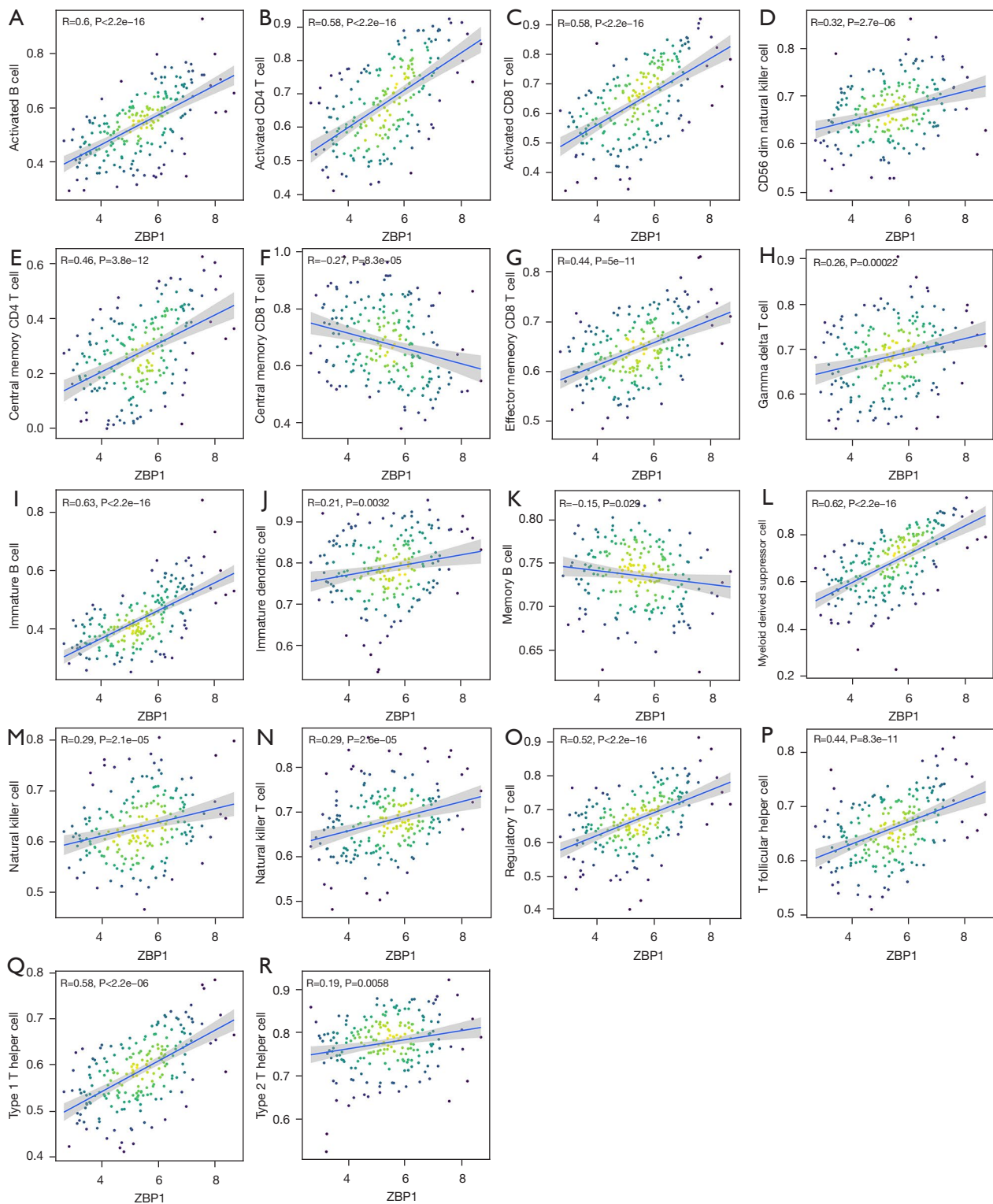


Figure 5 Correlation between *ZBP1* and immune cells. (A) Activated B cell; (B) activated CD4 T cell; (C) activated CD8 T cell; (D) CD56dim natural killer cell; (E) central memory CD4 T cell; (F) central memory CD8 T cell; (G) effector memory CD8 T cell; (H) gamma delta T cell; (I) immature B cell; (J) immature dendritic cell; (K) memory B cell; (L) myeloid-derived suppressor cell; (M) natural killer cell; (N) natural killer T cell; (O) regulatory T cell; (P) T follicular helper cell; (Q) type 1 T helper cell; and (R) type 2 T helper cell. *ZBP1*, Z-DNA Binding Protein 1.

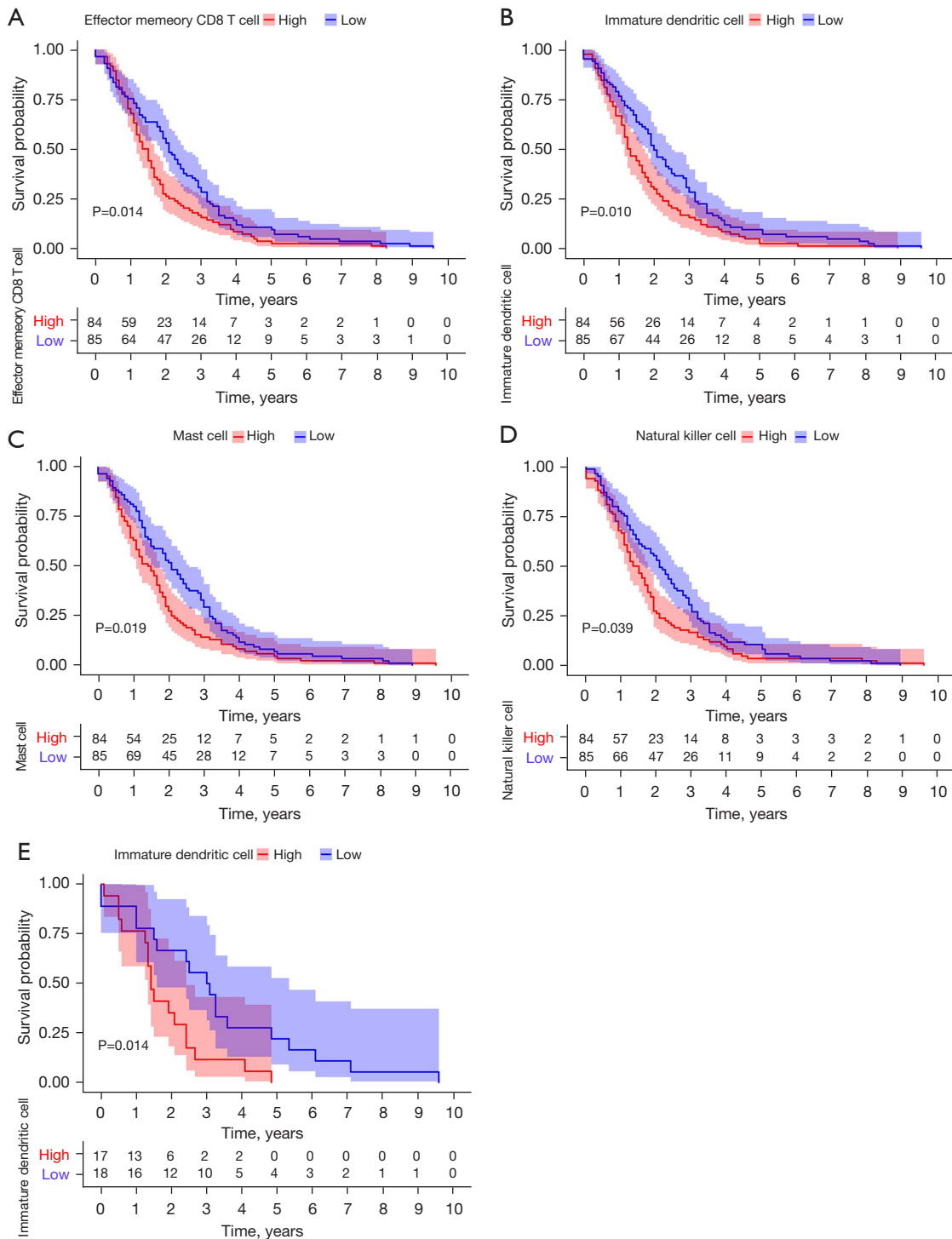


Figure 6 Immune cell survival analysis. Patients with primary TNBC, (A) effector memory CD8 T cell; (B) immature dendritic cell (P=0.001); (C) mast cell (P=0.019); and (D) natural killer cell. Patients with TNBC BMs, (E) immature dendritic cell. TNBC BMs, triple-negative breast cancer with brain metastases.

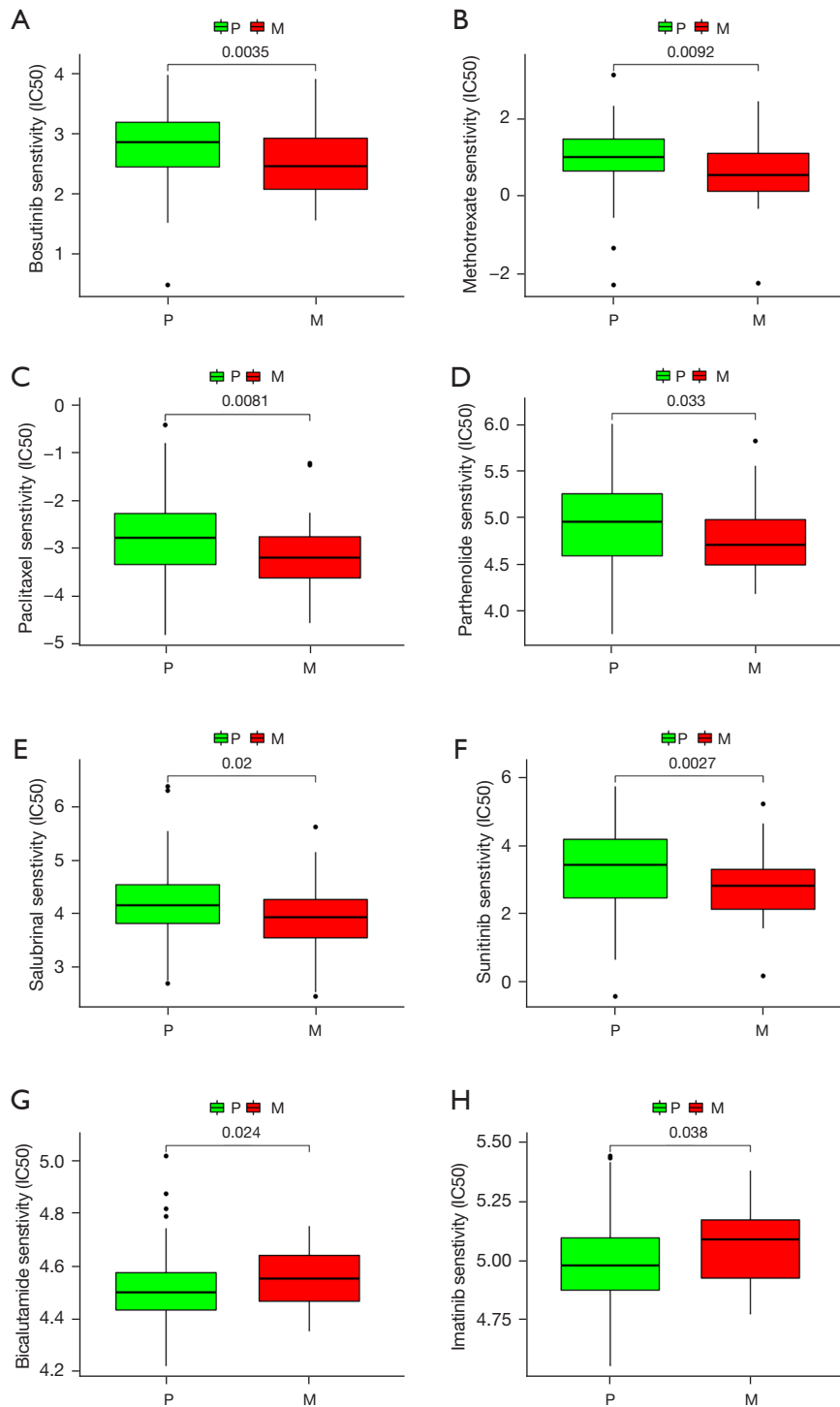


Figure 7 Drug sensitivity analysis. (A) Bosutinib; (B) Methotrexate; (C) Paclitaxel; (D) Parthenolide; (E) Salubrinal; (F) Sunitinib; (G) Bicalutamide; and (H) Imatinib. P, original TNBC. M, brain metastases.

of metastatic breast cancer cells (29). *ZBP1* is essentially a major mRNA regulator that plays an important role in maintaining the stability of non-metastatic phenotypes (30). All these findings suggest that *ZBP1* plays an important role in the metastatic behavior of breast cancer cells; however, its role in TNBC had not previously been examined.

Tumor cells and their microenvironment are a functional together. Tumor cells and the TME interact and influence the occurrence and development of tumors. With the development of TME research, researchers have shown that immune cells in the TME play a complex and important role in tumor progression. In this study, *AIM2* and *ZBP1* were identified as important genes related to TNBC BM. The results showed that *AIM2* was positively correlated with tumor-associated macrophages and most T cell types, while *AIM2* was negatively correlated with CD56 bright natural killer cells and central memory CD8 T cells. The relationship between expressed level of *ZBP1* and infiltration level of immune cells was similar to that of the *AIM2*, the difference is that there was a negative correlation between *ZBP1* and the infiltration level of central memory CD8 T cells and memory B cells. Among numerous immune cells, tumor-associated macrophages account for more than 50% of solid tumor components and participate in the whole process from the occurrence to the metastasis of TNBC, and thus have potential value in the prognosis of TNBC (31-33). In the TME, the abundant invasive level of tumor-associated macrophages suggests a higher risk of tumor invasion and metastasis (34).

Tumor-associated macrophages perform phenotypic transformation in the TME, driving the TME from an anti-tumor state to an immunosuppressive state, and tumor-associated macrophages are expected to become an important link in the regulation of tumor behavior and efficacy of treatments. In this study, the analysis of immune cell infiltration in primary TNBC tumors and TNBC BMs showed that most immune cells in TNBC BM were significantly increased. The prognosis analysis of TNBC patients without BM showed that the high expression of effector memory CD8 T cells, immature dendritic cells, mast cells, and natural killer cell indicated a poor prognosis, and an increase in immature dendritic cell expression in TNBC BM patients indicated a poor prognosis. Thus, there is a complicated relationship between the presence of BM and the expression of immune cells.

There has been a trend of integration in the treatment of TNBC BM, and drug therapy plays an important role in the treatment of TNBC. Due to the existence of the

blood-brain barrier and a lack of sensitive drugs, a number of challenges face the treatment of TNBC BM. In this study, the drug sensitivity analysis showed that TNBC BM patients were more sensitive to Bicalutamide and Imatinib compared with TNBC without BM patients. A series of preclinical and clinical studies have shown that Bicalutamide can improve the prognosis of some androgen antibody-positive patients with metastatic TNBC (35), and Imatinib has a certain effect on radiotherapy and chemotherapy in breast cancer (36). These reports are consistent with the findings of the present study.

Our study had some limitations. First, the TNBC and TNBC BM patients selected from the population were limited in terms of their representativeness, and subsequent studies with large samples need to be conducted. Second, molecular cytology experiments need to be conducted to confirm that the proposed biomarkers of TNBC BM.

Conclusions

AIM2 and *ZBP1* are an important pair of pyroptosis genes that can be used as biomarkers for TNBC BM. The expression of *AIM2* and *ZBP1* may be effective at predicting patients' responses to treatment and prognosis.

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Footnote

Reporting Checklist: The authors have completed the STREGA reporting checklist. Available at <https://dx.doi.org/10.21037/tcr-21-2182>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/tcr-21-2182>). 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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