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Original Research

The Role of *CD8A* in the Immune Microenvironment of Breast Cancer

Jian Chu[1], Shan Tang[1], Tangya Li[1], Huiwen Fan[1,*]

[1]Department of General Surgery, Yancheng First Hospital, Affiliated Hospital of Nanjing University Medical School, 224005 Yancheng, Jiangsu, China
*Correspondence: ycsyyrxwk@163.com (Huiwen Fan)

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Abstract

Background: Triple-negative breast cancer (TNBC) is an aggressive form of breast cancer (BC), and it is often associated with a high tumor grade, a younger age at diagnosis, and a low survival rate. Conventional endocrine and anti-HER-2 therapies are usually ineffective against TNBC, creating treatment challenges and resulting in a poor prognosis. Hence, new targets and treatment strategies for TNBC are urgently required. **Methods**: The GSE102818 dataset was used to identify differentially expressed genes (DEGs) between primary BC and metastatic BC lesions. The Cancer Genome Atlas and the cBioPortal platform were employed to explore mutations in candidate genes. Utilizing the Tumor IMmune Estimation Resource (TIMER), the relationship between the expression of candidate genes and immune cell infiltration was assessed. Additionally, the cell-specific expression of the candidate genes was examined in the immune microenvironment of primary BC and metastatic BC lesions using the single-cell RNA sequencing (scRNA-seq) datasets GSE118389 and GSE202695. Finally, the protein expression of the candidate genes in clinical TNBC samples was evaluated. **Results**: *CD8A* was identified as a hub gene in the DEG network and was found to be down-regulated in metastatic BC lesions. *CD8A* expression was highly correlated with the infiltration of $CD8^+$ T cells, and elevated *CD8A* expression was correlated with improved survival. Interestingly, scRNA-seq data revealed that *CD8A* was down-regulated in $CD8^+$ T cells in the immune microenvironment of metastatic BC lesions. Finally, the evaluation of clinical samples confirmed the down-regulation of *CD8A* in the immune microenvironment of BC lung metastases. **Conclusions**: In patients with metastatic TNBC, high levels of *CD8A* (especially in the immune microenvironment) are associated with a good prognosis.

Keywords: triple-negative breast cancer; differentially expressed genes; breast cancer; tumor immune microenvironment

1. Introduction

Triple-negative breast cancer (TNBC) is a highly heterogeneous type of breast cancer (BC) [1]. Compared with other subtypes of BC, TNBC is aggressive and often has a high tumor grade and poor prognosis [2]. Notably, TNBC is associated with a high risk of metastasis, with the lungs, liver, and brain being the three most common sites of metastasis [3].

Owing to a lack of target receptors, TNBC is typically unresponsive to endocrine therapy and targeted therapy. Hence, chemotherapy is often employed as the primary treatment strategy for TNBC. At present, adjuvant chemotherapy with anthracyclines plus taxanes is typically used to treat TNBC. Further, it is recommended as a first-line therapeutic option for TNBC per international guidelines. However, some patients with BC do not respond to anthracyclines and taxanes, which results in a poor prognosis [4]. Owing to limited treatment options and drug resistance in TNBC, research on this malignancy has become a key priority in the field of oncology. However, so far, studies on the treatment of TNBC have largely focused on the discovery of new targets and the use of endocrine therapy and immunotherapy.

Tumor immunotherapy aims to enhance the body's immune response against tumors, thereby reducing tumor immunosuppression and enhancing antitumor effects. Insights into the interaction between cancer and the immune system have helped in maximizing the benefits of immunotherapy. Notably, immunotherapy has achieved significantly stronger antitumor responses than monotherapy in several patients. Therefore, several immunotherapy-based treatment strategies have been proposed. The principle of immunotherapy is multifaceted. Immune checkpoint dysregulation is an important event during malignant transformation. This process allows tumor cells to resist immune responses, reduces the activation of T cells, prevents tumor surveillance, and enhances tumor survival. In this context, the clinical application of immune checkpoint inhibitors blocks immune checkpoint-related pathways, thus reactivating immune cells. Hence, treatment with immune checkpoint inhibitors disrupts immune resistance in tumor cells, strengthens the activity of T cells against cancer cells, and boosts the immune response [5]. Research has shown that both tumor-infiltrating lymphocytes (TILs) and checkpoint molecules can serve as indicators for the effectiveness of immune checkpoint inhibitors in BC [6]. Higher TIL levels can lead to improved immunotherapy outcomes, and the number of TILs is positively correlated with progression-free survival [7].

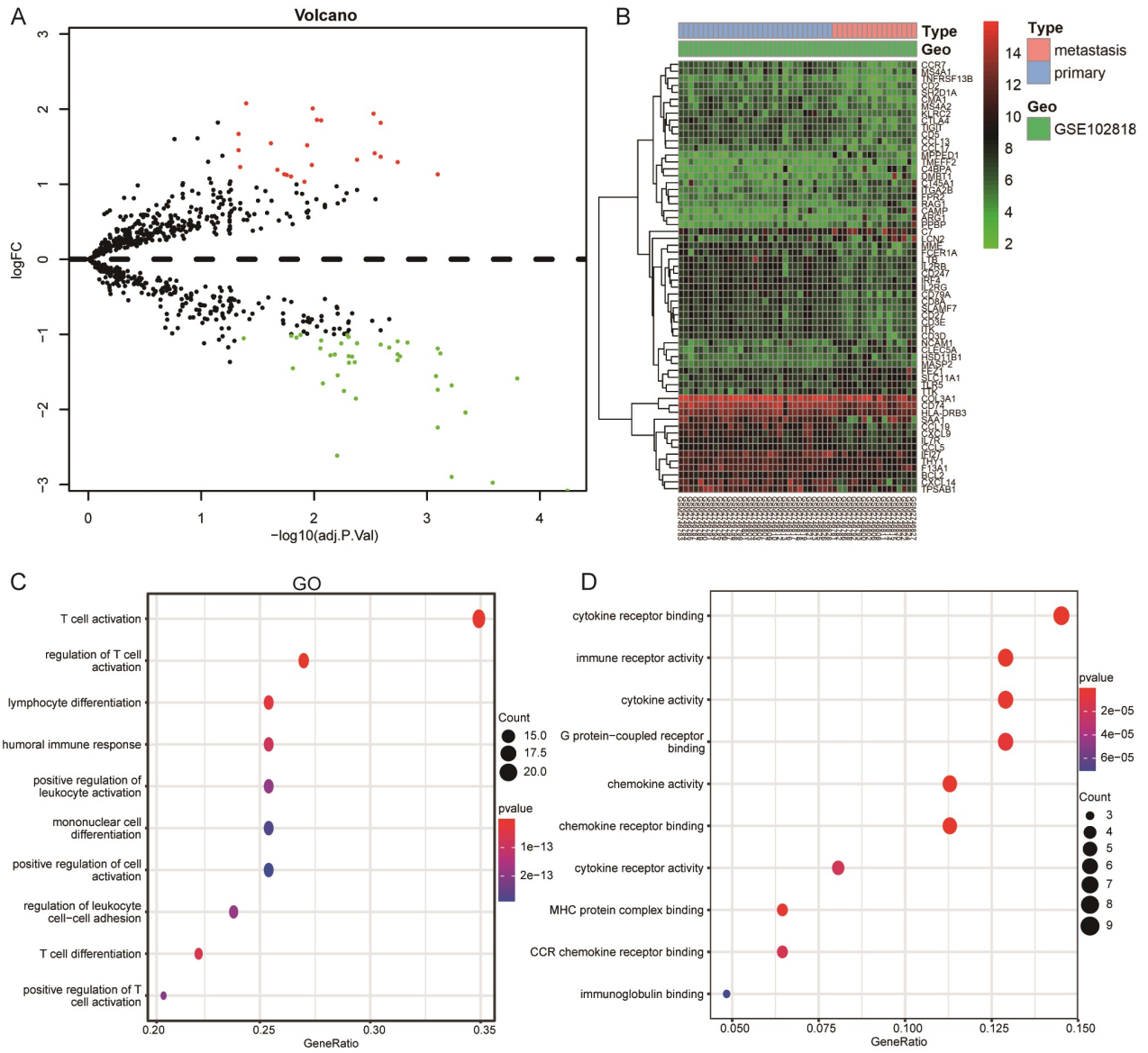

Fig. 1. Genes differentially expressed between primary and metastatic breast cancer (BC) lesions. (A) Volcano plot showing the distribution of differentially expressed genes (DEGs). (B) Heatmap denoting the DEGs (primary vs. metastatic BC lesions). (C,D) Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) gene enrichment analysis of the DEGs.

Interestingly, TNBC is the most immunogenic type of breast malignancy because it exhibits higher levels of TILs than other BC subtypes. The inherent heterogeneity of TNBC has important implications for drug development, clinical diagnosis, and treatment in this type of cancer. According to Oura *et al.* [8], the tumor immune microenvironment (TIME) plays a key role in TNBC metastasis. Identifying TIME biomarkers can help elucidate the causes of tumor heterogeneity in TNBC, enabling the development of targeted treatment strategies.

In this study, differentially expressed genes (DEGs) between primary and metastatic BC lesions were identified, with a focus on genes related to the TIME. After identifying the hub gene *CD8A*, immune cell infiltration (ICI) analysis was performed. The results showed that *CD8A* expression was closely related to the infiltration of $CD8^+$ T cells. Finally, single-cell RNA sequencing (scRNA-seq) data were analyzed to visualize the distribution of *CD8A* expression in $CD8^+$ T cells within the TIME of primary and metastatic BC. Notably, the expression of *CD8A* was found to be significantly down-regulated in cases of metastasis. Together, the findings confirmed that *CD8A* is a potential prognostic factor in metastatic BC.

2. Materials and Methods

2.1 Acquisition of Gene Expression Data

All gene expression data were obtained from the Gene Expression Omnibus (GEO) database. RNA-seq expression data from 31 patients with primary BC and 17 patients

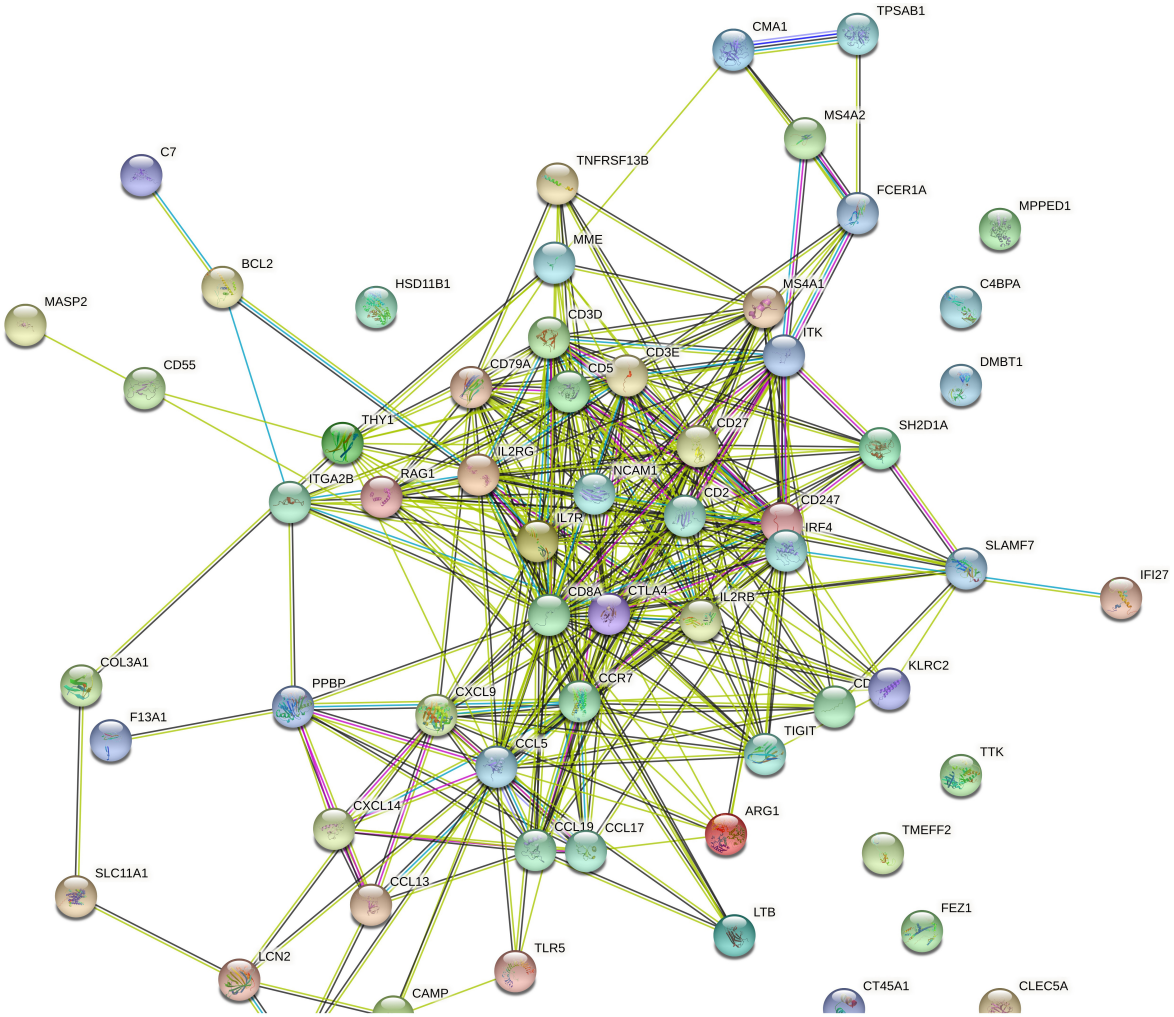

Fig. 2. Protein–protein interaction network of differentially expressed genes.

with metastatic BC were obtained from the GSE102818 dataset. Additionally, the scRNA-seq data of 1534 cells from six TNBC samples (GSE118389), as well as the scRNA-seq data of patient-derived BC xenograft tumors and matched lung macrometastases (GSE202695), were acquired.

2.2 DEG Screening

We used the "Limma" package and GSE102818 data to identify the genes differentially expressed between primary and metastatic BC lesions. DEGs were identified by comparing gene expression between the 31 primary BC samples and 17 metastatic BC samples.

2.3 Protein–Protein Interaction (PPI)

To explore the functional relationships and interactions among the identified DEGs, we constructed a PPI network using the STRING database. This network analysis helped us understand the potential roles of these genes in tumor-related biological processes.

2.4 Gene Enrichment Analysis

We conducted gene enrichment analysis on the identified DEGs using R software (version 4.3.2, The R Foundation, https://www.r-project.org/). The analysis was performed using the "clusterProfiler" package, "org.Hs.eg.db" package, and "enrichplot" package. Accordingly, we explored the biological functions and pathways associated with the identified DEGs.

2.5 Microsatellite Instability (MSI) and Tumor Mutational Burden (TMB)

We obtained MSI and TMB scores from The Cancer Genome Atlas (TCGA). Spearman's method was employed to analyze the relationship of MSI and TMB with the expression of specific genes.

2.6 Online cBioPortal Analysis

We used the cBioPortal platform and BC data from TCGA for gene mutation analysis and explored the genetic alterations in the genes of interest to better understand their potential roles in BC.

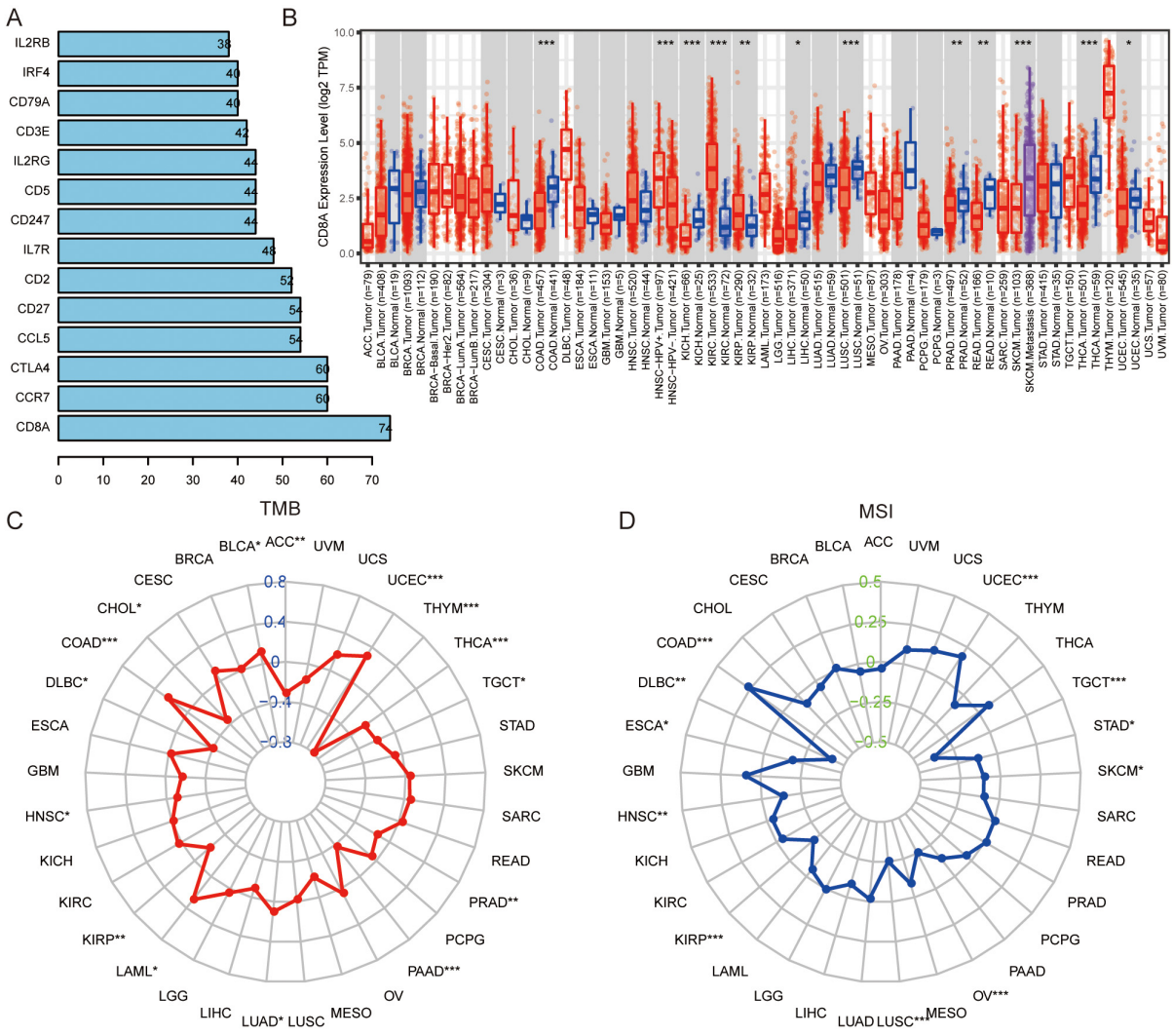

Fig. 3. *CD8A* is an important hub gene. (A) Node analysis of the differentially expressed gene–protein network. (B) Expression levels of *CD8A* in 33 types of tumors. (C) Association of *CD8A* expression with tumor mutational burden in 33 types of tumors. (D) Association of *CD8A* expression with microsatellite instability in 33 types of tumors. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

2.7 Online Tumor IMmune Estimation Resource (TIMER) Analysis

To investigate the correlation between ICI and *CD8A* expression, we utilized the TIMER platform (https://cistrome.shinyapps.io/timer/). Pearson correlation coefficients were calculated to explore the role of *CD8A* in the modulation of immune responses.

2.8 scRNA-seq Analysis

We applied the "SingleR" package to analyze the scRNA-seq datasets GSE118389 and GSE202695. This analysis allowed us to determine the specific expression patterns of *CD8A* within the TIME, as previously described [9].

2.9 Immunohistochemical Staining

Immunohistochemical staining was performed to examine primary and metastatic BC tissue. Tissue sections were dewaxed, and antigen retrieval was performed in citrate buffer (pH 6.0) at 95 °C for 20 min. Subsequently, the sections were treated with 3% peroxidase for 20 min and then use endogenous peroxidase to blocked for 30 min. Finally, the sections were incubated with the anti-*CD8A* antibody (ab237709, Abcam) overnight at 4 °C. The following day, the sections were washed and incubated with HRP-Conjugated Streptavidin and biotinylated goat anti-mouse IgG (H+L). DAB staining was performed, followed by hematoxylin counterstaining. The protein expression levels were assessed by five pathologists.

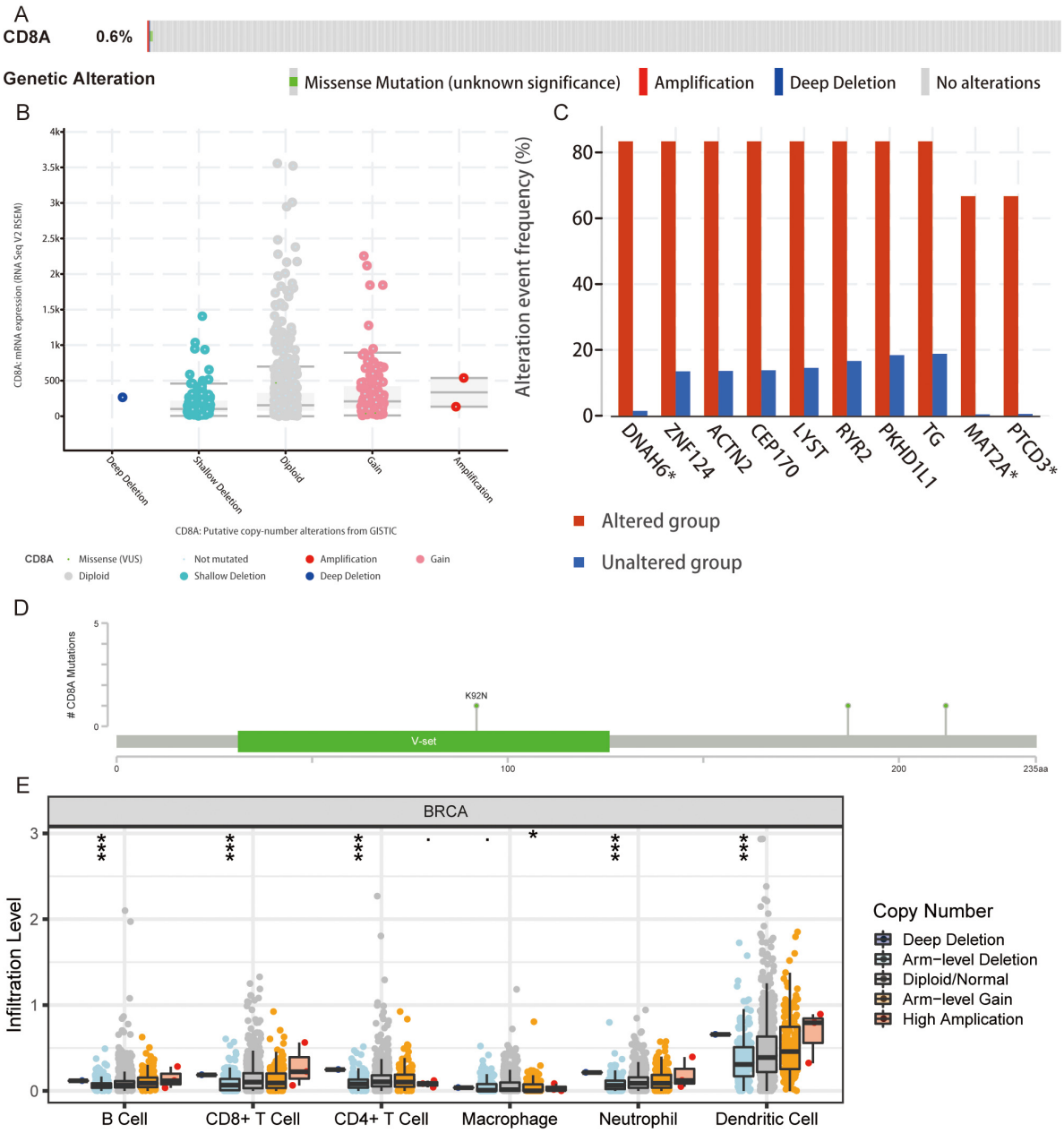

Fig. 4. Mutation status of *CD8A* in breast cancer (BC). (A) Oncoprint of *CD8A* in BC. (B) Types of *CD8A* mutations in BC. (C) Frequency of gene alterations in the presence and absence of *CD8A* mutations. (D) Site of *CD8A* mutations in BC. (E) Association of *CD8A* mutations in BC with copy number alterations in immune cells. *$p < 0.05$, ***$p < 0.001$.

2.10 Statistical Analysis

All data were subjected to statistical analysis using R software (version 4.0.3, https://www.r-project.org/). Statistical tests were performed, as appropriate, for the specific analyses conducted. A p value of 0.05 or below indicated statistical significance.

3. Results

3.1 Screening of DEGs Between Primary and Metastatic BC Lesions

To understand the differences in gene expression between primary BC and metastatic lesions, we analyzed differential gene expression using data from 31 primary BC samples and 17 metastatic BC lesions (obtained from the GSE102818 dataset). The analysis yielded 63 DEGs (Fig. 1A,B). Subsequently, we attempted to identify the potential biological functions of these DEGs. Gene enrichment analyses—including both Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG)

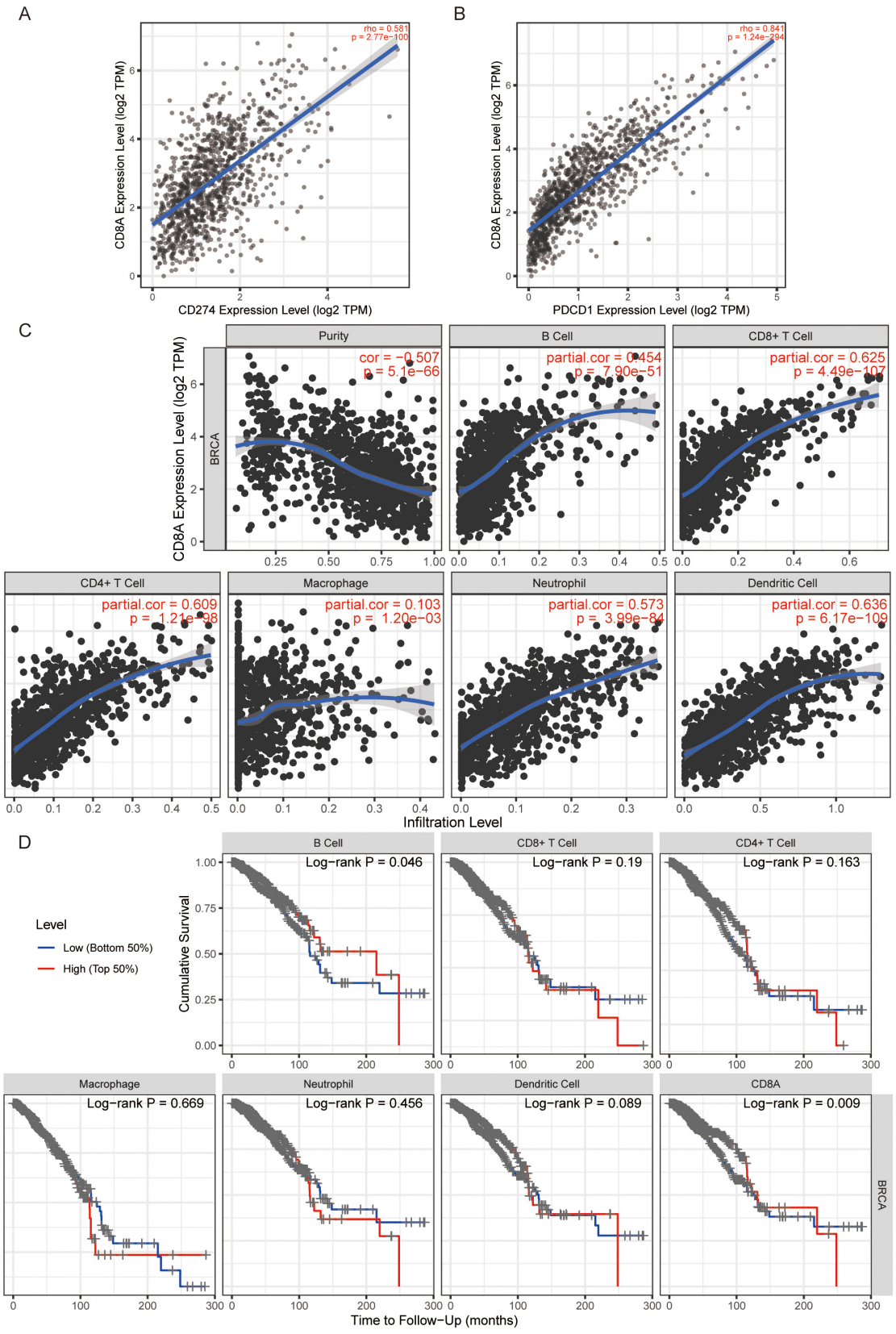

Fig. 5. Correlation between *CD8A* expression and immune cell infiltration (ICI). (A) Correlation between *CD8A* and PD-L1 (CD274) expression. (B) Correlation between *CD8A* and PD1 (PDCD1) expression. (C) Correlation between *CD8A* expression and ICI. (D) Survival analysis based on *CD8A* expression and ICI in BC patients.

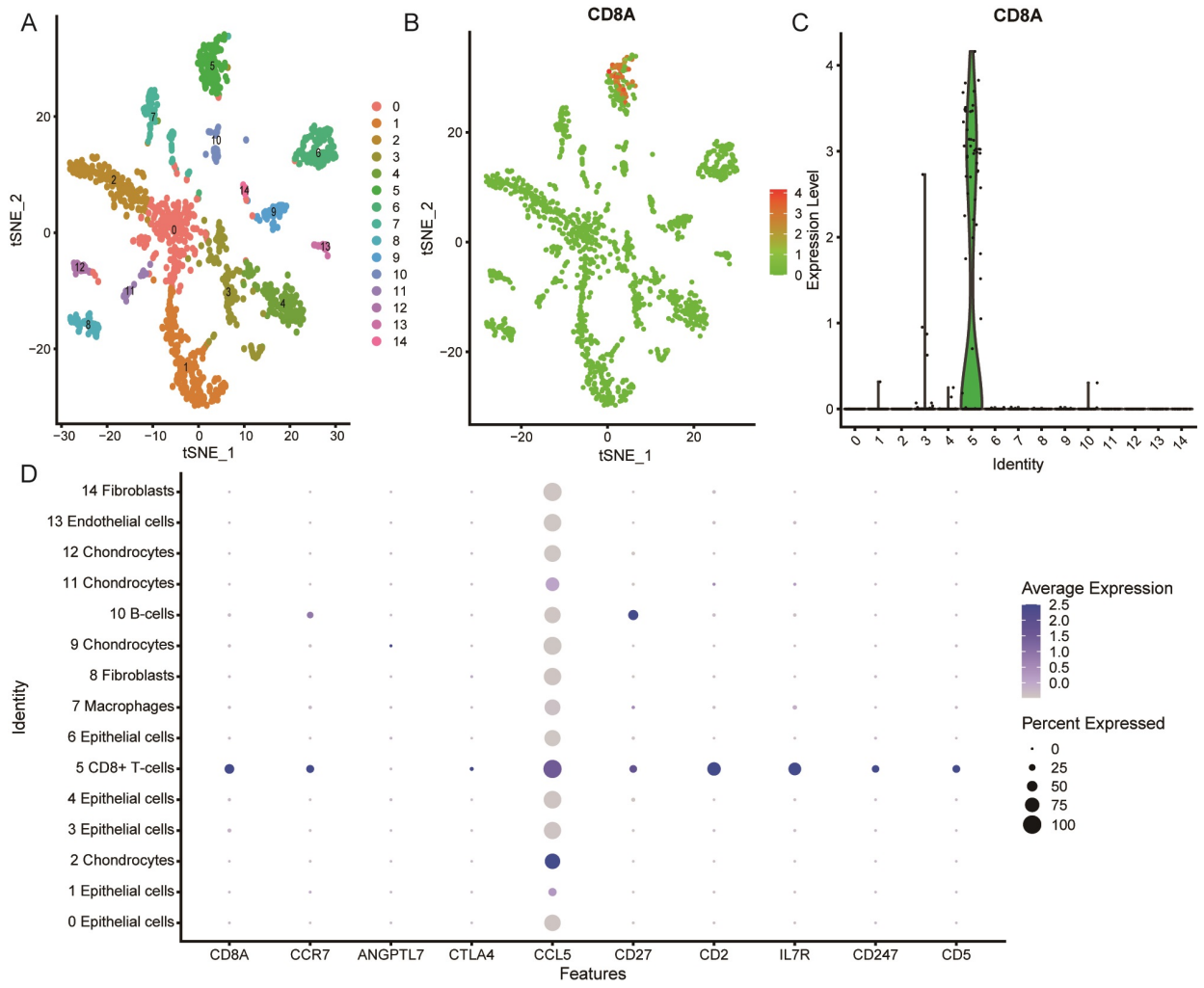

Fig. 6. Immune cell distribution of *CD8A* in primary breast cancer (BC) lesions. (A) tSNE plot illustrating the scRNA-seq data obtained from immune cells in the BC microenvironment. (B,C) Cellular distribution of *CD8A* expression in the BC immune microenvironment. (D) Cellular distribution of the top 10 differentially expressed genes in the BC immune microenvironment.

analyses—revealed the association of DEGs with various tumor-related processes. These included the humoral immune response, lymphocyte differentiation, G protein-coupled receptor binding, and chemokine activity (Fig. 1C,D).

3.2 Expression and Role of CD8A in BC

After the DEGs were identified, we visualized their interactions using a PPI network (Fig. 2). In-depth analysis revealed that *CD8A* was a key hub gene in this network and had the highest number of nodes (i.e., 74), which highlighted its centrality within this network (Fig. 3A). Furthermore, we validated the expression of *CD8A* using TCGA data. Interestingly, our analysis indicated that there was no significant difference in *CD8A* expression levels between BC and normal tissues (Fig. 3B). Moreover, in our pan-cancer analysis, *CD8A* expression was not significantly correlated with TMB or MSI (Fig. 3C,D).

3.3 CD8A Gene Mutations in BC

To further understand the role of *CD8A* in BC, we explored the mutations in this gene using the cBioPortal platform. The overall mutation rate of *CD8A* was 0.6% (Fig. 4A), and a detailed examination revealed various mutation types (Fig. 4B). Moreover, several genes showed co-alterations in the presence of *CD8A* mutations, including *DNAH6*, *MAT2A*, and *PTCD3* (Fig. 4C). Notably, K92N was the primary type of *CD8A* mutation (Fig. 4D). Interestingly, *CD8A* mutations were correlated with copy number alterations such as deep deletions, high amplifications, and arm-level gains in BC macrophages (Fig. 4E).

3.4 Correlation between CD8A Expression and ICI

We explored the correlations between *CD8A* expression and ICI. *CD8A* expression exhibited a strong correlation with PD-L1 (CD274, R = 0.581) and PD1 (PDCD1, R = 0.841) expression (Fig. 5A,B). Additionally, we analyzed the association between *CD8A* expression and the infiltra-

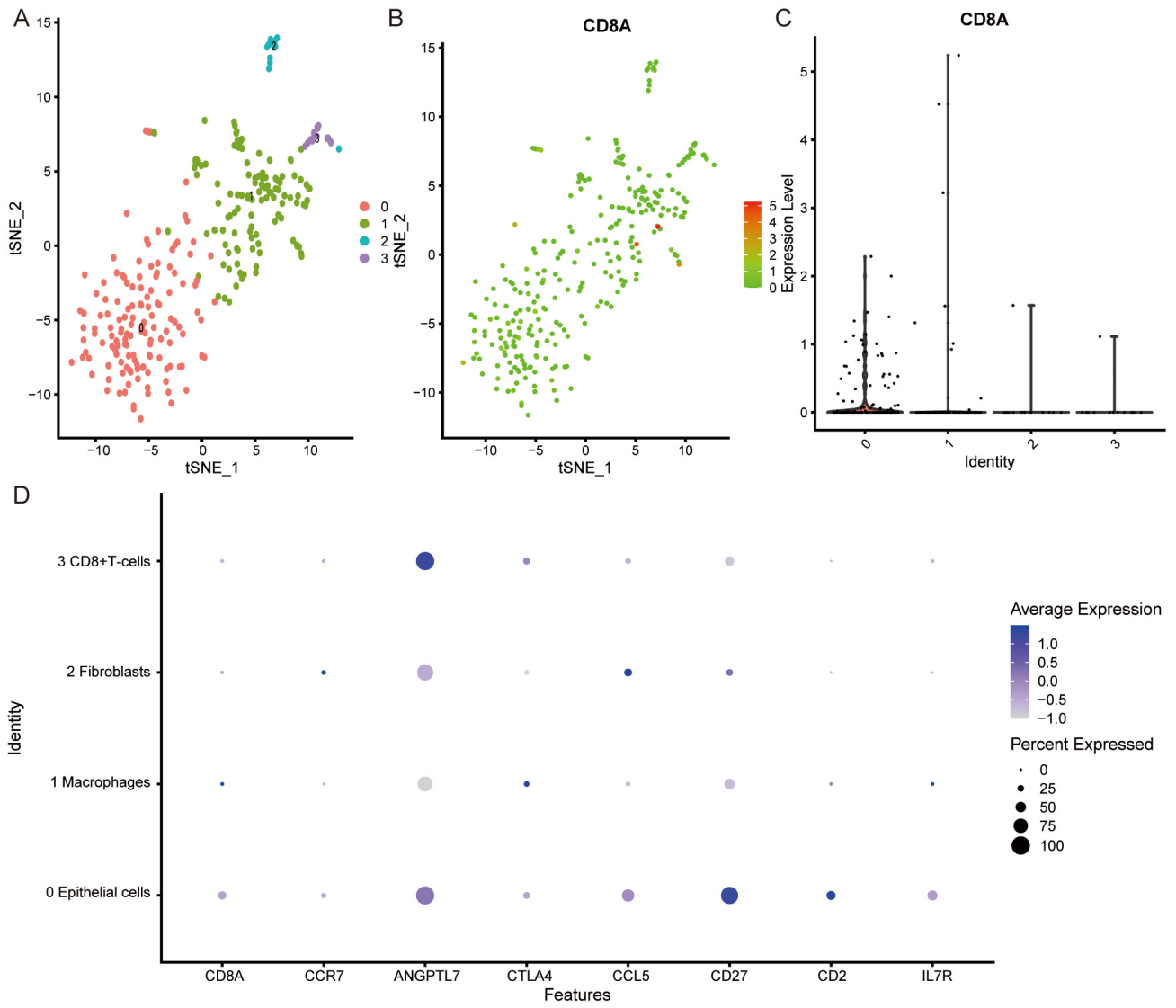

Fig. 7. Immune cell distribution of *CD8A* in lung metastases of breast cancer (BC). (A) tSNE plot illustrating the scRNA-seq data obtained from immune cells in BC lung metastases. (B,C) Cellular distribution of *CD8A* in the the Tumor IMmune Estimation Resource (TIME) of BC lung metastases. (D) Cellular distribution of DEGs in the TIME of BC lung metastases.

tion of various immune cells. *CD8A* expression exhibited a negative correlation with tumor purity. Moreover, it exhibited a positive correlation with the infiltration of B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells (Fig. 5C). Importantly, survival analysis revealed that BC patients with higher B cell infiltration and *CD8A* levels had a more favorable prognosis (Fig. 5D).

3.5 Distribution of CD8A in Immune Cells within the TIME of Metastatic and Primary BC Lesions

Using scRNA-seq data, we identified *CD8A*-expressing immune cells to understand the distribution of *CD8A* expression within the TIME of BC. Our findings showed that *CD8A* was predominantly expressed in $CD8^+$ T cells within primary BC foci (Fig. 6A–C). Interestingly, other hub genes also exhibited similar expression patterns in $CD8^+$ T cells (Fig. 6D). However, in BC lung metastases, *CD8A* expression was significantly down-regulated in $CD8^+$ T cells (Fig. 7A–D).

3.6 Validation of CD8A Expression in Primary and Metastatic BC Lesions

Finally, we validated our findings using clinical BC tissue samples. The results were consistent with our previous analyses, revealing high *CD8A* expression levels in the TIME of primary BC foci (Fig. 8A) and a significant down-regulation of *CD8A* expression in the TIME of BC lung metastases (Fig. 8B). These results confirmed the significance of *CD8A* in BC progression.

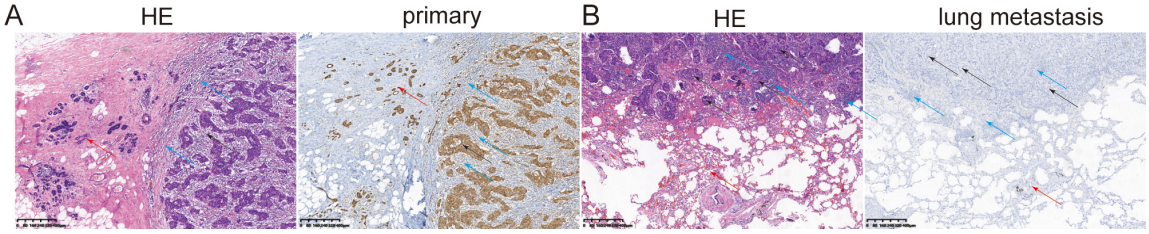

Fig. 8. Immunohistochemical staining for *CD8A* in clinical breast cancer and lung metastasis samples. Black box, cancerous tissue; red box, adjacent normal tissue; blue box, immune cells. High *CD8A* expression levels in the TIME of primary BC foci (A) ,down-regulation of *CD8A* expression in the TIME of BC lung metastases (B).

4. Discussion

TNBC, which accounts for 15% to 20% of all BC cases, is a highly aggressive breast malignancy. Pathologically invasive ductal carcinoma is the most common subtype of TNBC [10]. Clinically, TNBC is often accompanied by lung and brain metastasis, which typically occurs within the first 3 years after the initial diagnosis. TNBC is prone to recurrence even after neoadjuvant and adjuvant treatments, including chemotherapy, and it has a lower overall survival rate than other types of BC [11].

The present study demonstrates that *CD8A* plays a critical role in the progression of TNBC. *CD8A*, which is primarily expressed in cytotoxic T lymphocytes and natural killer cells, is a key mediator of the immune system's defense against cancer cells. In this study, we discovered that *CD8A* is significantly up-regulated in metastatic TNBC. This highlights the role of *CD8A* in the advanced stages of TNBC, particularly during its spread to distant sites.

In this study, we re-mined high-throughput data on tumor characteristics from existing public databases. We analyzed and integrated these data to obtain novel insights regarding TNBC [12]. Previously, a genomic study of 158 TNBC patients (TCGA dataset) revealed that some TNBC tumors only exhibited a few genomic changes, while others contained hundreds of somatic mutations and showed the simultaneous activation of multiple signaling pathways. This suggests that a single drug or therapeutic agent would likely be ineffective against TNBC [13]. Further detailed RNA-seq showed that about 36% of mutations in TNBC were expressed, and the gene mutations were most commonly concentrated in *TP53* (80%), followed by *PIK3CA* and *PTEN*.

The increased expression of *CD8A* in metastatic TNBC is not merely a correlative finding. Instead, it has profound prognostic implications. Our analysis revealed that higher *CD8A* expression levels are associated with more favorable survival outcomes. This finding suggests *CD8A* could serve as a valuable prognostic marker in TNBC. Hence, patients with elevated *CD8A* expression may have a better prognosis, potentially due to the heightened immune response against cancer cells in the metastatic microenvironment. The presence of *CD8A*-expressing $CD8^+$ T cells within the tumor microenvironment is clinically significant, given that $CD8^+$ T cells are essential for the immune system's defense against cancer. These cells are responsible for recognizing and targeting tumor-specific antigens, thus enabling the direct elimination of cancer cells. Furthermore, $CD8^+$ T cells secrete cytokines such as granzyme B and interferon-gamma (IFN-γ), which can inhibit the growth of cancer cells and even induce apoptosis. This dual functionality of CD8+ T cells makes them instrumental in the suppression of tumor progression [14].

Immune evasion is a well-established strategy that allows tumors to survive and spread in the body. In this context, the role of *CD8A* in recognizing and eliminating cancer cells is quite important. Antigen-presenting cells present cancer antigens to tumor-infiltrating $CD8^+$ T cells, activating their cytotoxic potential. These activated $CD8^+$ T cells then destroy tumor cells, inhibiting tumor progression [15]. Together, these mechanisms modulate antitumor responses to prevent tumor growth and progression [16]. In TNBC, TILs can predict the response to immunotherapy, and high levels of TILs are associated with more inert tumor behavior [17]. An increase in TILs is associated with pathological complete response after neoadjuvant chemotherapy. Further, it has been linked to improved prognosis in both patients treated with and without neoadjuvant therapy. Moreover, TNBC patients with high levels of TILs also tend to show reduced rates of disease recurrence [18].

Subgroup analysis can reveal the relationship between the number/proportion of different subtypes of TIL and the prognosis of cancer patients. Hence, different subgroups of TILs have different prognostic values for cancer. Of all the TIL subgroups, tumor-infiltrating $CD8^+$ T cells are the most closely related to cancer prognosis, and this finding has been extensively confirmed in various types of solid tumors. Tumor-infiltrating $CD8^+$ T cells play an active role in adaptive immune defense and kill cancer cells via both direct and indirect pathways [19]. More than 60% of TILs in TNBC are lymphocytes, and ICI in these tumors is dominated by $CD8^+$ T cells [20]. Interestingly, studies have confirmed that a high level of $CD8^+$ T cell infiltration in tumor tissue predicts a longer recurrence-free survival [21–23].

Consistent with these results, we found that *CD8A* is specifically expressed in CD8+ T cells within the TIME of primary BC. Moreover, high *CD8A* expression leads to better patient outcomes in BC, and *CD8A* expression is significantly related to PD1 expression, PD-L1 expression, and ICI. Subsequently, we explored the relationship between *CD8A* expression and genetic alterations in infiltrating immune cells, especially deep deletions. Examinations of a patient-derived xenograft model using scRNA-seq technology revealed that the temporal and spatial evolution of tumor genomes largely conforms to Darwinian evolutionary laws during *in vivo* BC progression. That is, the change in genome copy number is relatively stable even during the malignant transformation of BC cells. However, somatic mutations exhibit great heterogeneity during tumor development, and this acquired heterogeneity is even more pronounced in TNBC [24,25].

In this study, we also analyzed the distribution of *CD8A* expression using scRNA-seq data from a patient-derived TNBC xenograft tumor and matched lung metastases model. The findings showed that *CD8A* expression is down-regulated in BC lung metastases. These results suggest that high *CD8A* expression in tumor-infiltrating $CD8^+$ T cells may improve survival and prognosis in TNBC patients. Tumor-infiltrating $CD8^+$ T cells can improve survival in cancer patients through two key mechanisms, as mentioned previously. First, antigen-presenting cells can present the cancer antigens to tumor-infiltrating $CD8^+$ T cells, causing them to transform into cytotoxic T cells. These cytotoxic T cells can then eliminate cancer cells. Furthermore, $CD8^+$ T cells can also secrete cytokines such as granzyme B and IFN-γ, which inhibit the growth of cancer cells and induce tumor cell apoptosis, enabling tumor suppression [26,27].

The strength of this study lies in the combined transcriptome and scRNA-seq analysis of TNBC metastasis-associated biomarkers. Nevertheless, this study also has certain shortcomings. First, it was difficult to find a comparable BC metastasis transcriptome dataset for validation. Second, we only verified protein expression using clinical samples of primary and secondary BC lesions and did not establish an independent BC metastasis model to further clarify the role of *CD8A* in BC metastasis. Further experiments to clarify the mechanisms underlying the role of *CD8A* during BC metastasis are warranted.

5. Conclusions

Here, we comprehensively analyzed transcriptome and scRNA-seq datasets to identify the key genes involved in TNBC. Our results indicated that *CD8A* expression was correlated with BC metastasis, and a high level of *CD8A* expression was associated with improved survival outcomes in TNBC patients. Hence, *CD8A* might be a potential prognostic marker in TNBC and could also play a role in BC metastasis.

Abbreviations

TNBC, triple-negative breast cancer; BC, breast cancer; DEGs, differentially expressed genes; scRNA-seq, single-cell RNA sequencing; TIME, tumor immune microenvironment; TILs, tumor-infiltrating lymphocytes; GEO, Gene Expression Omnibus; PPI, protein–protein interaction; TMB, tumor mutational burden; MSI, microsatellite instability; ICI, immune cell infiltration.

Availability of Data and Materials

The corresponding author will provide the datasets upon reasonable request.

Author Contributions

JC and ST drafted the manuscript and revised it critically and finally approved the version to be published; JC, ST, TL, and HF designed the study and analyzed the data. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study's protocol was approved by the Affiliated Hospital of Nanjing University Medical School (protocol No. 2021220374).

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Conflict of Interest

The authors declare no conflict of interest.

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