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Exploring immune-related genes with prognostic value in microenvironment of breast cancer from TCGA database

Hao Yang, MD, Kankan Zhao, MD, Houlong Kang, MD, Mengchuan Wang, MD*, Aiguo Wu, MD*

Abstract

Breast cancer is one of the most common malignancies in women worldwide. Many studies have shown that tumor microenvironment cells, immune cells, and stromal cell infiltration have an important impact on prognosis, so it is important to identify biomarkers for achieving better treatment and prognosis.

To better understand the relationship between immune and stromal cell-related genes and prognosis, we screened patients with breast cancer in The Cancer Genome Atlas (TCGA) database and divided them into high and low groups based on immune/stromal scores. We next identified differentially expressed immune-related genes that are significantly associated with the prognosis of patients with breast cancer for functional enrichment analysis and protein–protein interaction networks, respectively. Finally, we selected a separate breast cancer cohort in gene expression synthesis (GEO) for validation.

Both immune scores and stromal scores are meaningful in the correlation of subtype classification. Disease-free survival of cases with the high score group of immune scores is statistically longer than the cases in the low score group. Differentially expressed immune-related genes extracted from the comparison can effectively evaluate the prognosis of patients with breast cancer and these genes are primarily involved in immune responses, extracellular matrix, and chemokine activity. At last, we obtained a series of verified tumor immune-related genes that predict the prognosis of patients with breast cancer.

Combining the Estimation of Stromal and Immune Cells in Malignant Tumor Tissues using Expression database and the TCGA database to extract the list of tumor microenvironment related genes which may help to outline the prognosis of patients with breast cancer. Some previously overlooked genes have the potential to become additional biomarkers for breast cancer. Further research on these genes can reveal a new understanding of the potential relationship between tumor microenvironment and breast cancer prognosis.

Abbreviations: BP = biological processes, CC = cellular components, DEGs = differentially expressed genes, DFS = disease-free survival, ESTIMATE = Estimation of Stromal and Immune Cells in Malignant Tumor Tissues using Expression data, ECM = extracellular matrix, GEO = gene expression synthesis, KEGG = Kyoto Encyclopedia of Genes and Genomes, MCODE = molecular complex detection, MF = molecular function, PPI = protein–protein interaction, TCGA = The Cancer Genome Atlas, DAVID = The Database for Annotation, Visualization, and Integrated Discovery, TME = tumor microenvironment.

Keywords: disease-free survival, gene expression omnibus, immune scores, The Cancer Genome Atlas, tumor microenvironment

1. Introduction

Breast cancer is the most common malignant tumor in women worldwide. The number of women who die of breast cancer every year ranks 2nd among female malignant tumor deaths. More than 500,000 women die of breast cancer each year, of which China accounts for 9.6%.[1,2] Since the 1990s, the rate of breast cancer incidence in China has increased twice as fast as the rate of global breast cancer incidence. It is estimated that the number of patients with breast cancer in China will reach 2.5 million by 2021.[2] The high death toll and the rapid increase of incidence rate make the further research of breast cancer more urgent. To better understand the impact of tumor gene composition on clinical outcomes, researchers have established genome-wide gene expression sets such as The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) to classify a large number of genomic abnormalities worldwide. In the TCGA database, breast cancer is initially classified into 4 subtypes based on global gene expression profiles: luminal A, luminal B, Her-2 positive, base-like. With these advances, gene expression profiles are increasingly being incorporated into clinical diagnostic criteria and accepted. Tumor cell intrinsic genes, especially transcription factors, determine the occurrence, development, and evolution of

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General Surgery Department, Zhujiang Hospital, Southern Medical University/The Second School of Clinical Medicine, Guangzhou, Guangdong, China.

** Correspondence: Aiguo Wu, and Mengchuan Wang, General Surgery Department, Zhujiang Hospital, Southern Medical University/The Second School of Clinical Medicine, Guangzhou, Guangdong, China (e-mails: wagtyz@sina.com, dr.mengchuan@outlook.com).*

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breast cancer.[3,4] On the contrary, the tumor microenvironment (TME) has also been reported to have an important impact on tumor tissue gene expression, thereby affecting clinical outcomes.[5,6] The TME is the cellular environment in which the tumor is located. Due to the complexity of the interaction of tumor cells with TME, it is important to identify biomarkers that can distinguish which patients are more likely to benefit from these treatments, thereby achieving better prognosis. It consists of immune cells, mesenchymal cells, endothelial cells, inflammatory mediators, and extracellular matrix (ECM) molecules.[7,8] In the TME, immune cells and stromal cells are 2 major nontumor components, which play a critical role in regulating both the initiation and development of disease, as well as cellular response to therapies. Previous studies suggest that high levels of immune cell infiltration are associated with favorable outcomes,[9] which means assessing the biomarkers predicting response and prognosis has great potential for improving the success rate of immunotherapy.

Algorithms[10,11] have been developed to predict the tumor purity using gene expression data in the TCGA database. For instance, Yoshihara et al[10] designed an algorithm called ESTIMATE (Estimation of Stromal and Immune cells in Malignant Tumor tissues using Expression data). The algorithm calculates the immune and stromal scores by analyzing the specific gene expression characteristics of immune cells and stromal cells, and predicts the infiltration of nontumor cells. Subsequent reports quickly applied this ESTIMATE algorithm to prostate cancer[12] and colon cancer,[13] showing the effectiveness of such big data-based algorithms, although utility on immune and/or stromal scores of breast cancer has not been investigated in detail.

In the current work, we combined the TCGA database of breast cancer groups and ESTIMATE algorithm to explore the factors of microenvironment associated with breast cancer and further we identified immune-related biomarkers for breast cancer prognosis. Importantly, we have verified this correlation in different breast queues in the GEO database.

2. Methods

2.1. Database and statistical analysis

This study uses data from the public domain and does not require the approval of an ethics committee. Gene expression profiles of patients with breast cancer were obtained from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/). Clinical characteristics such as gender, histological type, survival time, and outcome were also obtained from TCGA data portal. Applying the ESTIMATE algorithm to calculate scores for the level of stromal cells and immune cells in tumor tissues.[10] The immune score and stromal score of breast cancer were retrieved from ESTIMATE website (http://bioinformatics. mdanderson.org/estimate/). This website provides an easy access to predict infiltration of immune cell and stromal cells in TME. For validation, gene expression profiles and clinical information of patients with breast cancer were downloaded from the Gene Expression Omnibus (GEO) data portal (http://www.ncbi.nih.gov/geo).

Comparing the immune/stromal scores in different subtypes by using 1-way analysis of variance, Kaplan–Meier survival curves were generated to illustrate the relationship between patients' disease-free survival (DFS) and gene expression levels of differentially expressed genes (DEGs). The relationship was tested by log-rank test. Above plots were drawn using GraphPad Prism 7. Spearman rank correlation coefficient package in R software (version 3.5.2) was used to show the relevancy between the final verified genes.

2.2. Differential analysis of expressed genes

Data analysis was performed in TCGA data sets using package limma.[14] Using limma package in R software (version 3.5.2), we identified the differentially expressed genes between high immune score group and low immune score group. Fold change >1.0 and adjusted $P<.05$ were set as the cut-offs to screen for DEGs, the results were shown by volcano plot. Heat maps and clustering were generated using an open source web tool ClustVis.[15]

2.3. Functional analysis

The protein–protein interaction (PPI) network was built using STRING database[16] and reconstructed via Cytoscape software.[17] Only individual networks with 20 or more nodes were selected for further analysis and calculated the connectivity degree of each node of the network. Using the Molecular Complex Detection (MCODE) to find clusters based on topology to locate densely connected regions.

Functional enrichment analysis of DEGs was performed by The Database for Annotation, Visualization, and Integrated Discovery (DAVID)[18] to identify gene ontology (GO) categories by their biological processes, molecular functions, or cellular components (CC). The DAVID database was also used to perform Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. False discovery rate <0.05 was used as the cut-off.

3. Results

3.1. Association of immune and stromal scores with breast cancer subtypes and prognosis

Gene expression profiles and clinical data of all 723 patients with primary pathological diagnosis of breast cancer were obtained from the TCGA database. Among them, 702 (97.1%) patients were females, 6 (0.8%) cases were males, and 15 (2.1%) patients were of unclear gender. About 346 (47.9%) cases of luminal A subtype, 176 (24.3%) luminal B subtype, 66 (9.1%) Her-2 positive subtype, and 126 (17.4%) cases of basal-like subtype, 14 patients were of unknown pathological subtype. After excluding patients with incomplete clinical information and male patients, 498 women with breast cancer were eventually included in our analysis. Based on ESTIMATE algorithm, stromal scores ranged from -2282.33 to 1958.16, and immune scores were distributed between -1343.3 to 3487.52. The average immune scores of basal-like subtype cases ranked the highest of all 4 subtypes, followed by that of Her-2 positive subtype, and luminal A subtype. The luminal B subtype cases had the lowest immune scores (Fig. 1A, $P<.0001$). Similarly, the rank order of stromal scores across breast cancer subtypes from highest to lowest is luminal A > Her-2 positive > luminal B > basal-like (Fig. 1B, $P<.0001$), indicating that both immune scores and stromal scores are meaningful in the correlation of subtype classification.

To mine the potential connection between DFS and immune/stromal scores, we divided 498 patients with breast cancer into upper and lower halves based on median immune/stromal scores as the cut-off criteria. Kaplan–Meier survival curves (Fig. 1C) were used to show that DFS of cases with the high score group of

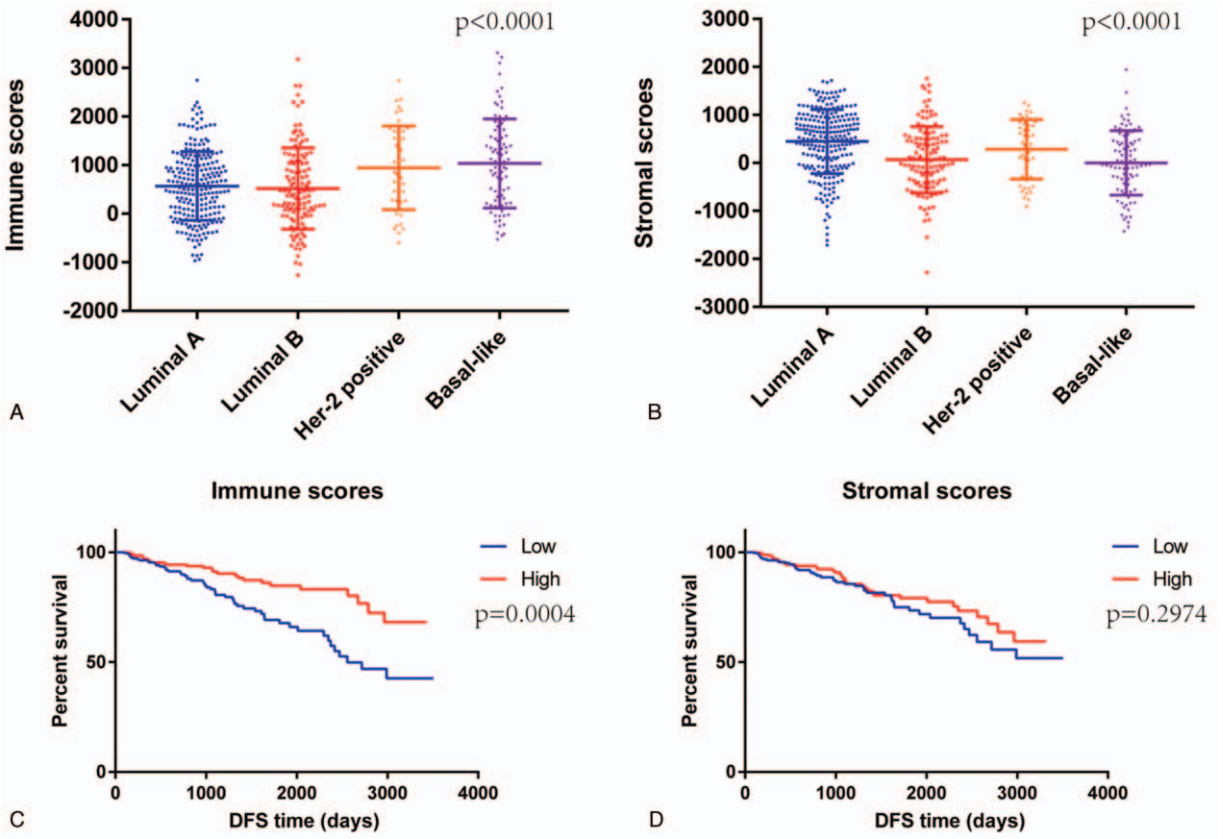

Figure 1. Immune scores and stromal scores are associated with breast cancer subtypes and their overall survival. (A) Distribution of immune scores of breast cancer subtypes. Box-plot shows that there is significant association between breast cancer subtypes and the level of immune scores. (B) Distribution of stromal scores of breast cancer subtypes. Box-plot shows that there is significant association between breast cancer subtypes and the level of stromal scores. (C) Breast cancer cases were divided into 2 groups based on their immune scores. As shown in the Kaplan–Meier survival curve, median survival of the high score group is longer than low score group. (D) Similarly, breast cancer cases were divided into 2 groups based on their stromal scores. The median survival of the high score group is longer than the low score group. DFS = disease-free survival.

immune scores is statistically longer than the cases in the low score group ($P = .0004$ in log-rank test). Although it was not statistically significant, cases with higher stromal scores showed longer overall survival compared to patients with lower stromal scores (Fig. 1D, $P = .2974$ in log-rank test).

3.2. Comparison of gene expression profile with immune scores in breast cancer

We can see from the previous analysis that the association between DFS and immune scores was statistically significant. Immune-related genes were worthwhile to be explored by comparing high and low score groups. These DEGs (Supplementary File 1, http://links.lww.com/MD/D942) extracted from the comparison can effectively evaluate the prognosis of patients with breast cancer. Therefore, we decided to focus on these DEGs for use in subsequent analysis in this article (Fig. 2). The volcano plot in Figure 2A shows genes associated with prognostic differences between high and low immune scores groups. Heat maps in Figure 2B showed immune-related genes were selected by comparing high and low score groups; 18 genes were upregulated and 307 genes downregulated (fold change >1.5, $P < .05$).

Furthermore, we performed functional enrichment analysis of the 325 DEGs to mine the potential function. Plasma membrane, immune and inflammatory response, chemokine activities, and transmembrane signaling receptor activity were indicated by GO analysis. Cytokine–cytokine receptor interaction and chemokine signaling pathway were indicated by KEGG analysis (Fig. 2C–F).

3.3. Survival analysis of individual DEGs

We generated Kaplan–Meier survival curves to explore the potential link between individual DEGs and DFS. Median was used as the cut-off for high or low expression chosen for the DEGs. Among the 325 DEGs, a total of 259 DEGs (Supplementary File 2, http://links.lww.com/MD/D943) were shown to significantly predict DFS ($P < .05$, selected genes are shown in Fig. 3A–F). These genes were considered to be potential prognostic immune-related genes and the focus of further research.

3.4. Functional analysis of genes of prognostic value

To better understand the relationship and function of prognostic genes, PPI networks were revealed using the STRING tool. Seven modules including 295 nodes and 3811 edges together form this network. Top 4 significant modules were selected from this network to further analysis (Fig. 4A–D). PTPRC, ITGB2, LCP2, and IL10RA modules were remarkable for having many connections with other genes, so that we named these modules,

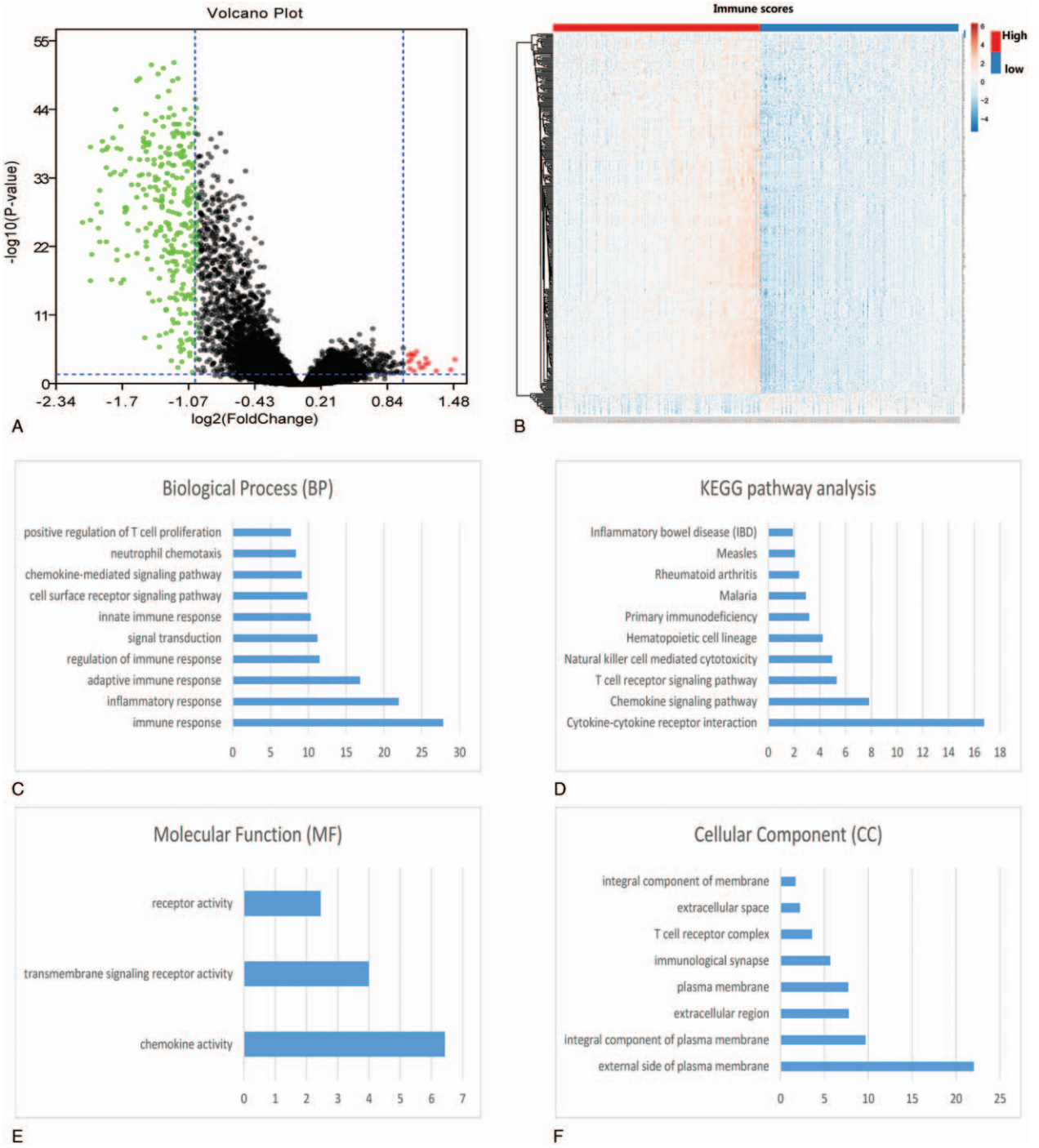

Figure 2. Genes associated with prognostic differences between high and low immune score groups were shown in the volcano plot. Heat maps were drawn based on the comparison of gene expression profile with immune scores in breast cancer. (A) Volcano plot of the differentially expressed genes (DEGs) of immune scores of top half (high score) vs bottom half (low score). (B) Heat map of the DEGs of immune scores of top half (high score) vs bottom half (low score). (C–F) Top gene ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. False discovery rate (FDR) of GO analysis was acquired from The Database for Annotation, Visualization, and Integrated Discovery functional annotation tool.

respectively, module A to D. In the module A (Fig. 4A), 826 edges involving 46 nodes were generated in the network, PTPRC, CCR5, IL6, SELL, CCR7, CD2, and TLR8 were the significant nodes, as they had more connections with other parts of the module. In the module B (Fig. 4B), ITGB2, CD53, VAV1, LAPTM5, CD3D, ITK, MNDA, and CXCL13 had higher degree values. For the module C (Fig. 4C), there are several critical immune-related genes in the center, including LCP2, PLEK, BTK, IKZF1, and FYB. In the module D (Fig. 4D), IL10RA, CD48, VCAM1, CD247, and S1PR4 are also connected to immune response genes.

The functional enrichment clustering of genes of prognostic value has a significant correlation with immune response, and this result is basically consistent with the analysis of PPI network. Top

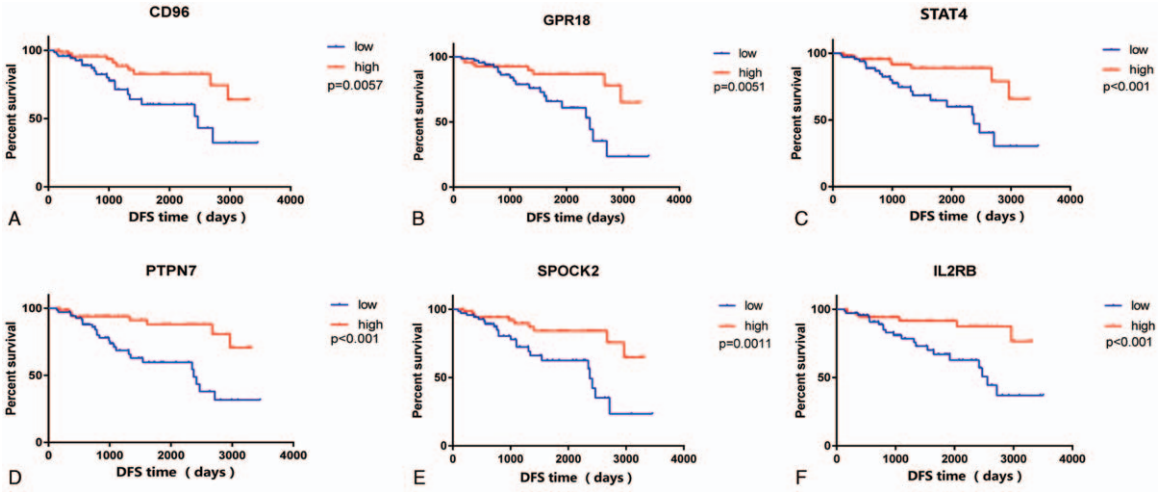

Figure 3. Correlation of expression of individual differentially expressed genes (DEGs) in disease-free survival in The Cancer Genome Atlas. (A–F) Kaplan–Meier survival curves were generated for selected DEGs extracted from the comparison of groups of high (red line) and low (blue line) gene expression. $P < .05$ in log-rank test. DFS = disease-free survival in days.

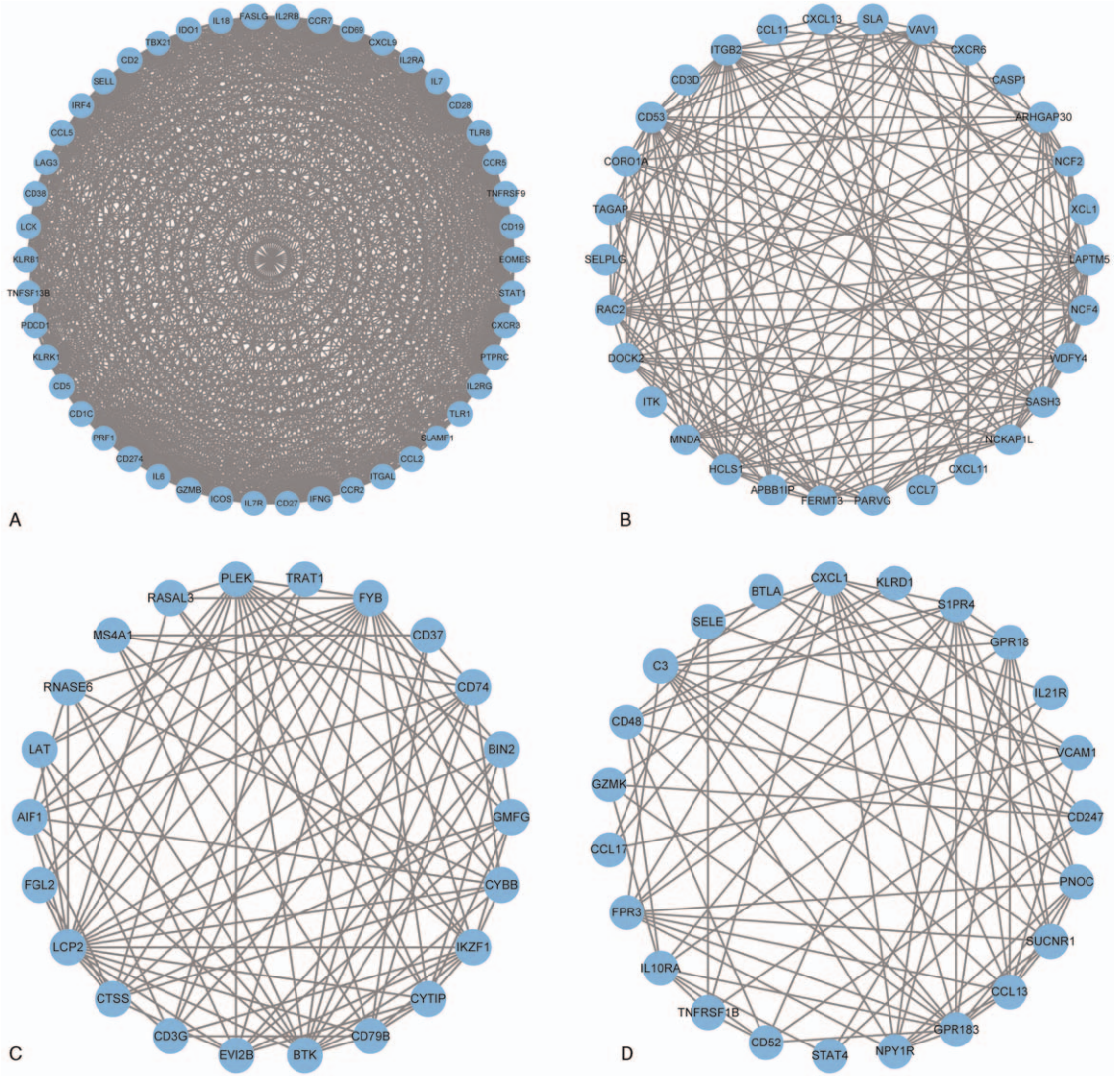

Figure 4. Top 4 protein–protein interaction networks of (A–D) modules A to D. PTPRC, ITGB2, LCP2, and IL10RA occupy the center of their modules, respectively.

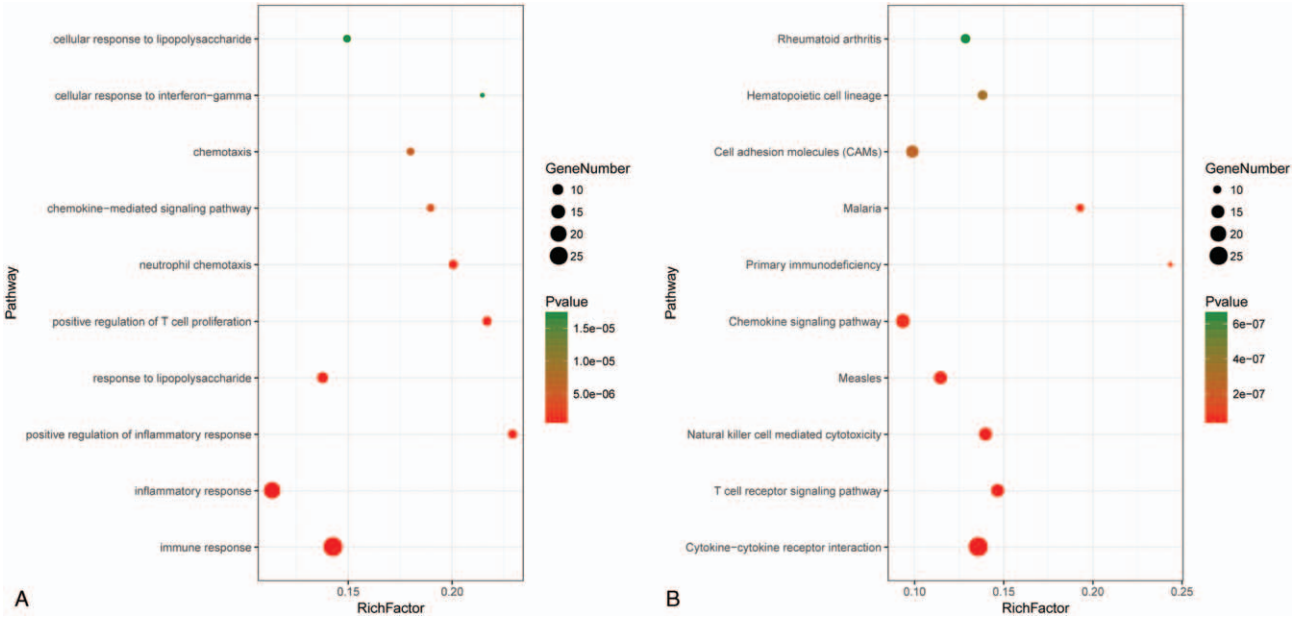

Figure 5. Gene ontology term and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for differentially expressed genes significantly associated with disease-free survival. Top pathways with false discovery rate <0.05 are shown: (A) biological process, (B) KEGG pathway.

GO terms (Fig. 5A) included immune/inflammatory response, chemotaxis, and chemokine activity. In addition, all the pathways that were yielded from the KEGG analysis (Fig. 5B) were associated with immune response.

3.5. Validation in the GEO database

To understand whether the genes found in the TCGA database have an impact on the prognosis of other patients with breast cancer, we downloaded and analyzed gene expression data from 139 patients with breast cancer from the GEO database (GSE45255). A total of 44 genes were validated (Fig. 6A–F) to be significantly linked to prognosis, Spearman rank correlation coefficient also showed strong relevancy between these verified genes (Fig. 7). By searching a large amount of literature, we found that 18 genes of these verified genes have never or rarely been connected with pathophysiology and prognosis in patients with breast cancer.

4. Discussion

In our present work, we attempt to identify genes associated with the TME in the TCGA database that contribute to DFS of breast cancer. First, we analyzed 325 DEGs generated by comparing

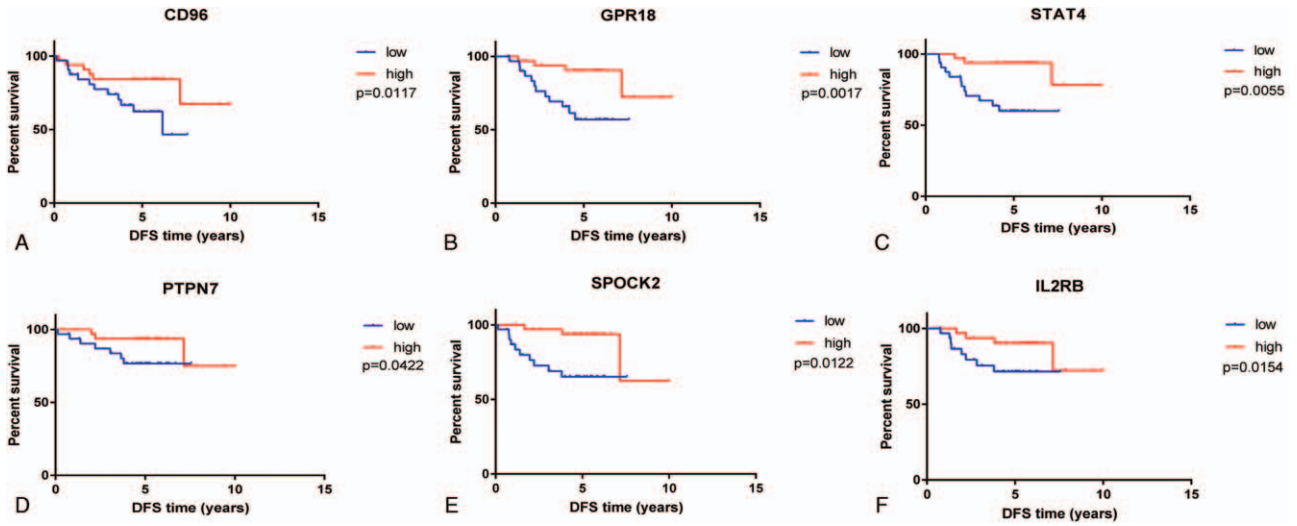

Figure 6. (A–F) Validation of correlation of differentially expressed genes (DEGs) extracted from The Cancer Genome Atlas database with disease-free survival in gene expression synthesis cohort. Kaplan–Meier survival curves were generated for selected DEGs extracted from the comparison of groups of high (red line) and low (blue line) gene expression. $P < .05$ in log-rank test. DFS = disease-free survival in years.

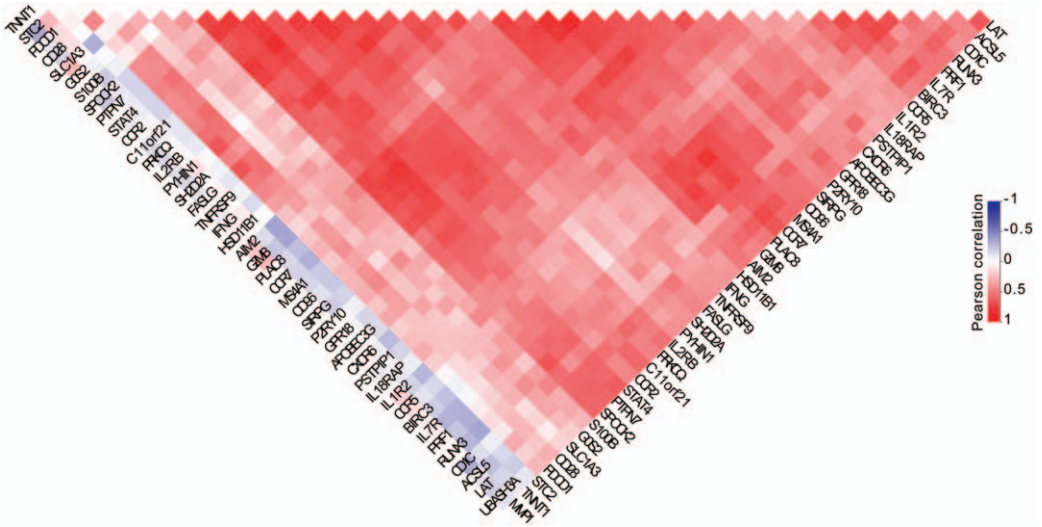

Figure 7. The Spearman correlation matrix of 44 verified genes.

high immune scores groups with low immune scores groups. Through GO term analysis, we can find many of these genes were involved in TME (Fig. 2C–F). This is consistent with the relationship between previously reported immune cell function and ECM molecules in the construction of breast cancer TME.[19–22] Next, we were able to perform a survival analysis of these 325 genes and found that 259 genes are associated with the prognosis of patients with breast cancer. Besides, we constructed 7 PPI modules which were related to immune and inflammation response (Fig. 4). PTPRC, ITGB2, LCP2, and IL10RA are highly interrelated nodes in the network, ITGB2 and IL10RA have been reported to promote proliferation, angiogenesis, migration, and invasiveness in Glioblastoma Multiforme (GBM) cell lines or patient samples.[23,24] Finally, by cross-validation with an independent cohort of 139 patients with breast cancer from GEO, we identified 44 TME-related genes whose expression was significantly associated with prognosis (Figs. 6 and 7). Of the 44 genes identified, 26 genes have been reported to be involved in breast cancer pathogenesis or significant in predicting survival, explaining that big data analysis based on TCGA and GEO has predictive value. The remaining 18 genes have not previously been linked with breast cancer pathophysiological mechanism and prognosis, and could serve as potential biomarkers for breast cancer. Among them, STAT4, SPOCK2, PTPN7, CD96, GPR18, and IL2RB aroused our interest.

Relevant studies have found that STAT4 can promote the metastasis of ovarian cancer by inducing the activation of cancer-associated fibroblasts.[25] It was also found that high expression of STAT4 in gastric cancer predicted better clinical outcomes.[26] SPOCK2 is involved in the progression of endometrial cancer by regulating the biological behavior of cancer cells in recent studies.[27] According to an article predicting miRNA targets in stomach adenocarcinoma, PTPN7 was identified as a valuable target genes.[28] As a new immunological checkpoint receptor target, CD96 has received extensive attention in recent years. Although the role of CD96 as an immunological checkpoint receptor just begun to be discovered, the accumulated data support the targeting of these receptors to improve antitumor immune responses.[29] GPR18 has recently been identified as a potential member of the cannabinoid family because it recognizes several endogenous, vegetal, and synthetic cannabinoids. Potential therapeutic applications for GPR18 include cancer.[30] IL2RB belongs to the member of interleukin. As shown in the latest literature, the autosomal recessive mutation of IL2RB was 1st observed, revealing the need for IL2RB in immune and peripheral immune tolerance.[31] In this study, all the above 6 genes showed a close relationship with prognosis and immunity. Based on the information obtained from literature search, we are interested in further studies on the relationship between these genes and breast cancer in the future.

Significant progress has been made in the study of the correlation between survival and gene expression in patients with breast cancer. Many of these experiments were performed in animal tumor models, in vitro tumor cell lines, or small populations of patient tumor samples. However, the complexity of breast cancer and the microenvironment of breast cancer require more comprehensive analysis and evaluation. Fortunately, the rapid development of high-throughput tumor databases, including TCGA and GEO, was developed and made available to the research community free of charge. These resources provide a solid foundation for big data analysis for large breast cancer populations.[32,33] The interaction of breast cancer and its TME seriously affects the evolution of tumors, which in turn affects subtype classification, recurrence, drug resistance, and prognosis of patients. Former reports have indicated activation of tumor-intrinsic genes can form a TME.[34] In the present work, we are concerned with the genetic characteristics of the microenvironment, which in turn affects the development of breast cancer, thus contributing to the survival of patients. Our findings may provide additional data to address the complex interactions between tumors and the tumor environment in breast cancer.

5. Conclusion

The TCGA data were analyzed by immune scores based on ESTIMATE algorithm to extract the list of TME-related genes and various biological analyses were performed on these genes. These genes have been validated in an independent breast cancer cohort, which may help to outline the prognosis of patients with breast cancer. Some previously overlooked genes have the

potential to become additional biomarkers for breast cancer. In addition, it would be very interesting to test whether this new set of genes provides a stronger survival prediction than a single gene. Finally, through further research on these genes, a new understanding of the potential relationship between TME and breast cancer prognosis can be achieved.

Author contributions

Conceptualization: Hao Yang, Kankan Zhao.
Data curation: Hao Yang, Kankan Zhao.
Formal analysis: Hao Yang, Kankan Zhao, Houlong Kang.
Investigation: Kankan Zhao.
Methodology: Hao Yang, Mengchuan Wang, Aiguo Wu.
Resources: Hao Yang.
Software: Hao Yang.
Supervision: Mengchuan Wang, Aiguo Wu.
Validation: Hao Yang, Kankan Zhao.
Visualization: Hao Yang, Kankan Zhao.
Writing – original draft: Hao Yang, Kankan Zhao.
Writing – review & editing: Hao Yang, Kankan Zhao, Houlong Kang, Mengchuan Wang, Aiguo Wu.
Aiguo Wu orcid: 0000-0002-6793-8779.

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