Original Article

# Identification of immune-related biomarkers for predicting neoadjuvant chemotherapy sensitivity in HER2 negative breast cancer via bioinformatics analysis

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**Background:** Neoadjuvant chemotherapy (NAC) is an important treatment for breast cancer (BC) patients. However, due to the lack of specific therapeutic targets, only 1/3 of human epidermal growth factor receptor 2 (HER2)-negative patients reach pathological complete response (pCR). Therefore, there is an urgent need to identify novel biomarkers to distinguish and predict NAC sensitive in BC patients.

**Methods:** The GSE163882 dataset, containing 159 BC patients treated with NAC, was downloaded from the Gene Expression Omnibus (GEO) database. Patients with pathological complete response (pCR) and those with residual disease (RD) were compared to obtain the differentially expressed genes (DEGs). Functional enrichment analyses were conducted on these DEGs. Then, we intersect the DEGs and immune-related genes to obtain the hub immune biomarkers, and then use the linear fitting model ("glm" package) to construct a prediction model composed of 9 immune biomarkers. Finally, the single sample gene set enrichment analysis (ssGSEA) algorithm was used to analyze immune cell invasion in BC patients, and the correlation between immune cell content and immune gene expression levels was analyzed.

**Results:** Nine immune-related biomarkers were obtained in the intersection of DEGs and immune-related genes. Compared with RD patients, *CXCL9*, *CXCL10*, *CXCL11*, *CXCL13*, *GZMB*, *IDO1*, and *LYZ* were highly expressed in pCR patients, while *CXCL14* and *ESR1* were lowly expressed in pCR patients. After linear fitting of the multi-gene expression model, the area under the curve (AUC) value of the ROC curve diagnosis of pCR patients was 0.844. Immunoinfiltration analysis showed that compared with RD patients, 15 of the 28 immune cell types examined showed high-infiltration in pCR patients, including activated CD8 T cells, effector memory CD8 T cells, and activated CD4 T cells.

**Conclusions:** This investigation ultimately identified 9 immune-related biomarkers as potential tools for assessing the sensitivity of NAC in HER2-negative BC patients. These biomarkers have great potential for predicting pCR BC patients.

**Keywords:** Breast cancer (BC); immune-related gene; pathological complete response (pCR)

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## Introduction

Breast cancer (BC) ranks number one among all female malignancies, with 281,550 new diagnoses in 2021, accounting for 30% of all female cancer cases (1). With recent advances in medical treatments, the mortality rate of BC patients continues to decrease, and in 2021, the mortality rate was 15% (1). One of the key treatment strategies for BC is neoadjuvant chemotherapy (NAC), which refers to chemotherapy before surgery. NAC aims to reduce the tumor size, thereby facilitating surgery in otherwise inoperable patients or patients for whom breast-conserving surgery is not an option (2,3). In recent years, increasing numbers of BC patients have undergoing NAC with favorable results (4).

Human epidermal growth factor receptor 2 (HER2) is an important therapeutic target for breast cancer (5). HER2 is a specific antigen expressed on breast cancer tumor cells. Combined with anti-HER2 drugs such as trastuzumab, it can mediate antibody-dependent cell-mediated cytotoxicity (ADCC) effect and kill tumor cells (6). In the past 10 years, anti-HER-2 target therapy has significantly improved the prognosis of breast cancer, and about 75% of metastatic HER2-positive patients who undergo NAC can achieve pathological complete response (pCR) (7). However, about 70–80% of BC patients are HER2-negative, and the response of these patients to NAC is much lower than that of HER2 positive patients (8,9). For HER2-negative patients, including intracavitary and triple-negative breast cancer (TNBC) patients, in the reason of the lack of specific therapeutic targets for breast cancer cells by traditional anthracycline and paclitaxel drugs, conventional chemotherapy regimens can only achieve pCR in about 20–40% of patients (10-12). The low pCR rate of HER2-negative BC patients necessitates the search for novel biomarkers to identify which patients are likely to acquire pCR after NAC. Cancer immunotherapy is a treatment that identifies and attacks cancer cells by manipulating the immune system (13). There have been some breakthroughs in a variety of solid malignant tumors and hematological malignant tumors (13). Immunotherapy in BC patients mainly aims to block antibodies against programmed death 1 (PD-1)/programmed death ligand 1 (PD-L1), and this has been shown to achieve strong local tumor control and lasting response in BC patients (14). While immunotherapy is suitable for a portion of TNBC patients, at present, there are no effective immunotherapy drugs for the treatment of luminal BCs (15,16). For patients with HER-negative BC, study has shown that the expression of maternal embryonic leucine zipper kinase (MELK) in breast cancer is related to immune cell infiltration and pCR after NAC (17).

From a previous study, pCR predictors such as prediction analysis of microarray 50 (PAM50), integrated clustering (intcluster), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3KCA) mutation or trastuzumab risk model (TRAR) are useful molecular tools to evaluate treatment response and prognosis (18). These predictive tools have limit investigate with tumor immunity. Therefore, present study wants to further explore the relationship between immunity and the prediction of NAC treatment effect in breast cancer, and analyze whether the expression of immune genes can predict pCR in BC patients with NAC. We identified differentially expressed genes (DEGs) between the 51 patients with pCR and the 108 patients with residual disease (RD), and constructed a prediction model to determine the accuracy of predicting the NAC sensitive in BC patients. We present the following article in accordance with the STARD reporting checklist (available at https://gs.amegroups.com/article/view/10.21037/gs-22-234/rc).

## Methods

### *Research process*

The raw data of GSE163882 was downloaded from the Gene Expression Omnibus (GEO) database. We compared pCR and RD patients to obtain the DEGs. Functional enrichment analyses were then conducted on these DEGs. We intersect the DEGs and immune-related genes to obtain the hub immune biomarkers, and then use the linear fitting model to construct a prediction model to predicting the NAC sensitive in BC patients. Finally, the single sample gene set enrichment analysis (ssGSEA) algorithm was used to analyze immune cell invasion in BC patients.

### *Raw data*

The GSE163882 dataset, containing 222 BC patients treated with NAC, was downloaded from the GEO database. There were 159 HER2 negative and 63 HER2 positive patients. The data from the 159 HER2 negative patients were used for subsequent analyses. The list of immune-related genes was obtained from ImPort share data

(https://www.immport.org/shared/home). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

### *Identification of the differentially expressed genes between pathological complete response samples and residual disease samples*

Among the 159 HER2 negative BC patients, 51 reached pCR and 108 reached RD after NAC. The pCR and RD patients were compared to obtain the DEGs using the "limma" package in R software, with the threshold set to log2 fold change |logFC| >1 and false discovery rate (FDR) <0.05. pCR means no residual invasive tumor cells were found in the surgical specimens of primary breast; RD means there's still residual invasive tumor cells residual in the surgical specimens of primary breast.

### *Functional enrichment analysis*

After obtaining the DEGs, the "clusterProfiler" package in R software was used to analyze the functional enrichment of the DEGs. Enrichment function analysis including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) were performed.

### *Construction of the protein-protein interaction (PPI) network*

The PPI network of DEGs was constructed through the STRING online website (https://string-db.org/) and visualized using the Cytoscape software.

### *The expression of immune-related differentially expressed genes in breast cancer and the construction of a single- and multi-index diagnostic model*

The intersection of the DEGs and immune-related genes was used to obtain the immune-related DEGs. The expression of these immune-related DEGs was compared between RD and pCR patients. The ROC curves of the expression levels of these immune-related DEGs were constructed using the "pROC" package and linearly combined to construct a multi-index diagnostic model using the "glm" R function.

### *Single sample gene set enrichment analysis (ssGSEA) of immune cell abundance in breast cancer patients*

The "GSEABase" and "GSVA" packages were used to identify the abundance of 28 types of immune cells in pCR and RD patients. Furthermore, the relationship between immune cells and immune-related DEGs in BC patients was analyzed.

### *Statistical analysis*

The R software was used for all data analysis in the present study. Differential analysis of pCR and RD BC patients was performed using the limma package. Wilcoxon non-parametric tests and Spearman's method were used to compare the differences and correlation between the two groups. A P value <0.05 (two-sided) was considered statistically significant.

## Results

### *The differentially expressed genes between pathological complete response and residual disease patients*

Compared with RD patients, a total of 45 DEGs were identified in pCR patients, of which, 17 were upregulated and 28 were downregulated (*Figure 1A*,*1B*).

### *Functional enrichment analysis*

The results of the GO functional enrichment analysis of the DEGs indicated that antimicrobial humoral response, antimicrobial humoral immune response mediated by antimicrobial peptide, lymphocyte chemotaxis, lung epithelial cell differentiation, and lung cell differentiation were the top five biological processes (BPs). Apical plasma membrane, apical part of the cell, chloride channel complex, specific granule lumen, and Golgi medial cisterna were the top five cell components (CC). Chemokine activity, chemokine receptor binding, cytokine activity, CXCR chemokine receptor binding, and dystroglycan binding were the top five molecular functions (MFs) (*Figure 2A*,*2B*).

KEGG analysis indicated that viral protein interaction with cytokine and cytokine receptors, chemokine signaling pathway, cytokine-cytokine receptor interaction, Toll-like receptor signaling pathway, cytosolic DNA-sensing

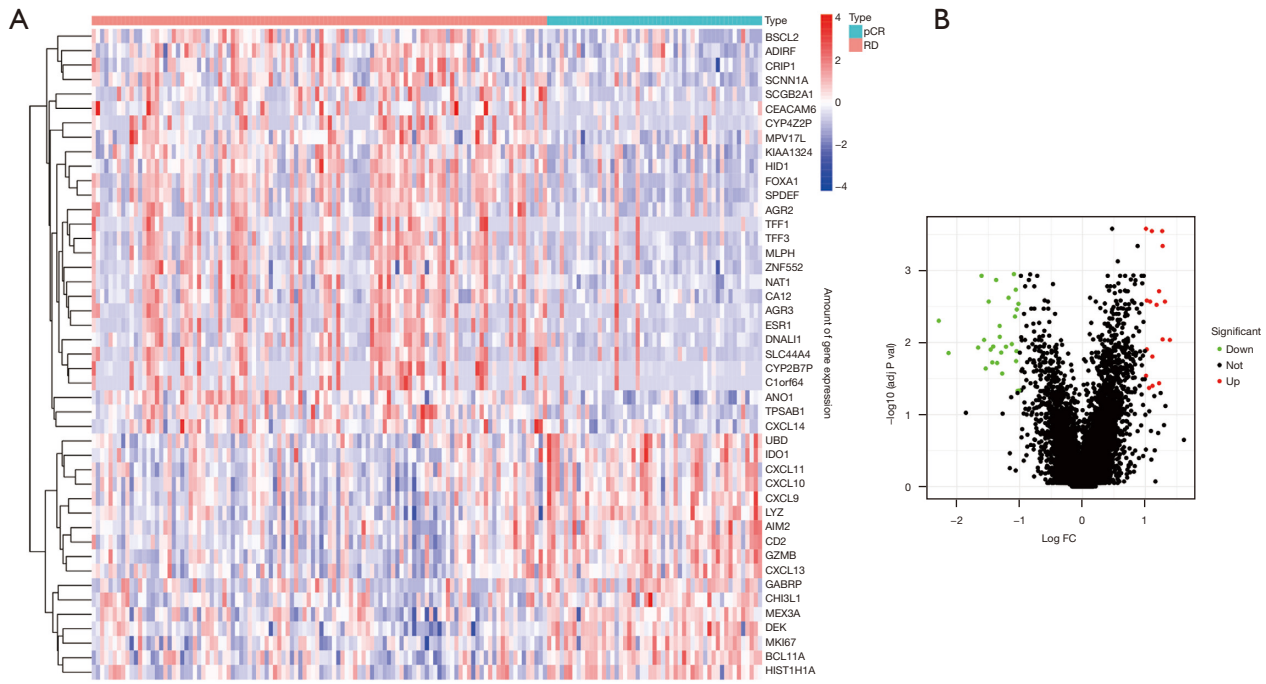


**Figure 1** The DEGs between pCR and RD patients. (A) A heatmap of the DEGs in BC patients. (B) A volcano plot of the DEGs in BC patients. DEGs, differentially expressed genes; pCR, pathological complete response; RD, residual disease; BC, breast cancer.

pathway, estrogen signaling pathway, influenza A, nitrogen metabolism, aldosterone-regulated sodium reabsorption, and African trypanosomiasis were the top 10 enriched KEGG signaling pathways (*Figure 2C,2D*).

### *Protein-protein interaction network of the differentially expressed genes and the immune-related differentially expressed genes*

The PPI network constructed with the 45 DEGs resulted in a total of 30 nodes and 56 edges. The top nodes were anterior gradient 2, protein disulphide isomerase family member (*AGR2*), C-X-C motif chemokine ligand 10 (*CXCL10*), *CXCL9*, and estrogen receptor 1 (*ESR1*) (*Figure 3A,3B*). Based on the intersection of the immune-related genes and the DEGs, 9 hub immune-related DEGs were obtained (*Figure 3C*).

### *The expression of the 9 immune-related genes in pathological complete response and residual disease patients*

Among the 9 immune-related DEGs, *CXCL9*, *CXCL10*, *CXCL11*, *CXCL13*, granzyme B (*GZMB*), indoleamine 2,3-dioxygenase 1 (*IDO1*), and lysozyme (*LYZ*) were highly expressed in pCR patients, while *CXCL14* and *ESR1* were lowly expressed in pCR patients compared to RD patients (*Figure 4A-4I*).

### *The diagnostic value of the 9 immune-related DEGs in pathological complete response breast cancer patients (ROC curve)*

At the single-gene expression level, the AUC values of the ROC curve for pCR patient diagnosis were as follows: *CXCL9* =0.722; *CXCL10* =0.690; *CXCL11* =0.654; *CXCL13* =0.707; *CXCL14* =0.659; *ESR1* =0.682; *GZMB* =0.760; *IDO1* =0.716; and *LYZ* =0.764 (*Figure 5A-5I*).

At the multi-gene expression model (model = −6.222−0.280* *CXCL9* +0.114* *CXCL10* −0.324* *CXCL11* −0.004* *CXCL13* −0.367* *CXCL14* −0.179* *ESR1* +0.405* *GZMB* +0.459* *IDO1* +0.769* *LYZ*), the AUC value of the ROC curve in the diagnosis of pCR patients was 0.844 (*Figure 5J*).

### *The relationship between immune cells and immune-related genes*

Among the 28 immune cell types examined, 15 immune cell

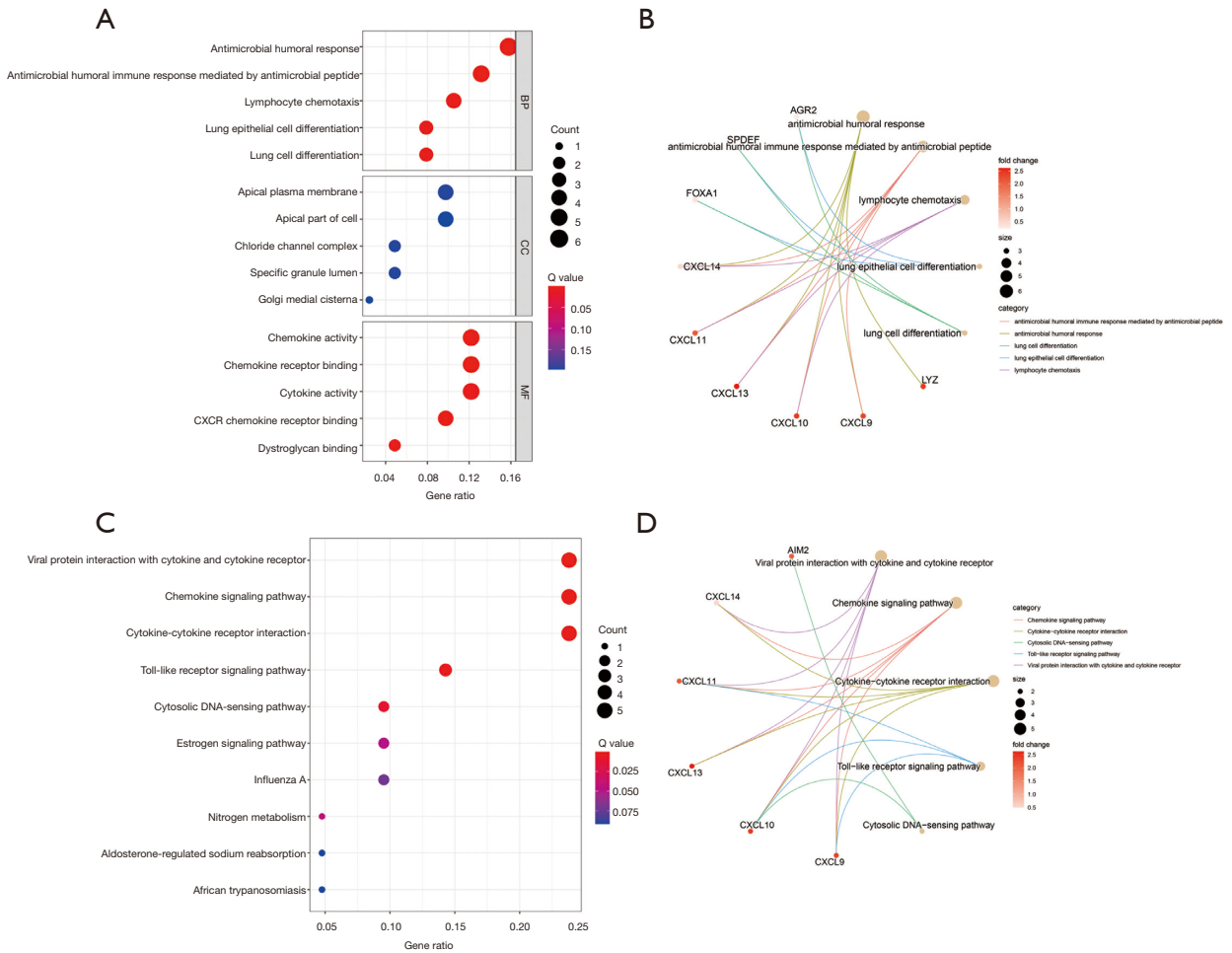


**Figure 2** Functional enrichment analysis of the DEGs. (A) Bubble diagram of GO enrichment function analysis. (B) A circle diagram of the top 5 biological process. (C) A bubble diagram of the top 10 KEGG signaling pathways. (D) A circle diagram of specific enrichment genes and the top 5 KEGG signaling pathways. The size of the bubble represents the number of enrichment genes, and the color represents the q value. DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cell components; MF, molecular function.

types showed high-infiltration in pCR patients compared to RD patients, including activated CD8 T cells, effector memory CD8 T cells, activated 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, memory B cells, myeloid derived suppressor cells, natural killer T cell, activated dendritic cells, and plasmacytoid dendritic cells. Meanwhile, CD56 dim natural killer cells showed low infiltration in pCR patients (*Figure 6A*).

There was a positive correlation between the expression levels of the 9 immune-related genes and the abundance of immune cell infiltration, especially with *CXCL9*, *CXCL10*, *CXCL11*, *CXCL13*, *CXCL14*, *IDO1*, *LYZ* and *GZMB*. However, *ESR1* was negatively correlated with immune cell infiltration levels (*Figure 6B*).

## Discussion

In patients with stage II or III BC, preoperative NAC has several advantages. First, chemotherapy can shrink and downgrade the tumor, enabling surgery or breast-conserving surgery in patients who would otherwise be unsuitable for such treatments. Second, the follow-up treatment plan can

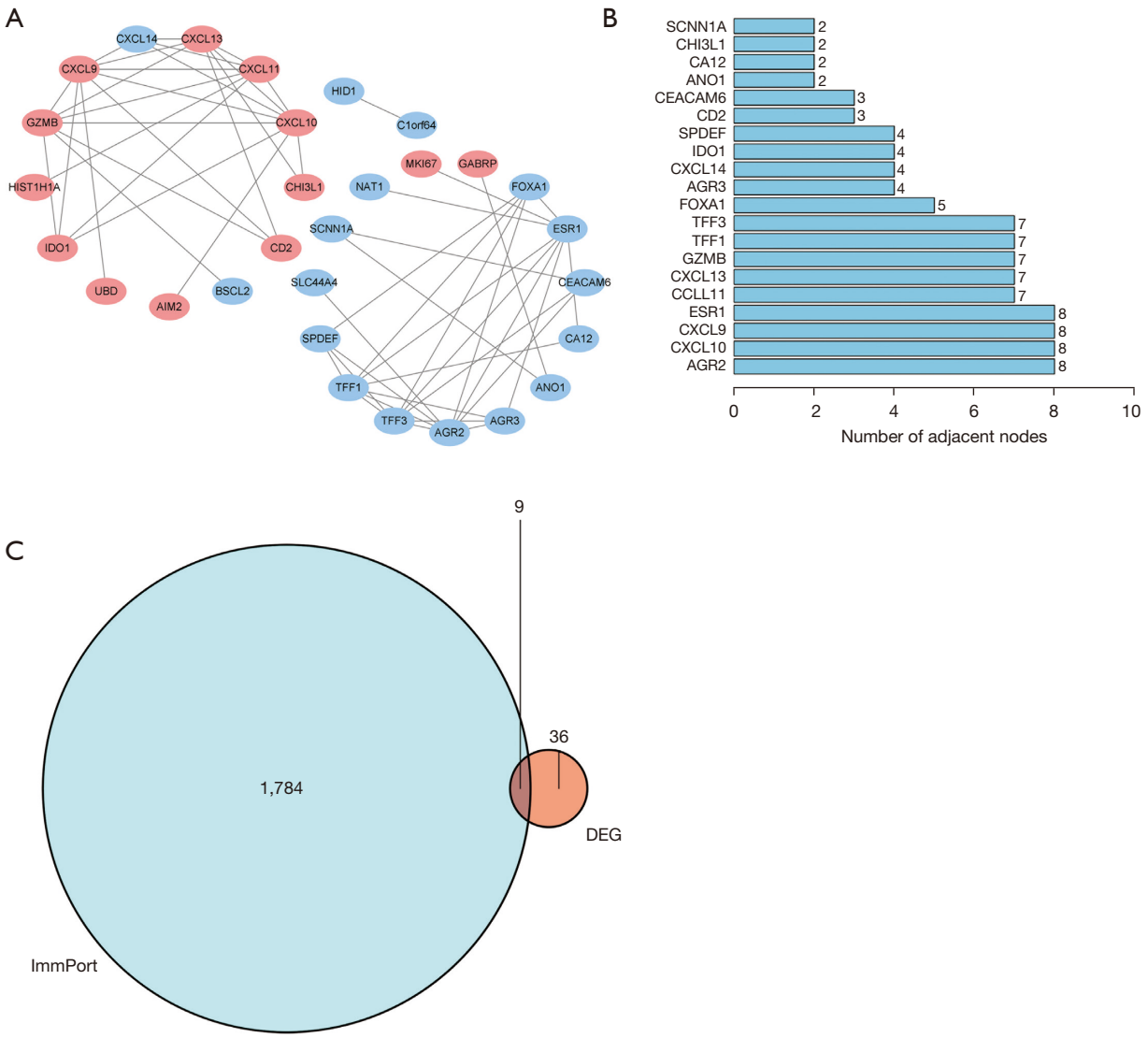


**Figure 3** PPI network of the DEGs and the immune-related DEGs of BC patients. (A) The PPI network of the DEGs. (B) A histogram showing the top 20 proteins in the number of PPI connections. The number on the right of the bar represents the number of connections to the protein. (C) A Venn diagram showing the intersection of immune-related genes and the DEGs. DEGs, differentially expressed genes; BC, breast cancer; PPI, protein-protein interaction.

be formulated according to the efficacy of chemotherapy, which can be used as a marker of prognosis (3,19). High pCR rates can be achieved with anti-HER2-targeted therapy in HER2-positive BC patients. However, HER2-negative is the most common pathological type of BC, especially estrogen receptor (ER) positive BC, which is associated with poorer response to chemotherapy (20). Hence, for HER2-negative patients, there is an urgent need to identify the biomarkers which respond to chemotherapy, and find effective HER2-negative diagnostic markers to identify which patients are likely to acquire pCR. This has important guiding significance for the selection of subsequent treatment options.

Epigenetics plays an important role in the pathogenesis of BC drug resistance. For example, changes in gene expression levels and DNA methylation silencing key genes can inhibit related signaling pathways, leading to drug resistance in BC (21,22). There is much evidence to show

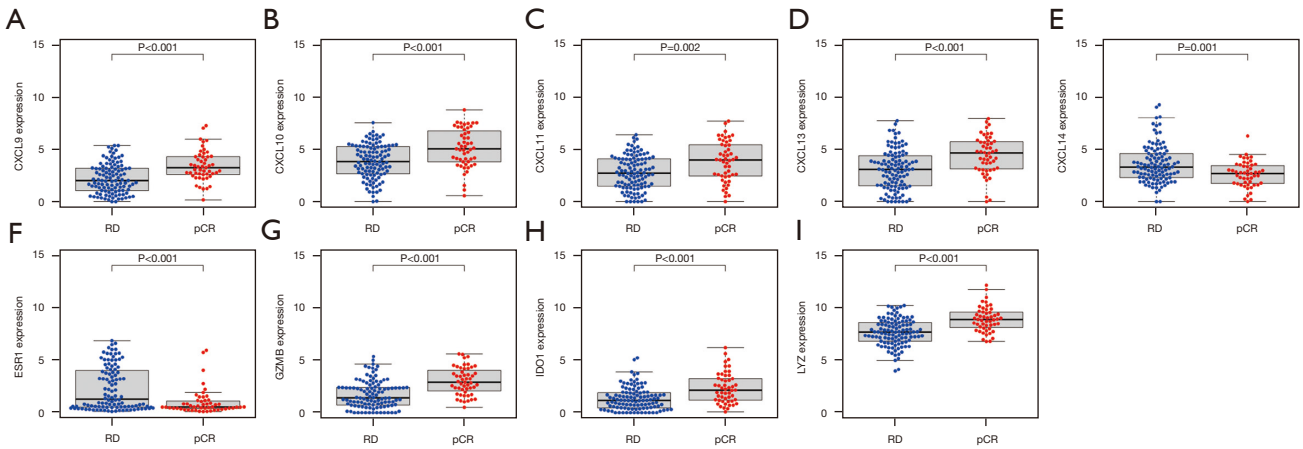


**Figure 4** The expression levels of the 9 immune-related genes in RD and pCR patients. (A) *CXCL9*; (B) *CXCL10*; (C) *CXCL11*; (D) *CXCL13*; (E) *CXCL14*; (F) *ESR1*; (G) *GZMB*; (H) *IDO1*; (I) *LYZ*. *CXCL*, C-X-C motif chemokine ligand; *ESR1*, estrogen receptor 1; *GZMB*, granzyme B; *IDO1*, indoleamine 2,3-dioxygenase 1; *LYZ*, lysozyme; pCR, pathological complete response; RD, residual disease.

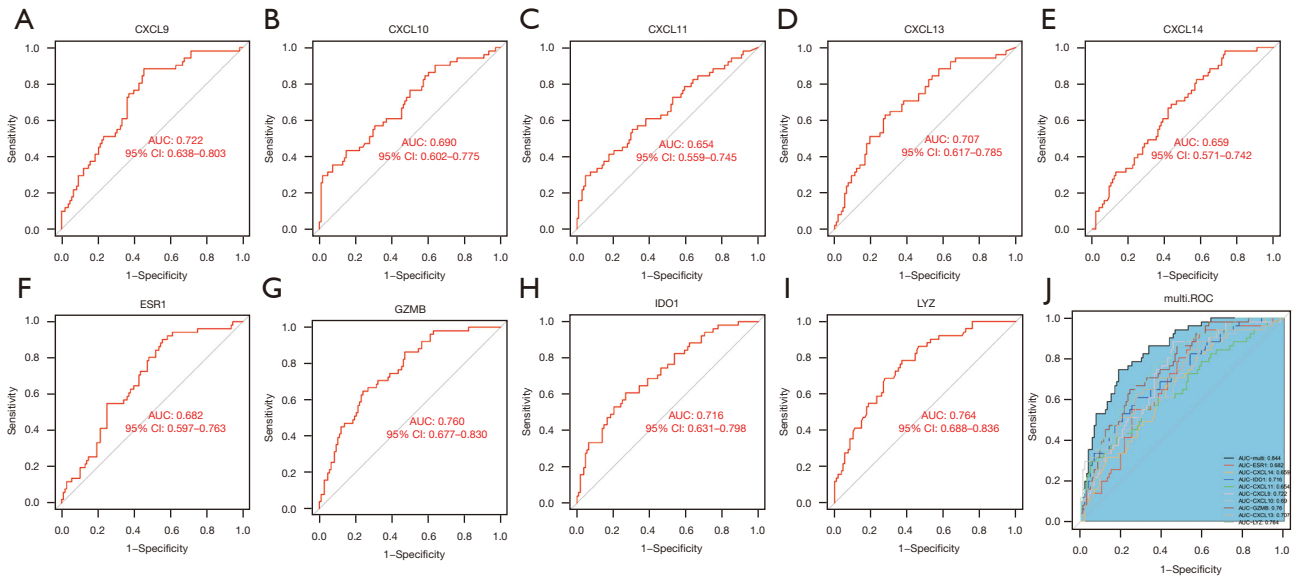


**Figure 5** The accuracy of the single- and multi-gene expression levels in the diagnosis of pCR BC patients (ROC curve). (A) *CXCL9*; (B) *CXCL10*; (C) *CXCL11*; (D) *CXCL13*; (E) *CXCL14*; (F) *ESR1*; (G) *GZMB*; (H) *IDO1*; (I) *LYZ*; (J) multi ROC. *CXCL*, C-X-C motif chemokine ligand; *ESR1*, estrogen receptor 1; *GZMB*, granzyme B; *IDO1*, indoleamine 2,3-dioxygenase 1; *LYZ*, lysozyme; pCR, pathological complete response; BC, breast cancer; ROC, receiver operator characteristic.

that cytokines play an important role in the treatment of drug resistance in BC. For instance, interleukin (IL)-6, IL-8, and tumor necrosis factor-α (TNF-α) can significantly regulate epithelial-mesenchymal transformation (EMT)-mediated drug resistance (23-26). In the present study, a total of 45 DEGs were screened from pCR and RD patients, including 17 upregulated and 28 downregulated genes. The results of functional enrichment analysis indicated that viral protein interaction with cytokine and cytokine receptors, chemokine signaling pathways, cytokine-cytokine receptor interaction, and Toll-like receptor signaling pathways are related to the most abundant DEGs. To further investigate the roles of key immune-related genes in these signaling pathways, 9 key immune genes were obtained by

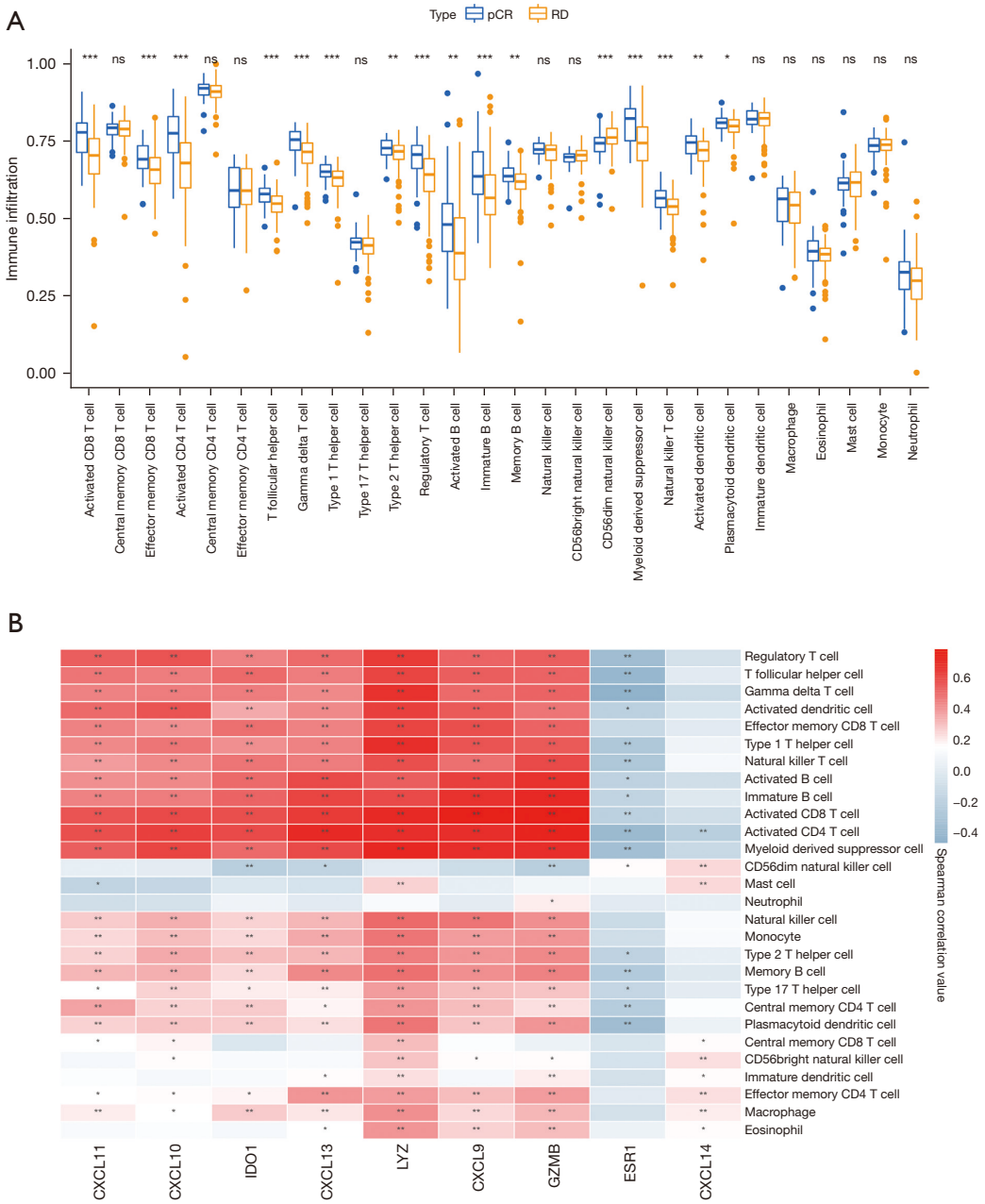


**Figure 6** The relationship between immune cells and immune-related DEGs. (A) A boxplot of the immune cell infiltration levels in RD and pCR patients. (B) A heatmap of the immune cell infiltration levels and the 9 immune-related genes, where red represents a strong positive correlation, and gray represents a weak positive correlation. ns, P>0.05; *, P<0.05; **, P<0.01; ***, P<0.001. pCR, pathological complete response; RD, residual disease.

intersecting immune-related genes and the DEGs. The results indicated that *CXCL9*, *CXCL10*, *CXCL11*, *CXCL13*, *GZMB*, *IDO1*, and *LYZ* were highly expressed in pCR patients compared to RD patients, while *CXCL14* and *ESR1* were lowly expressed in pCR patients. A study by Chen *et al.* demonstrated that high expression of *CXCL9* can not only inhibit the proliferation and apoptosis of BC cells, but can also reduce the $IC_{50}$ of taxanes, which indicates that *CXCL9* plays an important role in the resistance of chemotherapeutic drugs (27). Our results showed that

*CXCL9* is highly expressed in BCs with pCR, which is consistent with Chen's report. Dai *et al.* demonstrated *in vitro* that *CXCL10* was correlated with the NAC response on account of the hypoxia inducible factor-1α (HIF1α)/interleukin 17 (IL-17A)/*CXCL10* pathway affecting the sensitivity of BC cells to paclitaxel (28). The results of our study also revealed that *CXCL10* is highly expressed in pCR patients, indicating that a higher expression level of *CXCL10* may result in pCR in BC patients. Several studies have shown that *CXCL11*, *CXCL13*, and *CXCL14* biomarkers are closely related to chemotherapeutic resistance in BC (29-33). *ESR1* encodes estrogen receptor α (ERα) and plays an important role in the tumorigenesis of BC (34). The vast majority of BCs are ER positive and endocrine sensitive but not sensitive to chemotherapy (35,36). Our results showed that the expression of *ESR1* is low in pCR BCs, suggesting that high expression of *ESR1* may inhibit the sensitivity of tumor cells to chemotherapeutic drugs. The combined results of above previous studies and this current report suggests that the 9 hub immune-related genes identified in the present study may play an important role in the chemotherapeutic resistance of BC cells. Indeed, a linear model for predicting pCR in BC patients following NAC was established based on these hub genes. The model has high diagnostic value (AUC =0.844) and has the potential to be used in practical clinical applications.

The tumor microenvironment, including cancer-associated fibroblast (CAFs), mesenchymal cells, tumor-associated macrophages (TAMs), and endothelial cells, plays an important role in the occurrence and development, recurrence and metastasis, and chemotherapeutic resistance of BC (37,38). Macrophages are one of the most important types of immune cells in the TME stroma of BCs. Several *in vitro* and *in vivo* studies have shown that TAMs affect the response of tumor cells to paclitaxel, doxorubicin, and cyclophosphamide (39,40). The mechanisms may involve TAM-derived IL-10 increasing the resistance of BC cells to paclitaxel (41). However, in the present study, no significant difference was found in the abundance of TAMs between RD and pCR patients, as calculated by the ssGSEA algorithm. Nonetheless, the abundance of macrophages was positively correlated with the expression levels of key immune genes in this research (except *ESR1*), suggesting that the immune-related genes screened in this study may be potential biomarkers for chemotherapeutic drug resistance in BC. *In vitro* study by Gomes-Santos *et al.* demonstrated that exercise training in mice promoted the infiltration of CD8+T cells and made refractory breast cancer sensitive to treatment through the causal effect of the *CXCL9*/*CXCL11*/C-X-C motif chemokine receptor 3 (*CXCR3*) pathway. Exercise training can also normalize the tumor vascular system, reshape tumor microenvironment, and enhance CD8+T cell-mediated anti-tumor activity through *CXCR3* (33). In this study, the results of the immune cell infiltration analysis showed that activated CD8 T cells, effector memory CD8 T cells, and activated CD4 T immune cells were highly infiltrated in pCR compared to RD patients. Although tumor immunotherapy has made great achievements in non-small cell lung cancer, its effect on epithelial solid tumors, including breast cancer, has been limited (42,43). This current investigation was limited by the lack of experimental verification of the key genes identified. In the future, further gene functional verification experiments should be performed investigations should continue to identify novel and effective targets for the diagnosis and treatment of BC.

## Conclusions

In summary, this study ultimately identified 9 immune-related biomarkers as potential tools for assessing the sensitivity to NAC in BC patients, which will help clinicians predict a patient's likely response to chemotherapy based on model scores.

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## Footnote

*Reporting Checklist:* The authors have completed the STARD reporting checklist. Available at https://gs.amegroups.com/article/view/10.21037/gs-22-234/rc

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at https://gs.amegroups.com/article/view/10.21037/gs-22-234/coif). 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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