Original Article

Identification of *C4BPA* as biomarker associated with immune infiltration and prognosis in breast cancer

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Background: *C4BPA* is a gene that encodes the C4BP protein α chain and is involved in the complement system. *C4BPA* is regarded as a new biomarker for cancer, especially for non-small cell lung cancer and ovarian cancer. However, its role in breast cancer (BC) has not yet been determined.

Methods: In this research, we used a bioinformatics approach to assess the prognostic significance of C4BPA in BC. Utilizing a variety of databases and analysis tools, including The Cancer Genome Atlas (TCGA), Genotype-Tissue Expression (GTEx), Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), R, STRING, and the Kaplan-Meier plotter, we specifically assessed the connection between C4BPA and BC.

Results: *C4BPA* expression was markedly decreased in BC tissues compared to its expression in normal breast tissues ($P<0.05$). Additionally, a receiver operating characteristic (ROC) curve revealed that *C4BPA* has a significant capacity for prognostication and diagnostics. Additionally, *C4BPA* expression was linked to some immune infiltrating cells' functionality, according to gene set enrichment analysis (GSEA) and immune infiltration analysis. Low *C4BPA* expression was additionally related to poor progression-free interval (PFI) and overall survival (OS), according to the Kaplan-Meier method. We also found that *C4BPA* expression was independently connected to PFI and OS through Cox regression analysis. Finally, prognostic analysis of the various subgroups of breast invasive carcinoma (BRCA/BIC) in TCGA showed that patients with low *C4BPA* expression might have worse PFI and OS in patients with Luminal A compared to other BC subtypes.

Conclusions: In conclusion, these results revealed that *C4BPA* could potentially act as a diagnostic biomarker for BC patients indicating unfavorable prognoses and offers valuable knowledge for creating therapeutics and prognostic indicators.

Keywords: Breast cancer (BC); bioinformatics analysis; *C4BPA*; immune infiltration; prognosis

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Introduction

Breast cancer (BC) is one of the most common malignancies in women. Globally, approximately 1.3 million people are diagnosed with BC (1,2), and 600,000 die of BC every year (3). At present, the incidence and mortality rates of BC in China have gradually increased, suggesting that the disease burden of BC in Chinese women is becoming increasingly severe (4). Despite recent advances in diagnosis and therapy, patients with recurrent and metastatic BC have poor survival (5). Thus, it is of urgent importance to discover sensitive biomarkers and innovative therapeutics to improve BC's clinical diagnosis and treatment.

Numerous molecular indicators, including DNA mutations, abnormal messenger ribonucleic acid (mRNA) expression, and microRNAs, have been associated with the occurrence and progression of BC. *C4BPA*, also known as Complement Component 4 Binding Protein Alpha, Proline-Rich Protein, is a novel biomarker for multiple types of cancers (6). For example, C4BP has been linked to aggressive tumor characteristics and a poor prognosis in individuals with non-small cell lung cancer stage IIb and IIIa (7). Furthermore, the present study endeavors to elucidate the potential of *C4BPA* as an autophagy-immune-derived biomarker for prognostication and therapeutic responsiveness among patients diagnosed with lung adenocarcinoma (8). Additionally, *C4BPA* has been identified as a promising serum biomarker for the early detection of pancreatic ductal adenocarcinoma (PDAC) and the prediction of lymph node metastasis in PDAC cases (9,10). Reports have indicated that *C4BPA* harbors considerable biomarker potential in the context of patient outcomes in myeloma and suggests its prospective utility as a molecular signature in hepatocellular carcinoma (11,12). Besides, fully-silylated *C4BPA*, A2160, is a potentially novel serum biomarker for ovarian cancer (13). Furthermore, *C4BPA* has been identified in tissue and blood samples taken from BC patients (14). Yet, the precise functions of *C4BPA* in BC are still unknown.

In this research endeavor, we explored the correlation between *C4BPA* and BC as well as the prognostic value of *C4BPA* in BC from The Cancer Genome Atlas (TCGA) database. Furthermore, we performed gene set enrichment analysis (GSEA) function and pathway analysis to learn more about the molecular mechanism of *C4BPA* in BC pathogenesis. Additionally, the putative mechanism by which *C4BPA* modifies the occurrence and progression of BC was also thoroughly studied and addressed by investigating the connection between immune infiltration and *C4BPA* expression. Finally, we looked into the relationship between the clinical pathological characteristics of BC and the degree of *C4BPA* expression. We present this article in accordance with the TRIPOD reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1215/rc).

Highlight box

Key findings

- *C4BPA* expression is significantly decreased in breast cancer (BC) tissues compared to normal breast tissues (P<0.05).
- *C4BPA* has a notable prognostic and diagnostic capacity for BC.
- Low *C4BPA* expression is associated with poor progression-free interval and overall survival, particularly in patients with Luminal A subtype.
- *C4BPA* expression correlates with the functionality of immune infiltrating cells, indicating its potential role in the tumor microenvironment.

What is known and what is new?

- Previous research has highlighted *C4BPA* as a potential biomarker for certain cancers, and this study further establishes its significance in BC prognosis and diagnostics.

What is the implication, and what should change now?

- These findings emphasize the potential of *C4BPA* as a diagnostic biomarker for BC, with implications for therapeutic strategies and prognostic assessments in clinical settings.

Methods

RNA-sequencing (RNA-seq) data and bioinformatics analysis

TCGA (https://cancergenome.nih.gov/), a free public platform, is a source of abundant cancer-related dataset. The TCGA database was used to download the gene expression data (HTseq-Counts) and associated clinical data for BC for this analysis; the criteria for exclusion were normal BC samples and overall survival (OS) times of <1 month. The level 3 HTSeq-Counts data of 1,065 BC patients, the clinic data, and the RNA-seq gene expression were retained and subsequently investigated. Differential expression analysis and pan-cancer analysis data containing RNA-seq data in transcripts per million (TPM) format in TCGA and GTEx (https://www.gtexportal.org/) were uniformly processed by the Toil process downloaded from University of California Santa Cruz (UCSC) XENA (https://xenabrowser.net/

datapages/) (15). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

RNA-seq data preprocessing and differentially expressed genes (DEGs) analysis

The gene expression levels of *C4BPA* in TCGA breast invasive carcinoma (BRCA/BIC) were divided into high expression and low expression groups based on the median value. Then, the unpaired Wilcoxon rank-sum test within the DESeq2 (3.8) software and R were used to compare expression profiles (HTSeq-Counts) between high and low *C4BPA* expression groups in order to evaluate DEGs and assess the prognostic value of various C4BPA expression levels (16). As threshold values for the DEGs, |log2-Fold Change (FC)| >1.5 and adjusted P value <0.05 were taken into consideration.

Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analyses of DEGs

R package cluster Profiler was used to conduct pathway enrichment analysis of DEGs of BC significantly associated with *C4BPA* alterations (17). The GO terms for molecular functions (MFs), biological processes (BPs), and cellular components (CCs), as well as KEGG pathways, were enriched. Significant statistical differences were defined as P<0.01, the count ≥3, and the enrichment factor >1.5.

GSEA

The fundamental mechanisms of *C4BPA* in BC were also investigated by GSEA (18) [PMID: 16199517], performed by R package cluster Profiler (17) [PMID: 22455463]. This is a computational method for determining if a priori-defined list of genes displays statistically meaningful concordant variations between groups with high and low *C4BPA* expression. This study employed a permutation test with 1000 repetitions to identify the significantly altered pathways. The level of *C4BPA* expression was used as a phenotypic label. The analysis included gene sets from molecular signatures database (MSigDB) pathways. The pathways that were greatly enhanced for each phenotype were categorized using the adjusted P value (<0.1), false discovery rate (FDR) q-value (<0.25) and normalized enrichment score (NES; |NES| >1).

The correlation between C4BPA gene expression and immune infiltration

The single-sample GSEA (ssGSEA) method (an extension of GSEA) was used to analyze the immune infiltration of BC using the gene set variation analysis (GSVA) package in R (3.6.3) for 24 different types of immune cells in tumor samples. Genes predicting the number of 24 tumor-infiltrating immune cells in different tissue samples are part of the immune cell signature [PMID: 24138885] (19). The Wilcoxon rank sum test was used to compare the immune cell infiltration in the high-expression and low-expression groups of *C4BPA*. And the relationship between *C4BPA* and these immune cells was investigated by Spearman's correlation method.

Protein-protein interaction (PPI) network construction and molecular analysis

The creation of PPI networks was accomplished through the successive utilization of a sophisticated Search Tool for the Retrieval of Interacting Genes (20). Cytoscape software (version 3.8.0) was used in this work to view and analyze the data from the STRING database (https://cn.string-db.org/). The DEGs with a confidence score >0.4 were marked as significant.

Prognostic model generation and prediction

The ideal model was selected by utilizing a multivariate Cox regression analysis and the Akaike information criterion (AIC) approach. Subsequently, a nomogram was created with the rms package to assess the prognosis of BC patients, followed by a calibration plot to evaluate its predictive accuracy. The risk score for each patient was determined by summing the scores of individual parameters, which were obtained by multiplying the parameter value by its coefficient. Next, the TCGA BC cohort was segregated into two distinct groups, namely high-risk and low-risk, based on the median risk score value. The disparity in the OS between these groups was determined using the Kaplan-Meier method and analyzed via a two-sided log-rank test utilizing the survminer package. The predictive performance of the prognostic model was assessed by constructing a receiver operating characteristic (ROC) curve. Additionally, prognostic information comes from a *Cell* paper (21). This study set the statistical significance level for all two-tailed tests at 0.05.

Statistical analysis

R was used to conduct all statistical analyses (v.3.6.2). Wilcoxon rank-sum tests were utilized to compare the expression of *C4BPA* between the BC and normal groups, while Wilcoxon signed-rank tests were used to compare expression in adjacent groups. Clinical pathologic features and *C4BPA* were analyzed using the Wilcoxon signed-rank test and logistic regression. The Cox regression and the Kaplan-Meier method were used to evaluate clinicopathologic characteristics associated with OS in TCGA patients. Furthermore, multivariate Cox analysis was utilized to compare the influence of C4BPA expression on survival and other clinical features [including T stage, N stage, M stage, pathologic stage, progesterone receptor (PR) status, and estrogen receptor (ER) status]. The cut-off value for C4BPA expression was determined by its median value, and a P value of less than 0.05 was considered meaningful.

Results

C4BPA gene expression levels in pan-cancer and BC and identification of DEGs

According to the screening criteria, 1,065 RNA-seq data with clinical information were available, including 110 cases of BC with paired normal samples. To investigate differences in *C4BPA* expression between various cancer types and normal tissues, we analyzed the TCGA and GTEx databases. Our findings revealed that *C4BPA* expression was significantly lower in multiple malignancies compared to normal tissues (P<0.05, *Figure 1A*). Next, our team conducted a Wilcoxon rank-sum test to assess the influence of *C4BPA* expression in patients with BC in the TCGA and GTEx/TCGA. *C4BPA* expression levels were found to be substantially lower in 1,065 tumor tissues than in 111 normal tissues (P<0.001; *Figure 1B*), and *C4BPA* expression levels in 110 tumor tissues were much lower than that in 110 matched normal breast tissues in the TCGA cohort (P<0.001; *Figure 1C*). Moreover, the GTEx/TCGA cohort findings were aligned with those from the TCGA cohort, showing that *C4BPA* expression levels in tumor tissues were lower than that in normal tissue (P<0.001; *Figure 1D*). Additionally, we employed the ROC curve to assess the ability of *C4BPA* expression level to discriminate BC tumors from non-tumor tissues. The analysis revealed that *C4BPA* exhibited a remarkable area under the curve (AUC) value of 0.870, indicating its potential as a reliable biomarker for distinguishing BC tissues from non-tumor tissues (*Figure 1E*).

In addition, by dividing the expression levels of *C4BPA* in BRCA from TCGA into high-expression and low-expression groups based on the median value, and conducting differential gene expression analysis with the criteria of logFC >1.5 and adj.P <0.05, we identified a total of 335 DEGs, of which 235 were upregulated and 100 were downregulated (Figure S1A). Furthermore, in TCGA BRCA, when *C4BPA* was divided into high expression and low expression groups, the co-expression differential gene pattern is shown in Figure S1B: we selected the top 10 differential genes with the lowest and highest P values.

Gene function annotation and analysis of DEGs

We carried out an extensive gene function annotation using KEGG and GO with the aim of getting comprehensive information on gene function. Our analysis revealed significant findings, with 57 GO terms associated with BPs, three GO terms associated with CCs, and three GO terms associated with MFs (*Figure 2A*). The top three GO terms of each part are presented in Table S1. The BP findings showed that DEGs were primarily enriched in signal release, positive regulation of cell secretion, and positive regulation of secretion; the CC results revealed that DEGs were primarily enriched in multivesicular body, cornified envelope, and lamellar body; and the MF results indicated that DEGs were predominantly enriched in receptor-ligand activity, syntaxin-1 binding, and hormone activity. KEGG signaling pathway analysis identified the top five pathways, which included drug metabolism-cytochrome P450, metabolism of xenobiotics by cytochrome P450, chemical carcinogenesis, retinol metabolism, and complement and coagulation cascades (*Figure 2B*; Table S2).

To further study the biological functions of *C4BPA*, we conducted GSEA and discovered that low expression of *C4BPA* was notably linked with reactome acetylcholine neurotransmitter release cycle, BC, BC relapse signaling pathways (*Figure 3A-3D*; Table S3).

Correlation analysis of C4BPA gene and immune cell infiltration

To explore the relationship between *C4BPA* expression in BC and the abundance of immune infiltrates, we extracted 24 marker genes of immune cells from literature in *Immunity* [PMID: 24138885] (19). The infiltration of these 24 immune cells in BC was analyzed by ssGSEA method, and the difference in the infiltration level of

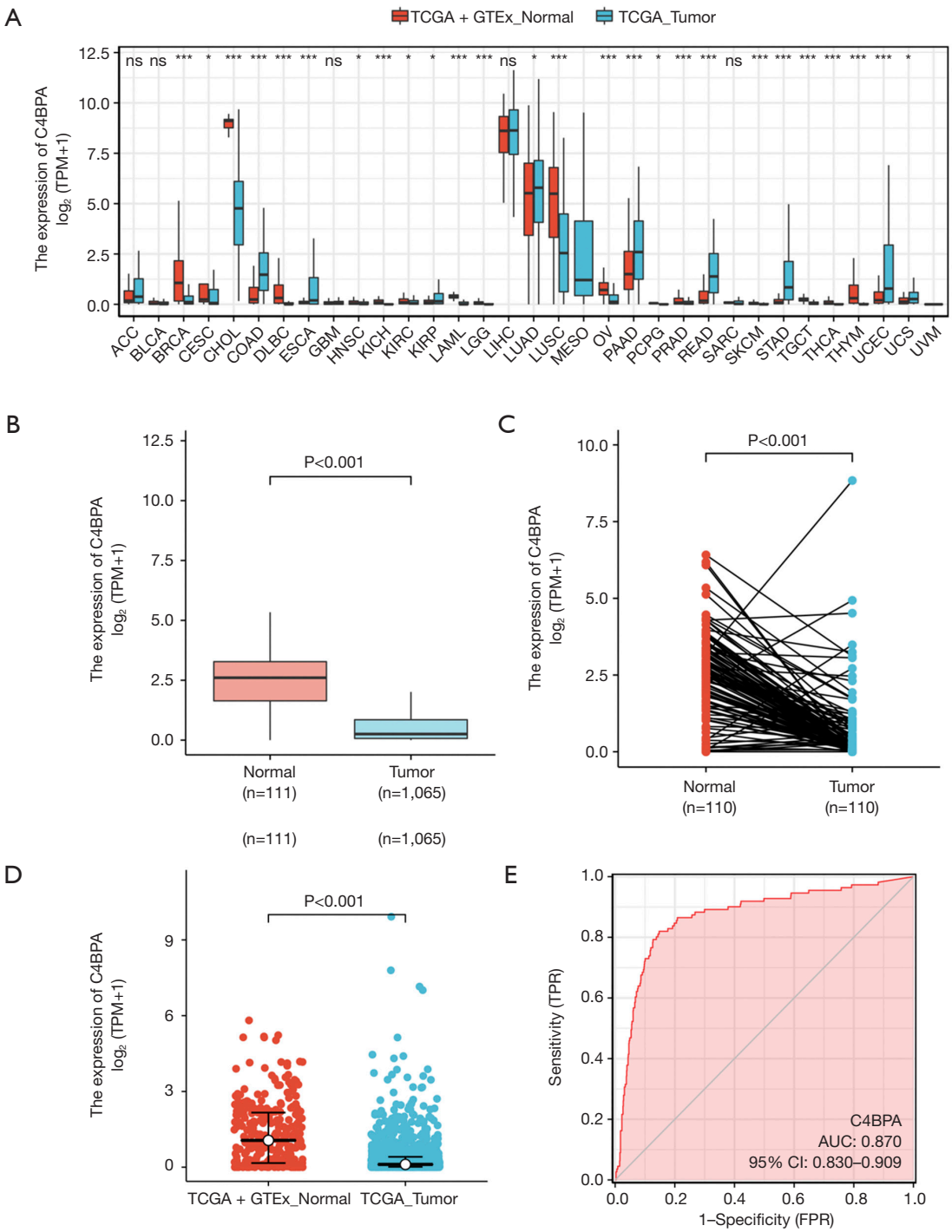

Figure 1 Association between *C4BPA* gene expression and BC. (A) Expression of *C4BPA* in normal samples of GTEx combined with TCGA and different multiple cancer samples corresponding to TCGA. (B-D) Wilcoxon rank-sum test was used to analyze the differential expression of *C4PBA* in BC tissues and adjacent breast tissues, in BC tissues and paired adjacent breast tissues, in normal breast tissues of GTEx combined with TCGA and BC tissues of TCGA, respectively. (E) ROC curve showing the efficiency of *C4BPA* expression level in distinguishing BC tissue from non-tumor tissue. ns, P≥0.05; *, P<0.05; ***, P<0.001. TCGA, The Cancer Genome Atlas; GTEx, Genotype-Tissue Expression; TPM, transcripts per million; AUC, area under the curve; CI, confidence interval; FPR, false positive rate; TPR, true positive rate; BC, breast cancer; ROC, receiver operating characteristic.

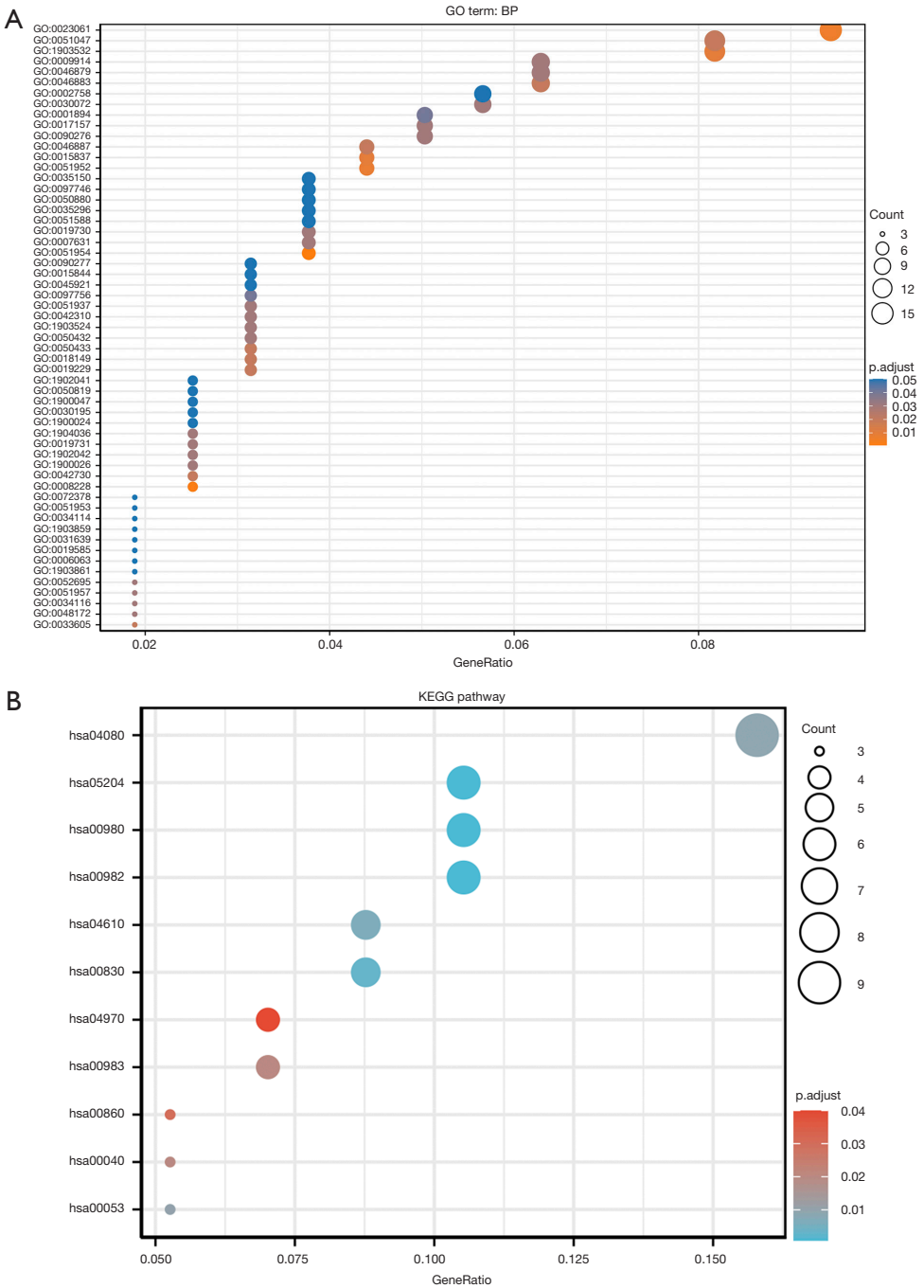

Figure 2 Functional enrichment analysis of consensus genes. (A) GO enrichment analysis results include 57 BP. (B) Enrichment analysis of KEGG pathway. GO, Gene Ontology; BP, biological process; KEGG, Kyoto Encyclopedia of Genes and Genomes.

these 24 immune cells between the high and low *C4BPA* expression groups was analyzed by Wilcoxon rank sum test. In addition, we utilized the Wilcoxon rank-sum test to conduct an in-depth examination of the relationship between the expression of *C4BPA* and immune infiltration.

Our findings indicate a significant association between the *C4BPA* signature and immune infiltration in cases of BC. Notably, *C4BPA* demonstrated a positive correlation with various immune cell types, notably B cells (P<0.001), neutrophils (P<0.001), plasmacytoid dendritic cells (pDCs)

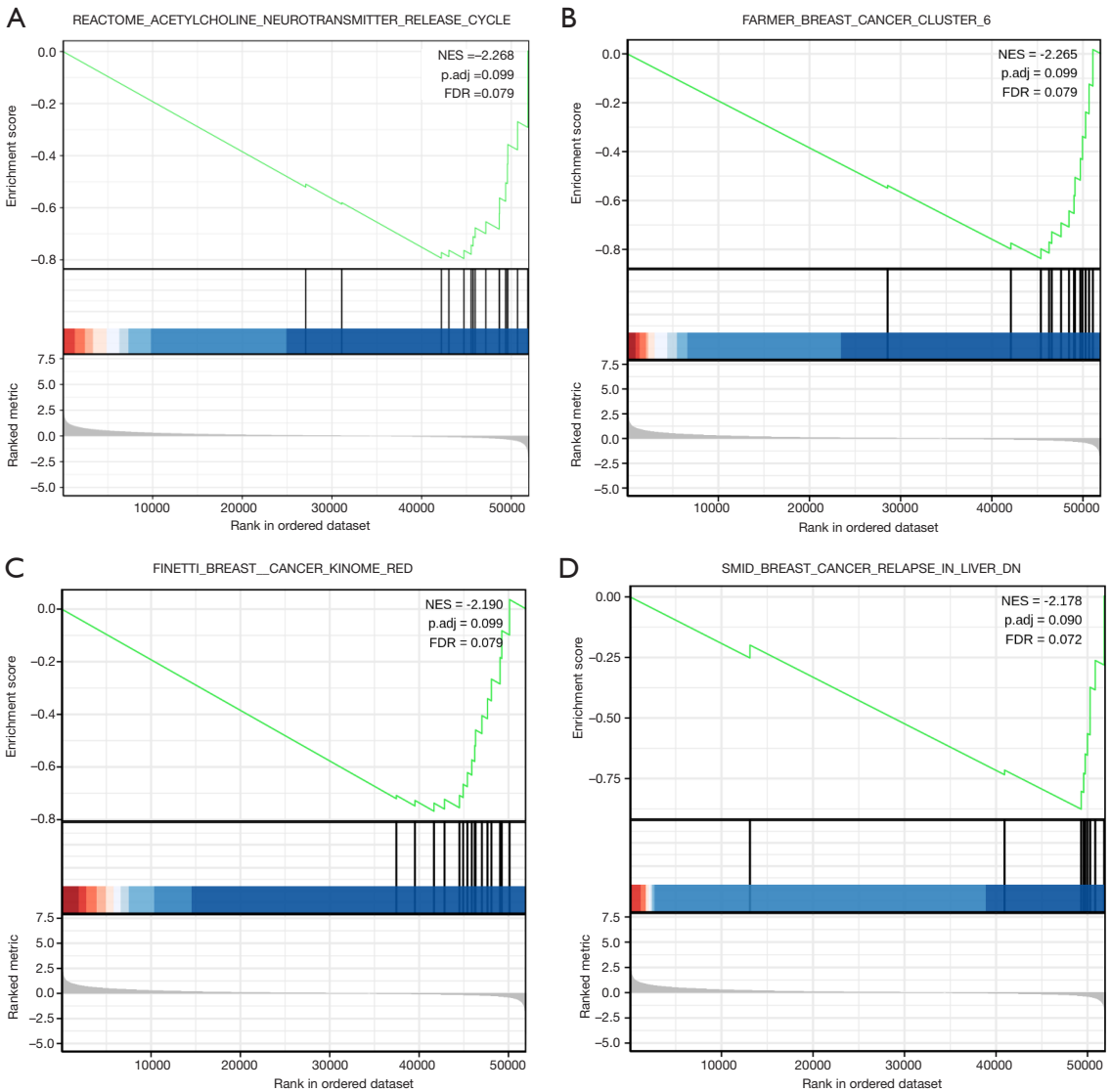

Figure 3 Enrichment plot from the GSEA. The data set was on the right significantly enriched in the blue area (*C4BPA* low expression group): (A) REACTOME_ACETYLCHOLINE_NEUROTRANSMITTER_RELEASE_CYCLE, NES =−2.268, p.adj =0.099, FDR =0.079. (B) FARMER_BREAST_CANCER_CLUSTER_6, NES =−2.265, p.adj =0.099, FDR =0.079. (C) FINETTI_BREAST_CANCER_KINOME_RED, NES =−2.19, p.adj =0.099, FDR =0.079. (D) SMID_BREAST_CANCER_RELAPSE_IN_LIVER_DN, NES =−2.178, p.adj =0.09, FDR =0.072. NES, normalized enrichment score; p.adj, adjust P value; FDR, false discovery rate; GSEA, gene set enrichment analysis.

(P<0.001), CD8 T cells (P<0.001), and T cells (P<0.001) (*Figures 4*,*5A-5E*). Intriguingly, an unexpected inverse relationship was observed between *C4BPA* expression and Th2 cells (P<0.001) (*Figure 5F*). Moreover, we employed the Spearman correlation method to further analyze the association between *C4BPA* expression and immune infiltration. Our results revealed that immune cell infiltration in BC was connected to the *C4BPA* signature. *C4BPA* was positively linked with immune cell types, the top five immune cell type were B cells (R=0.280, P<0.001), neutrophils (R=0.236, P<0.001), pDCs (R=0.236, P<0.001), CD8 T cells (R=0.231, P<0.001) and T cells (R=0.223, P<0.001) (*Figure 6A-6E*). Unexpectedly, our results also showed an inverse correlation between *C4BPA* expression

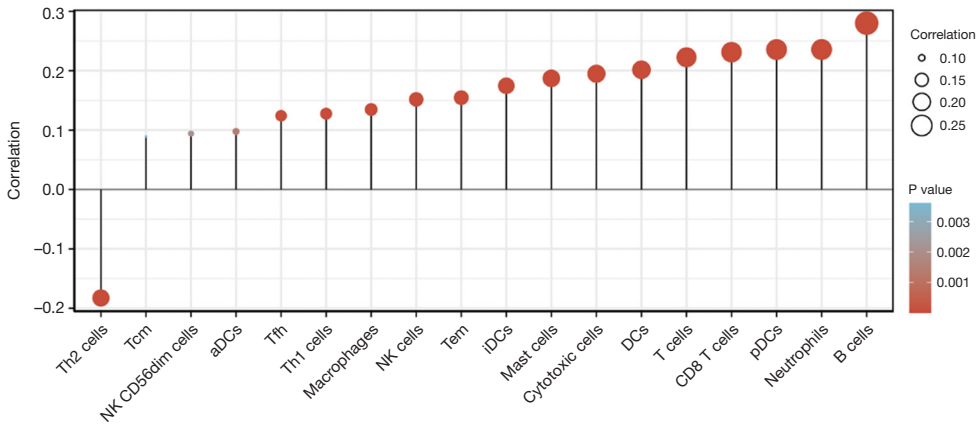

Figure 4 The forest plot showing the correlation between *C4BPA* expression level and 24 immune cells. aDCs, activated dendritic cells; iDCs, immature dendritic cells; DCs, dendritic cells; pDCs, plasmacytoid dendritic cells.

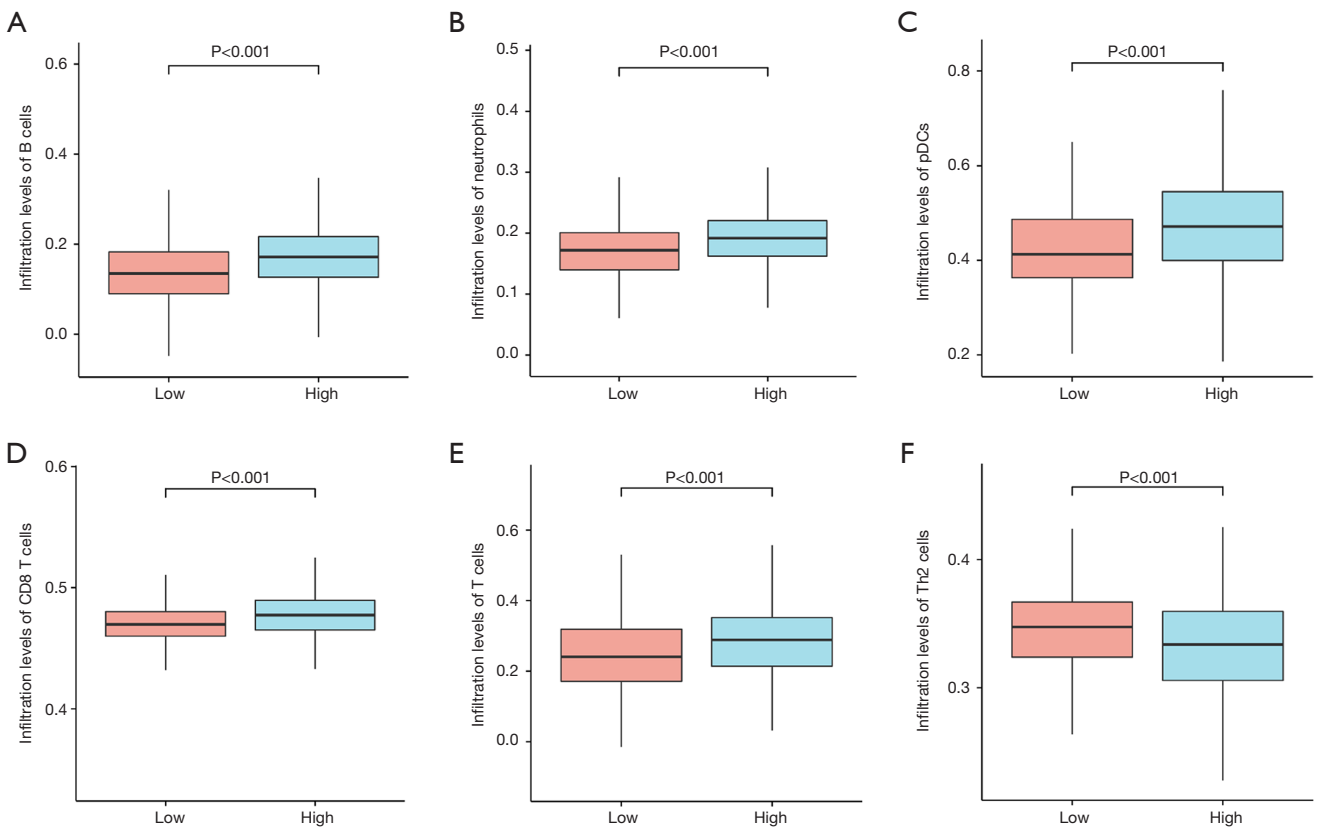

Figure 5 The expression level of *C4BPA* was related to the immune infiltration in the tumor microenvironment. (A-F) The Wilcoxon rank-sum test was used to analyze the difference of B cells, neutrophils, pDCs, CD8 T cells, T cells, Th2 cells infiltration between *C4BPA* high and low expression groups. pDCs, plasmacytoid dendritic cells.

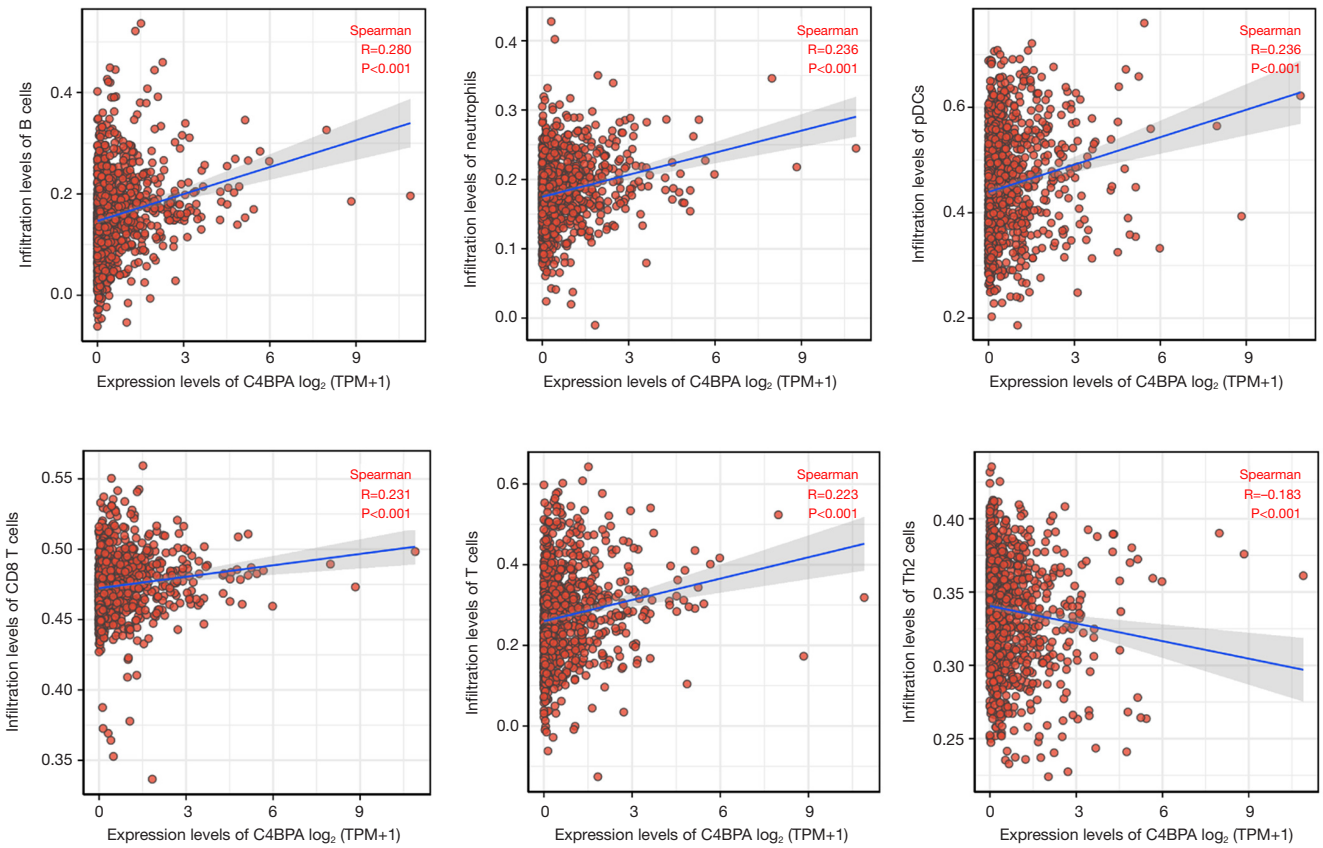

Figure 6 The expression level of *C4BPA* was related to the immune infiltration in the tumor microenvironment. (A-F) The correlation between *C4BPA* expression and B cells, neutrophils, pDCs, CD8 T cells, T cells, Th2 cells infiltration was analyzed by the Spearman correlation method. TPM, transcripts per million; pDCs, plasmacytoid dendritic cells.

and Th2 cells (R=−0.183, P<0.001) (*Figure 6F*).

PPI network analysis

The PPI network was established using PPI pairs derived from the STRING database. Utilizing a minimum required interaction score of >0.4, we identified 208 edges and 164 nodes within the PPI network (*Figure 7A*). In addition, based on the STRING library, we obtained molecules directly associated with *C4BPA* (*Figure 7B*): CD40, NFKBIA, RELA, NFKB1, C4BPB, and CFI.

Assessment of the prognostic value of C4BPA gene

The Kaplan-Meier analysis demonstrated that patients with low C4BPA expression had a significantly shorter progression-free interval (PFI) [hazard ratio (HR) =0.61; 95% confidence interval (CI): 0.43–0.85; P=0.003] and OS (HR =0.61; 95% CI: 0.44–0.85; P=0.004) compared to those with high *C4BPA* expression (*Figure 8A,8B*). The Cox univariate and multivariate analyses of PFI in BC patients are presented in *Table 1*. In the univariate analysis, several variables including T stage, N stage, M stage, pathologic stage, PR status, ER status, and *C4BPA* expression were found to have a significant association with PFI, with P values <0.01. Multivariate analysis revealed that M stage (P<0.001), pathologic stage (P=0.012), and *C4BPA* expression (P=0.003) were autonomous prognostic factors for PFI in BC patients.

Moreover, univariate Cox proportional hazards analyses showed that *C4BPA* expression (P=0.004), T stage (P=0.007), N stage (P<0.001), M stage (P<0.001) and pathologic stage (P<0.001) were independent prognostic factors for OS in BC patients. Multifactorial Cox regression further indicated

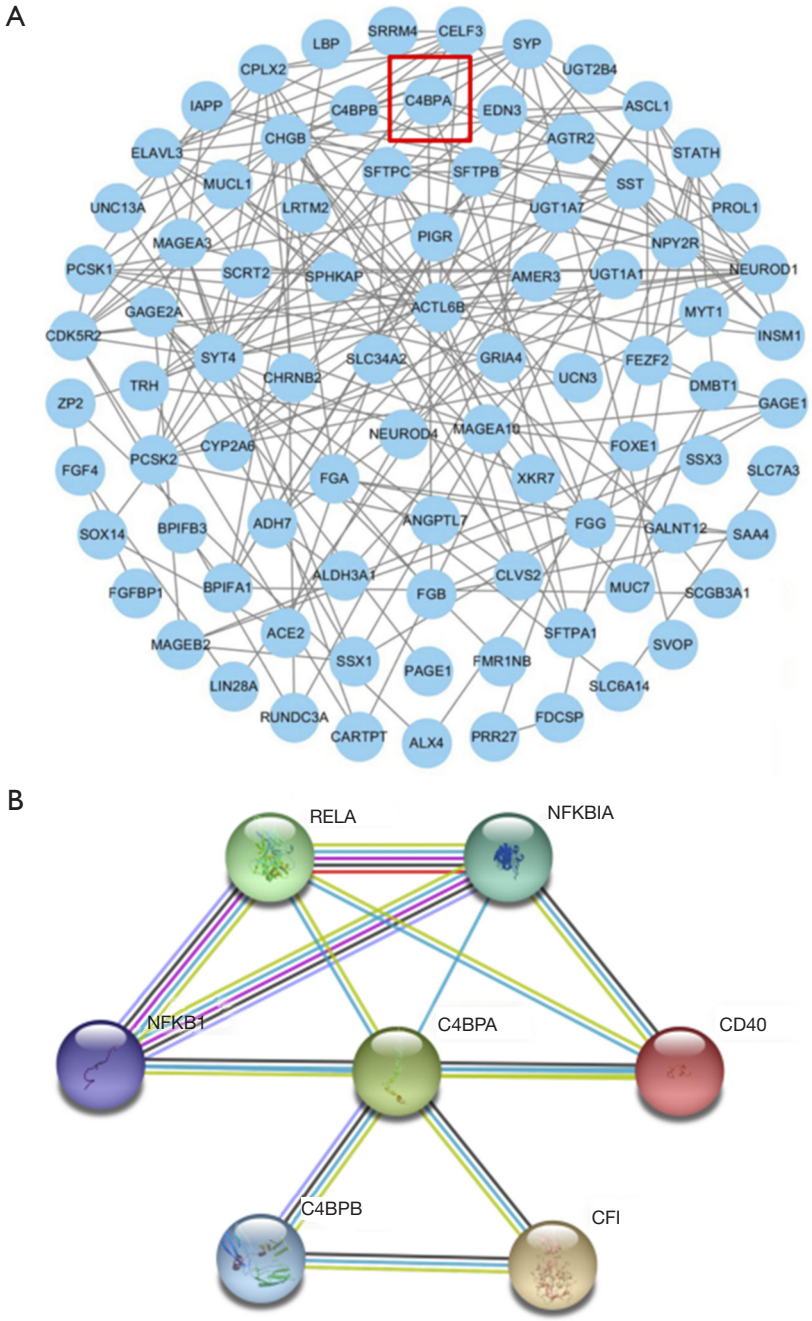

Figure 7 Visualization of PPI network analysis of DEGs in *C4BPA* high/low expression groups. (A) The PPI network of DEGs constructed by String and Cytoscape. (B) Relationship between C4BPA and other molecules. PPI, protein-protein interaction; DEGs, differentially expressed genes.

that *C4BPA* (P=0.013), M stage (P=0.002), pathologic stage (P=0.041) and ER status (P=0.004) were independent prognostic factors in OS (P<0.05) (*Table 2*).

In addition, the nomogram was constructed to evaluate the prognostic prediction model of *C4BPA*. The Concordance Index (C-index), which reflects the discrimination ability of the model, was used to assess the predictive accuracy of the nomograms. The C-indexes

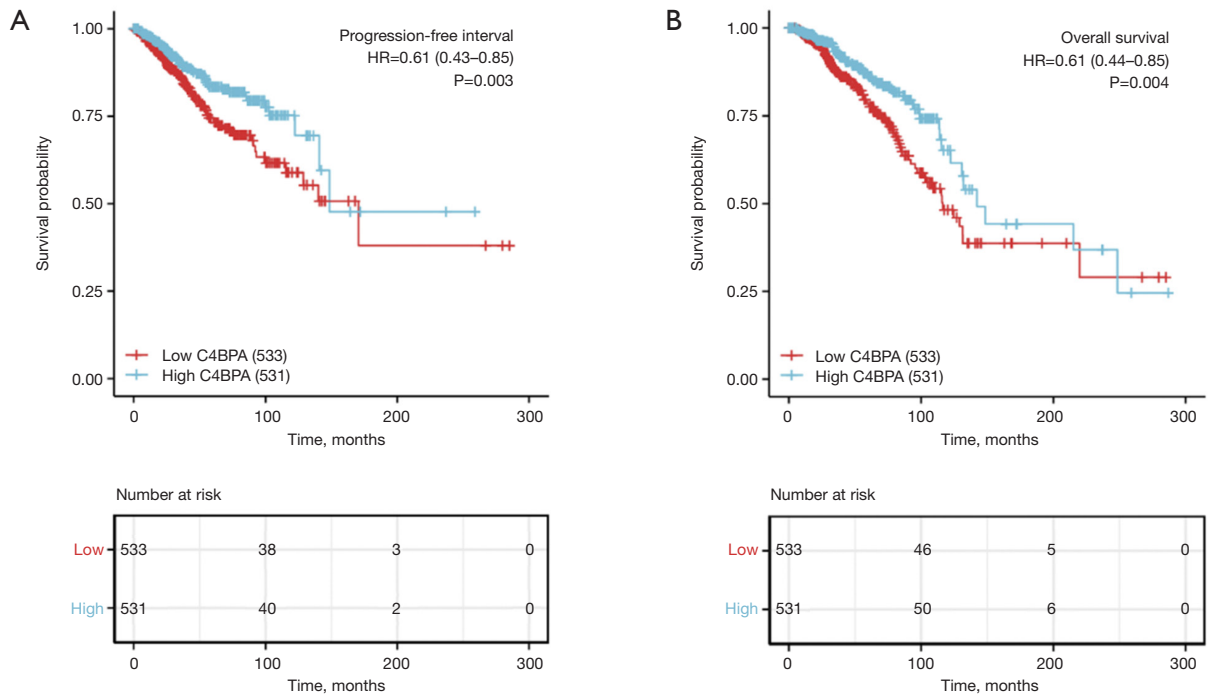

Figure 8 *C4BPA* expression and prognosis of BIC. (A,B) Kaplan-Meier method was used to evaluate the prognostic value of *C4BPA* in PFI and OS of BIC. HR, hazard ratio; BIC, breast invasive carcinoma; PFI, progression-free interval; OS, overall survival.

Table 1 Associations with PFI and clinicopathological characteristics in invasive breast cancer using Cox regression

Characteristics	Total, N	Univariate analysis		Multivariate analysis	
		HR (95% CI)	P value	HR (95% CI)	P value
T stage (T1&T2 *vs.* T3&T4)	1,061	0.472 (0.327–0.682)	<0.001	0.955 (0.554–1.644)	0.867
N stage (N1&N2&N3 *vs.* N0)	1,045	2.250 (1.559–3.245)	<0.001	1.273 (0.786–2.062)	0.326
M stage (M1 *vs.* M0)	909	8.288 (4.812–14.275)	<0.001	3.947 (1.969–7.913)	<0.001
Pathologic stage (stage III&stage IV *vs.* stage I&stage II)	1,041	2.962 (2.102–4.173)	<0.001	2.121 (1.176–3.826)	0.012
PR status (positive *vs.* negative)	1,011	0.567 (0.405–0.795)	<0.001	0.593 (0.342–1.026)	0.062
ER status (positive *vs.* negative)	1,014	0.599 (0.419–0.857)	0.005	0.656 (0.369–1.168)	0.152
HER2 status (positive *vs.* negative)	705	1.221 (0.707–2.109)	0.475	–	–
Race (White *vs.* Asian&Black or African American)	975	0.848 (0.572–1.256)	0.410	–	–
Histological type (infiltrating ductal carcinoma *vs.* infiltrating lobular carcinoma)	959	1.236 (0.772–1.979)	0.379	–	–
Anatomic neoplasm subdivisions (right *vs.* left)	1,064	0.850 (0.610–1.184)	0.337	–	–
TP53 status (Mut *vs.* WT)	955	1.258 (0.879–1.802)	0.210	–	–
PIK3CA status (Mut *vs.* WT)	955	0.838 (0.565–1.245)	0.382	–	–
C4BPA (high *vs.* low)	1,064	0.607 (0.434–0.848)	0.003	0.562 (0.382–0.827)	0.003

PFI, progression-free interval; HR, hazard ratio; CI, confidence interval; PR, progesterone receptor; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; Mut, mutation; WT, wild type.

Table 2 Associations with OS and clinicopathological characteristics in invasive breast cancer using Cox regression

Characteristics	Total, N	Univariate analysis		Multivariate analysis	
		HR (95% CI)	P value	HR (95% CI)	P value
T stage (T1&T2 *vs.* T3&T4)	1,061	0.598 (0.412–0.868)	0.007	0.586 (0.305–1.127)	0.109
N stage (N1&N2&N3 *vs.* N0)	1,045	2.145 (1.497–3.073)	<0.001	1.061 (0.576–1.957)	0.849
M stage (M1 *vs.* M0)	909	4.327 (2.508–7.465)	<0.001	4.294 (1.718–10.733)	0.002
Pathologic stage (stage III&stage IV *vs.* stage I&stage II)	1,041	2.519 (1.787–3.549)	<0.001	2.153 (1.034–4.483)	0.041
PR status (positive *vs.* negative)	1,011	0.762 (0.541–1.074)	0.120	–	–
ER status (positive *vs.* negative)	1,014	0.704 (0.487–1.017)	0.062	0.471 (0.281–0.787)	0.004
HER2 status (positive *vs.* negative)	705	1.611 (0.981–2.644)	0.059	1.294 (0.752–2.226)	0.353
Race (White *vs.* Asian&Black or African American)	975	0.880 (0.593–1.306)	0.526	–	–
Histological type (infiltrating ductal carcinoma *vs.* infiltrating lobular carcinoma)	959	1.162 (0.738–1.830)	0.516	–	–
Anatomic neoplasm subdivisions (right *vs.* left)	1,064	0.776 (0.559–1.077)	0.130	–	–
TP53 status (Mut *vs.* WT)	955	1.218 (0.858–1.730)	0.269	–	–
PIK3CA status (Mut *vs.* WT)	955	1.015 (0.696–1.479)	0.938	–	–
C4BPA (high *vs.* low)	1064	0.614 (0.441–0.854)	0.004	0.545 (0.337–0.882)	0.013

OS, overall survival; HR, hazard ratio; CI, confidence interval; PR, progesterone receptor; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; Mut, mutation; WT, wild type.

were 0.713 (0.684–0.741) and 0.712 (0.681–0.743) for PFI and OS, respectively. Both nomograms exhibited a high degree of concordance in the prediction value during validation (*Figure 9A*,*9B*). At the same time, we employed the calibration curve of the predictive model to validate the performance of Cox multi-factor model in predicting PFI and OS risks. The predictive model calibration curve exhibited the degree of concordance between the risk probabilities projected by the Cox multifactor model and the corresponding actual probabilities (*Figure 10A*,*10B*).

Correlation between C4BPA gene expression and clinicopathological characteristics in BC

We identified the potential clinical significance of *C4BPA* in BC. To achieve this goal, we separated patients into two groups (*C4BPA* low expression group and *C4BPA* high expression group) based on the relative expression levels of *C4BPA* in cancer/adjacent tissues, with the median value as the cut-off. The relationship between *C4BPA* expression and clinicopathological characteristics is analyzed and presented in *Table 3*. Low *C4BPA* expression was positively correlated with race (P<0.001), age (P<0.001), T stage (P=0.021), ER status (P<0.001), histological type (P<0.001), PR status (P=0.019) as well as PAM50 (P<0.001) (Figure S2A-S2G). However, the *C4BPA* was not significantly associated with the N stage, M stage, pathologic stage, PR status, human epidermal growth factor receptor 2 (HER2) status, anatomic neoplasm subdivisions, TP53 status, and PIK3CA status (all P>0.05).

The logistic regression method was also employed to demonstrate the association between the clinicopathological features of BC and the degree of *C4BPA* expression. *C4BPA* was found to be substantially connected with ER status (P=0.002) and histological type (P<0.001) (*Table 4*).

Prognostic analysis of C4BPA gene in various subgroups of BIC

To more accurately predict the prognostic factors for survival of *C4BPA* in BIC, we performed a prognostic analysis of various subgroups of BRCA in TCGA BRCA.

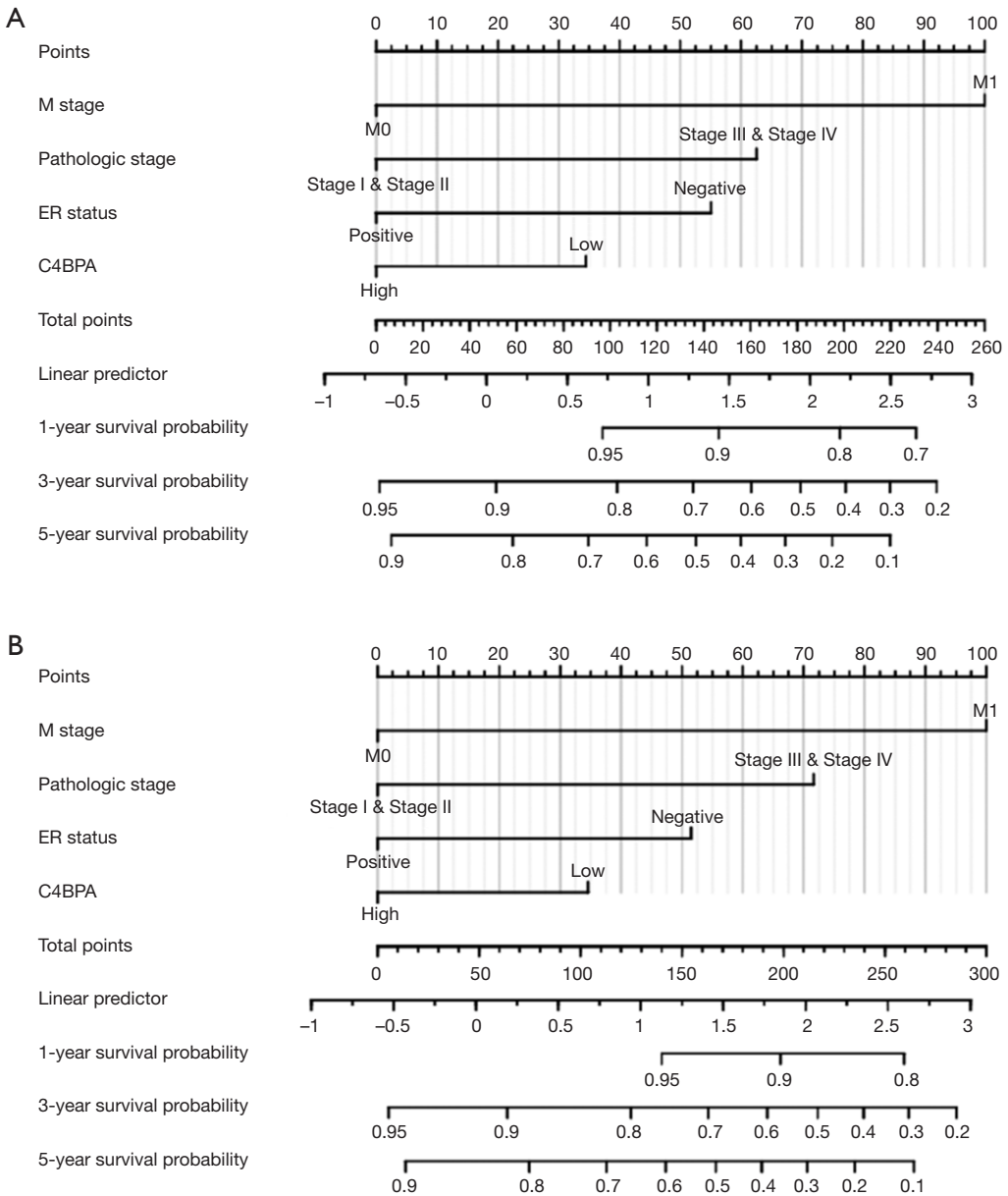

Figure 9 The prognosis prediction model of *C4BPA* was evaluated by calibration curve. (A,B) The prediction models for PFI and OS in BC patients evaluated using a calibration curve. ER, estrogen receptor; PFI, progression-free interval; OS, overall survival; BC, breast cancer.

Figure 11A depicts the remarkable prognostic value of *C4BPA* in predicting PFI in multiple subgroups of BRCA in TCGA BIC; *C4BPA* was found in ER-positive (HR =0.448; 95% CI: 0.288–0.697; P<0.001), PR-positive (HR =0.465; 95% CI: 0.287–0.753; P=0.002), HER2-negative (HR =0.471; 95% CI: 0.268–0.825; P=0.009) and Luminal A (HR =0.578; 95% CI: 0.351–0.955; P=0.032) subgroups of the PAM50. Similarly, the remarkable prognostic value of *C4BPA* for OS in various subgroups of BRCA in TCGA BIC is shown in *Figure 11B*; *C4BPA* was found in ER-positive (HR =0.449; 95% CI: 0.291–0.691; P<0.001), PR positive (HR =0.411; 95% CI: 0.259–0.653; P<0.001), and Luminal A (HR =0.587; 95% CI: 0.355–0.972; P=0.038) and Luminal B (HR =0.214; 95% CI: 0.051–0.899; P=0.035) subgroups of the PAM50.

To further evaluate the prognostic significance of *C4BPA*

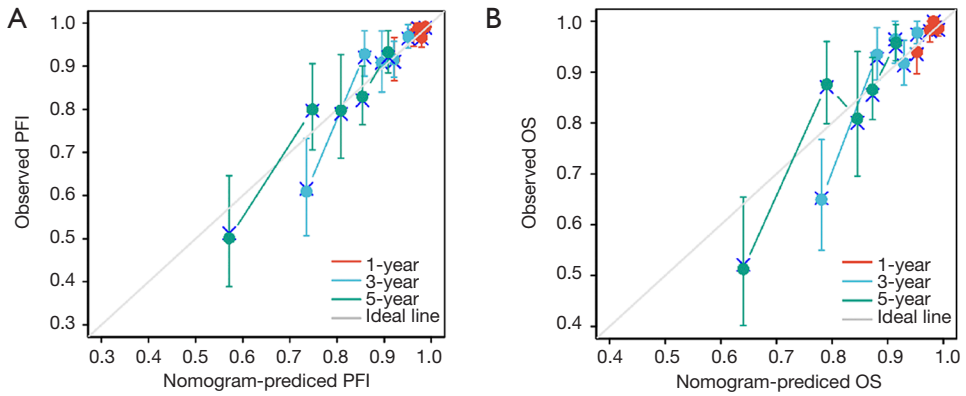

Figure 10 Calibration curve was used to verify the PFI and OS risk prediction efficiency of Cox multifactor model. (A,B) The PFI, and OS risk prediction effectiveness of the Cox multi-factor model verified by the Calibration curve. PFI, progression-free interval; OS, overall survival.

Table 3 Correlation between *C4BPA* expression and clinicopathological characteristics in breast cancer

Characteristics	Level	Low expression of *C4BPA*	High expression of *C4BPA*	P value
T stage, n (%)	T1	123 (23.1)	152 (28.6)	0.010
	T2	328 (61.5)	287 (53.9)	
	T3	58 (10.9)	79 (14.9)	
	T4	22 (4.1)	13 (2.4)	
N stage, n (%)	N0	247 (47.5)	260 (49.4)	0.626
	N1	183 (35.2)	166 (31.6)	
	N2	56 (10.8)	60 (11.4)	
	N3	34 (6.5)	40 (7.6)	
M stage, n (%)	M0	450 (97.4)	439 (98.2)	0.546
	M1	12 (2.6)	8 (1.8)	
Pathologic stage, n (%)	Stage I	84 (16.2)	96 (18.3)	0.481
	Stage II	309 (59.8)	297 (56.6)	
	Stage III	113 (21.9)	125 (23.8)	
	Stage IV	11 (2.1)	7 (1.3)	
PR status, n (%)	Negative	155 (31.2)	183 (35.5)	0.162
	Positive	342 (68.8)	332 (64.5)	
ER status, n (%)	Negative	96 (19.2)	141 (27.3)	0.003
	Positive	403 (80.8)	375 (72.7)	
HER2 status, n (%)	Negative	269 (74.7)	279 (80.9)	0.061
	Positive	91 (25.3)	66 (19.1)	

Table 3 (*continued*)

Table 3 (*continued*)

Characteristics	Level	Low expression of *C4BPA*	High expression of *C4BPA*	P value
PAM50, n (%)	Basal	78 (14.6)	112 (21.1)	<0.001
	Her2	44 (8.3)	38 (7.1)	
	LumA	247 (46.3)	304 (57.1)	
	LumB	161 (30.2)	41 (7.7)	
	Normal	3 (0.6)	37 (7.0)	
Histological type, n (%)	Infiltrating ductal carcinoma	402 (85.4)	355 (72.7)	<0.001
	Infiltrating lobular carcinoma	69 (14.6)	133 (27.3)	
Race, n (%)	Asian	39 (8.4)	21 (4.1)	<0.001
	Black or African American	65 (13.9)	114 (22.4)	
	White	363 (77.7)	374 (73.5)	
Anatomic neoplasm subdivisions, n (%)	Left	267 (50.1)	286 (53.8)	0.256
	Right	266 (49.9)	246 (46.2)	
TP53 status, n (%)	Mut	173 (35.5)	162 (34.5)	0.802
	WT	314 (64.5)	307 (65.5)	
PIK3CA status, n (%)	Mut	151 (31.0)	163 (34.8)	0.244
	WT	336 (69.0)	306 (65.2)	
Age (years), mean (SD)	–	59.61 (13.87)	57.10 (12.36)	0.002

PR, progesterone receptor; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; Mut, mutation; WT, wild type; SD, standard deviation.

Table 4 *C4BPA* expression associated with clinicopathologic characteristics (logistic regression)

Characteristics	N	Odds ratio (95% CI)	P value
T stage (T1&T2 *vs.* T3&T4)	1,062	1.18 (0.85–1.64)	0.318
N stage (N1&N2&N3 *vs.* N0)	1,046	0.93 (0.73–1.18)	0.532
M stage (M1 *vs.* M0)	909	0.68 (0.27–1.67)	0.409
Pathologic stage (stage III&stage IV *vs.* stage I&stage II)	1,042	1.06 (0.80–1.41)	0.664
PR status (positive *vs.* negative)	1,012	0.82 (0.63–1.07)	0.143
ER status (positive *vs.* negative)	1,015	0.63 (0.47–0.85)	0.002
HER2 status (positive *vs.* negative)	705	0.70 (0.49–1.00)	0.050
Histological type (infiltrating ductal carcinoma *vs.* infiltrating lobular carcinoma)	959	2.18 (1.58–3.03)	<0.001
TP53 status (Mut *vs.* WT)	956	0.96 (0.73–1.25)	0.750
PIK3CA status (Mut *vs.* WT)	956	1.19 (0.90–1.55)	0.217

CI, confidence interval; PR, progesterone receptor; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; Mut, mutation; WT, wild type.

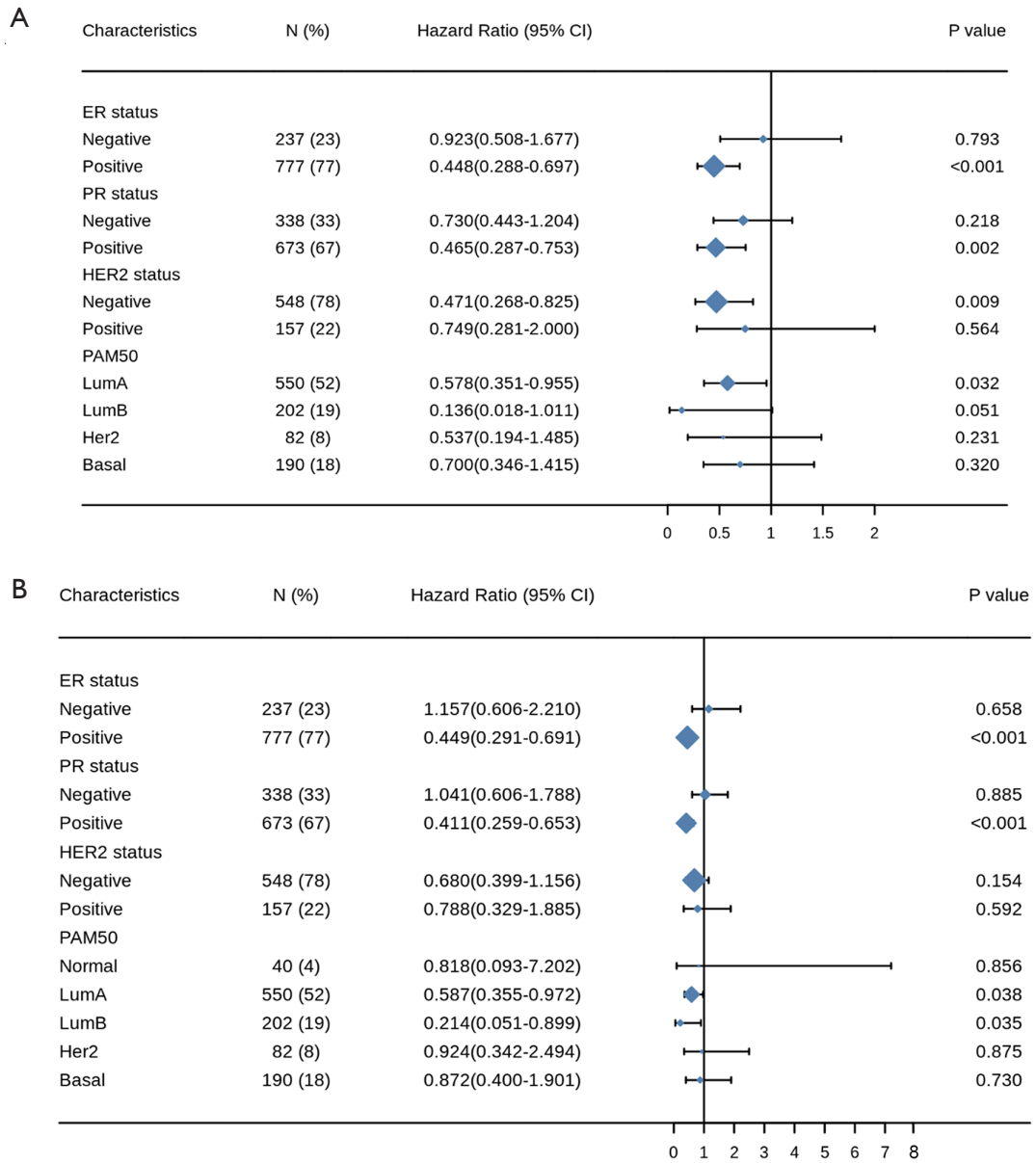

Figure 11 Forest map visualization of *C4BPA*'s prognostic value in PFI and OS in TCGA subsets of BIC BRCA. (A,B) Forest map visualization of the prognostic value of *C4BPA* in PFI and OS in TCGA subsets of BIC BRCA. P<0.05 was statistically significant. The x-axis is representing hazard ratio and its 95% CI. CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; PFI, progression-free interval; OS, overall survival; TCGA, The Cancer Genome Atlas; BIC/BRCA, breast invasive carcinoma.

for PFI and OS in each subgroup of BIC, we utilized the survminer software to construct Kaplan-Meier plots. The analysis revealed that BC patients with low expression of *C4BPA* had a shorter PFI in the ER-positive (HR =0.45; 95% CI: 0.29–0.70; P<0.001, *Figure 12A*), PR positive (HR =0.46; 95% CI: 0.29–0.75; P=0.002, *Figure 12B*), HER2-negative (HR =0.47; 95% CI: 0.27–0.83; P=0.009, *Figure 12C*), and Luminal A in PAM50 (HR =0.58; 95% CI: 0.35–0.95; P=0.032, *Figure 12D*) compared to the ones with high *C4BPA* expression. In addition, they had a worse OS in ER-positive (HR =0.45; 95% CI: 0.29–0.69; P<0.001, *Figure 13A*), PR positive (HR =0.61; 95% CI: 0.44–0.86;

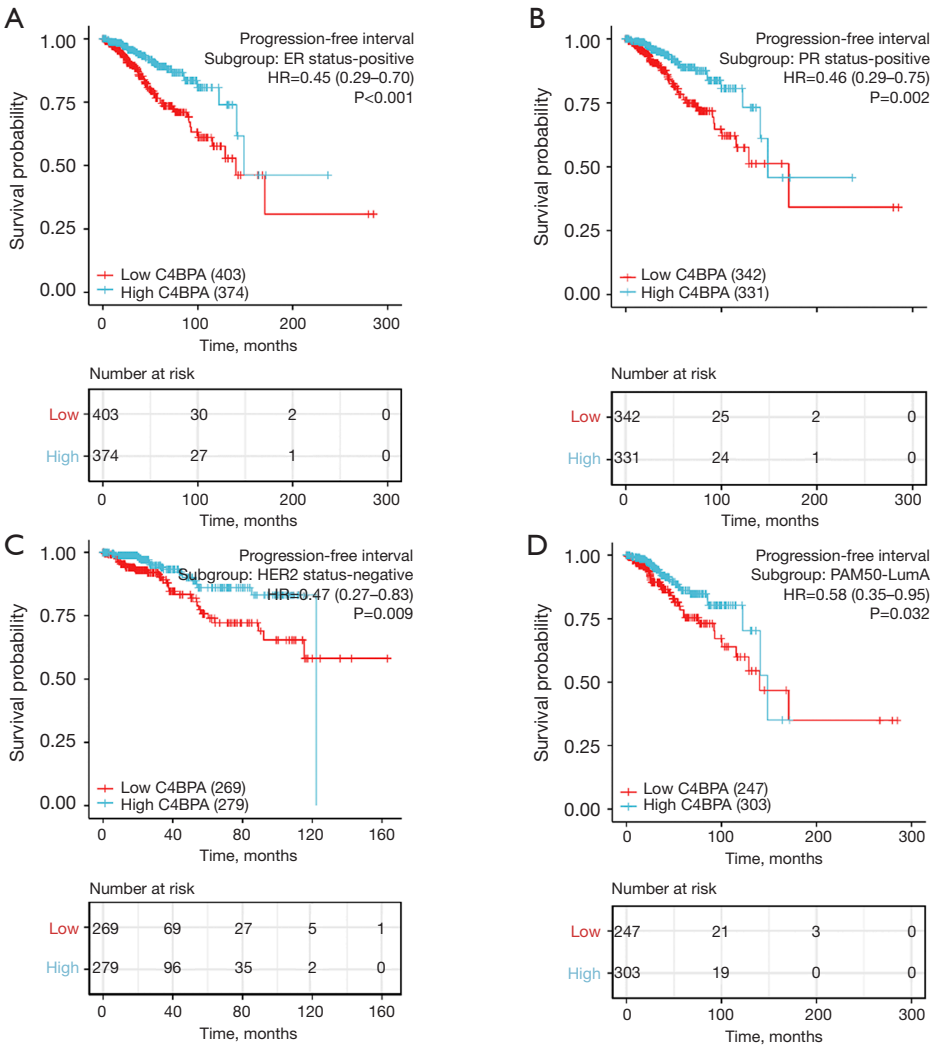

Figure 12 Prognostic value of *C4BPA* in PFI in BIC subgroups. (A-D) Kaplan-Meier was used to evaluate the prognostic value of *C4BPA* for PFI in ER-positive, PR-positive, HER2-negative and Luminal A of BIC patients. ER, estrogen receptor; HR, hazard ratio; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; PFI, progression-free interval; BIC, breast invasive carcinoma.

P=0.005, *Figure 13B*), Luminal A of PAM50 (HR =0.59; 95% CI: 0.36–0.97; P=0.038, *Figure 13C*), Luminal B of PAM50 (HR =0.21; 95% CI: 0.05–0.90; P=0.035, *Figure 13D*).

Discussion

C4BPA is thought to be a novel cancer biomarker. Its function has already been examined in pancreatic cancer, lung cancer, ovarian cancer, and so on. Yet, it is still unclear whether *C4BPA* could be used for effective early diagnosis and predicting prognosis in cancer patients. Herein, we explored the expression and prognostic significance of *C4BPA* in BC.

In this study, 335 DEGs (235 up-regulated and 100 down-regulated) were screened using the TCGA database and subjected to a co-differential gene analysis. The functional annotation and enrichment analyses of these DEGs revealed that most DEGs mainly affected the positive regulation of signal release and cellular secretion in the BPs and were mainly located in the intracellular part of the CCs. In addition, most DEGs primarily affected receptor ligand activity, syntaxin-1 binding activity, and hormone activity in the MFs. KEGG enrichment analysis revealed

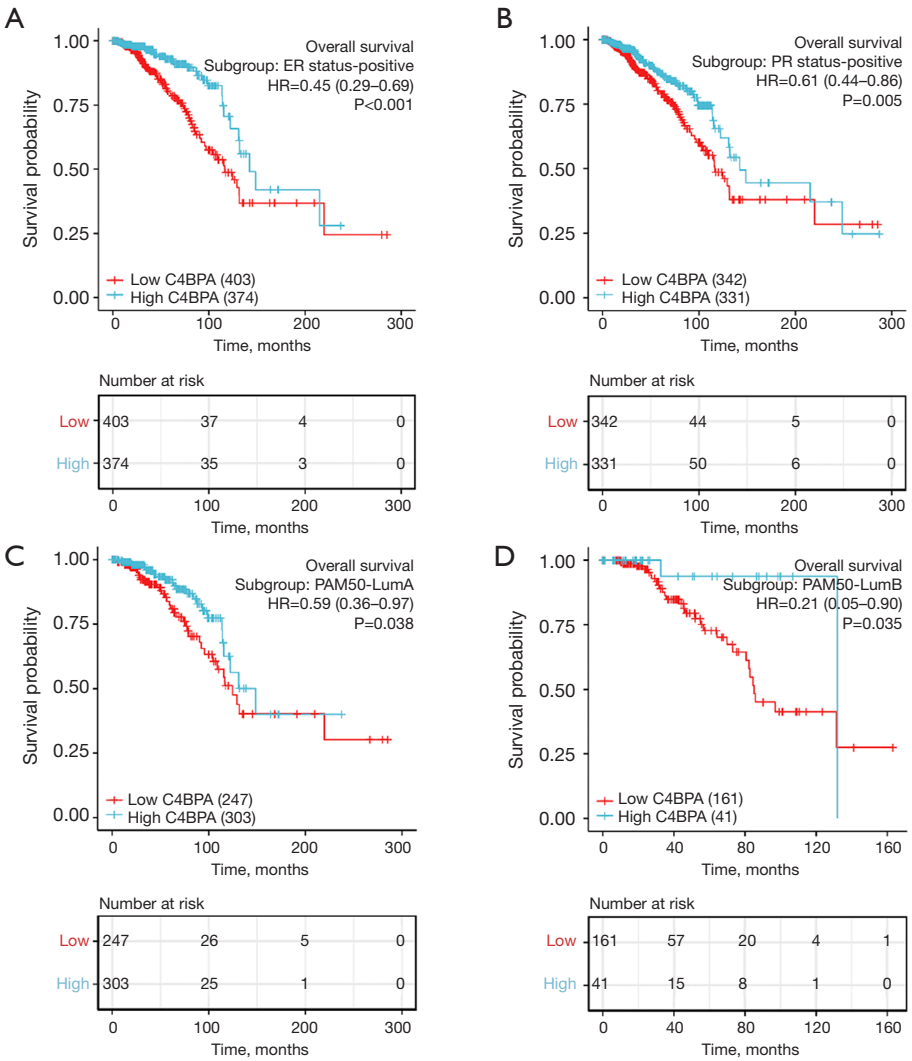

Figure 13 Prognostic value of *C4BPA* in OS in BIC subgroups. (A-D) Kaplan-Meier was used to evaluate the prognostic value of *C4BPA* for OS in ER-positive, PR-positive, Luminal A, and Luminal B of BIC patients. ER, estrogen receptor; HR, hazard ratio; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; OS, overall survival; BIC, breast invasive carcinoma.

that a significant proportion of the DEGs were enriched in several pathways including drug metabolism-cytochrome P450, chemotaxis, retinol metabolism, and complement and coagulation cascades. Cytochrome P450 genes can induce various solid tumors, such as colon cancer, by activating PI3K/Akt (22-24). Recent studies have identified a possible relationship between polymorphic variants of certain carcinogen-metabolizing enzyme genes and tumor susceptibility (25). Previous studies have also shown that polymorphisms in cytochrome P450 may increase the risk of BC (26,27).

We performed a GSEA enrichment analysis on the DEGs identified in the *C4BPA* high/low expression group to learn more about the function of the *C4BPA* protein in BC. We also discovered that there was a notable correlation between low expression of *C4BPA* with the acetylcholine neurotransmitter release cycle, as well as BC and BC metastasis signaling pathways. These results suggest that *C4BPA* may represent a promising candidate for prognostic stratification and therapeutic intervention in BC.

The primary goal of this research was to determine how *C4BPA* expression and various immune infiltration levels in BC relate to one another. According to our findings, *C4BPA* was negatively connected with Th2 cells

and favorably correlated with immune cell types like B cells, neutrophils, pDCs, CD8 T cells, and T cells. These correlations suggest that *C4BPA* may facilitate the function of B cells, neutrophils, pDCs, CD8 T cells, and T cells, thereby impeding the function of Th2 cells and exerting its inhibitory effect on BC. CD8 T cells are primarily cytotoxic T cells that can directly eliminate tumor cells and inhibit tumor growth through cell lysis and apoptosis, while Th2 cells play a dominant role in humoral immunity and promote tumor growth. In addition, research has shown that various malignant tumors, such as non-small cell lung cancer (28), BC (29), cervical cancer (30), bladder cancer (31), and lymphoma (32) have been found to be closely related to the drift from Th1 to Th2 cells; this drift becomes more pronounced as the malignancy of the tumor increases. Thus, low expression of *C4BPA* may promote the development of BC by inhibiting the function of B cells, neutrophils, pDCs, CD8 T cells, T cells, thereby promoting the function of Th2 cells.

In this study, we analyzed the interrelationships of *C4BPA* with other proteins by constructing a PPI network. We found a strong association between *C4BPA* and CD40, while *C4BPA* was part of the complement regulator C4BP. In particular, three NF-kB pathway members (RElA, NF-Kb1, and NF-KBIA) were estimated to be represented close to *C4BPA* at inter-action nodes. In addition, previous research using immunoprecipitation (IP) assays confirmed the connection between *C4BPA* and members of the NF-kB family, highlighting the critical role of *C4BPA* in the regulation of NF-kB-dependent apoptosis (33).

In this work, we also explored the relationship between clinicopathological characteristics, survival of BIC, and *C4BPA* expression in BIC. Firstly, we used bioinformatics methods, statistical methods, and relevant databases to evaluate *C4BPA* expression levels in BIC tissues and normal tissues. Our analysis revealed that *C4BPA* expression was substantially lower in BIC tissues compared to normal tissues. We also compared the expression levels of *C4BPA* in various human cancers and observed a downregulation of its expression in most cancers than in normal tissues, including BIC, which is consistent with the previous finding that *C4BPA* was of low expression in BC. Then, we used ROC curves to verify that *C4BPA* could be an ideal biomarker for differentiating BC from non-tumorous tissues. Next, by analyzing the correlation between *C4BPA* expression and the clinicopathological characteristics of BIC, we found that *C4BPA* expression was associated with T stage, ER status, PAM50, histological type, age, and race. The size and histological type of the malignant tumor are indicators of prognosis, which can be used to determine the recurrence and metastasis of the patient on a macroscopic level. Therefore, we further analyzed the relationship between the expression of *C4BPA* and PFI and OS by Kaplan-Meier survival curves. The survival curve demonstrated that low expression of *C4BPA* was associated with a shorter PFI and OS and was more likely to lead to tumor recurrence or distant metastases and a worse prognosis. Therefore, we set PFI and OS as our study endpoints. The Cox univariate results proved that the length of PFI was connected with the expression of *C4BPA*, T-stage, N-stage, M-stage, pathological stage, PR status, and ER status; the length of OS was associated with the expression of C4BPA, T-stage, N-stage, M-stage, and pathological stage.

Furthermore, the Cox multifactorial results demonstrated that the expression of C4BPA, M-stage, and pathological stage were associated with PFI, while the expression of C4BPA, M-stage, pathological stage, and ER status were associated with OS. Therefore, this suggests that *C4BPA* could be a unique prognostic factor influencing the long-term survival of patients with BIC. T-stage, N-stage, M-stage, TNM-stage, and ER status are recognized as prognostic factors. The correlation between the differential expression of *C4BPA* and PFI and OS suggested that *C4BPA* might be a new target for improving BIC prognosis. In addition, we evaluated the prognostic prediction model of *C4BPA* by constructing a nomogram that transformed the complex Cox multi-factor regression model into a visual graph. It made the results of the predicted model concrete and facilitated the assessment of patients. Thus, the nomogram improved the prognostic model for BC in general. At the same time, we used calibration curves to validate the PFI and OS risk prediction efficacy of the Cox multi-factor model and obtained a highly accurate prognostic model for BC.

BC is a heterogeneous tumor, and different subtypes of BC may have different gene expressions and biological behavior (34). In this study, the prognosis of BRCA subgroups in TCGA BIC was analyzed. In HER2-negative, Luminal A BC patients with low *C4BPA* expression had a shorter PFI compared to those with high *C4BPA* expression. In addition, in Luminal A and Luminal B BC patients with low *C4BPA* expression had worse OS compared to those with high *C4BPA* expression. Therefore, we can conclude that Luminal A BC patients with low *C4BPA* expression may have a worse prognosis compared to other BC subtypes.

This study has a few limitations. Experimental validation

was not included in this study; we plan to further examine the expression of C4BPA in BC by quantitative polymerase chain reaction (qPCR) and immunohistochemistry and investigate the biological function mechanism of *C4BPA* in BC by employing cellular and animal experiments.

Conclusions

In conclusion, this study concluded that the expression of *C4BPA* was lower in BC tissues compared to normal breast tissues, which suggested that *C4BPA* has potential as a reliable biomarker for differentiating BC from non-tumor tissue. In addition, low expression of *C4BPA* may be linked to poor prognosis in BC, and it may be a promising prognostic biomarker in BC. Also, Luminal A BC patients with low *C4BPA* expression may have worse PFI and OS compared to patients with other BC subtypes.

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Footnote

Reporting Checklist: The authors have completed the TRIPOD reporting checklist. Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1215/rc

Peer Review File: Available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1215/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-1215/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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