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# Comprehensive bioinformatics analysis of CYB561 expression in breast cancer: Link between prognosis and immune infiltration

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**Abstract: Background:** Cytochrome b561 (CYB561) plays a critical role in neuroendocrine function, cardiovascular regulation, and tumor growth; however, the prognostic value of CYB561 in patients with breast cancer and the relationship between CYB561 expression and immune infiltration in breast cancer remain unclear. **Methods:** The mRNA expression and clinical data of patients with breast cancer were obtained from The Cancer Genome Atlas database. Functional enrichment analysis was used to explore underlying biological functions associated with CYB561. The methylation status of CYB561 was analyzed using the MethSurv database. The enrichment score of immune cell infiltration for CYB561 in breast cancer was calculated using single-sample gene set enrichment analysis. The prognostic value of CYB561 was evaluated using Kaplan-Meier method and Cox regression analysis. Based on the results of the multivariate Cox analysis, a nomogram was constructed to predict the effect of CYB561 expression on overall survival (OS). **Results:** The results showed that CYB561 was highly expressed in breast cancer tissues. Hypomethylation of CYB561 is associated with an unfavorable prognosis. In multivariate Cox regression analysis, CYB561 was an independent prognostic factor for OS. Functional enrichment analysis indicated that estrogen signaling pathway, inflammatory response, KRAS signaling pathway, epithelial-mesenchymal transition, leukocyte migration, and regulation of lymphocyte activation were strongly enriched in the low CYB561 expression group. Additionally, CYB561 expression was negatively correlated with immune infiltration of B cells, plasmacytoid dendritic cells, dendritic cells, and neutrophils. **Conclusion:** CYB561 may serve as a potential biomarker for breast cancer diagnosis and prognosis.

## Introduction

According to the Global Burden of Cancer Report, breast cancer (BRCA) surpassed lung cancer as the most common cancer worldwide by 2020, with an estimated 2.3 million new cases and 685,000 deaths (Sung *et al.*, 2021). Although treatment strategies for BRCA are well-developed, the tumor-related mortality rate remains relatively high. Owing to the limitations of individualized tumor therapy, the

development of new methods to screen high-risk groups and provide accurate prognoses has become an urgent need for BRCA patients.

Cytochrome b561 (CYB561) is a protein family composed of six transmembrane helix proteins, and their structural characteristics and sequence are conserved at the protein level (Bashtovyy *et al.*, 2003). CYB561 is a prototype of the diheme cytochrome family, which is widely distributed in plant and animal species (Tsubaki *et al.*, 2005). Ascorbate (ASC) is the main electron donor for enzymatic reactions in the redox processes of plants and animals (Foyer and Noctor, 2011). For the maintenance of normal neuroendocrine function, CYB561 supports the regeneration of ASC, which is an indispensable process for dopamine β-hydroxylation (Berczi *et al.*, 2005; van den

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Berg *et al.*, 2018). CYB561 is also involved in tumor suppression and cooperates with ASC to inhibit lung cancer cell growth (Ohtani *et al.*, 2007). In a meta-analysis of single-gene prognostic biomarkers for ovarian cancer, low CYB561 expression levels indicated a poor prognosis (Willis *et al.*, 2016). Additionally, an alternate promoter of CYB561 was associated with clinical outcomes in patients with endometrial cancer, and decreased CYB561 expression was predictive of inferior overall survival (OS) (Sun *et al.*, 2022). In contrast, CYB561 was observed to be highly expressed in metastatic prostate cancer cells and to promote the proliferation and metastatic potential of prostate cancer cells (Olarte and Bagamasbad, 2020). However, the prognostic value of CYB561 expression and its correlation with immune infiltration in BRCA remain unclear.

In the present study, our aim was to investigate the relationship linking CYB561 expression and patient outcomes, pathological characteristics, and infiltration abundances of immune cells using bioinformatics, which may aid in designing individualized therapeutic strategies.

## Materials and Methods

### Data collection
The RNAseq data with clinical information of BRCA patients were collected from The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/), and the Genotype Tissue Expression Project (GTEx) databases (https://commonfund.nih.gov/gtex). Subsequently, the fragments per kilobase of transcript per million fragments mapped (FPKM) data (level 3) were converted to transcripts per million (TPM) reads. To verify the prognostic value of CYB561 expression in BRCA patients, the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) dataset was also obtained from the cBioPortal database (http://www.cbioportal.org/). The mRNA expression data of various types of cancer were collected from the UCSC Xena (https://xenabrowser.net/datapages/) and GTEx databases.

### Differentially expressed gene (DEG) analysis
All BRCA patients in the TCGA cohort were divided into two groups (high *vs.* low expression) based on the median CYB561 expression level. The DEGs between the two groups were identified using the R package DESeq2 with an adjusted *p*-value < 0.05 and |log2-fold-change (FC)| > 1.5 as the threshold (Love *et al.*, 2014). Spearman's correlation analysis was applied to assess the correlations between the CYB561 expression and the top 10 DEGs in the two groups.

### Functional enrichment analysis
Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were applied to calculate the enrichment terms of the DEGs by using the R package GO plot (version 1.0.2), based on $p < 0.05$, |log2FC| > 1. We used the R package ClusterProfiler for Gene set enrichment analysis (GSEA) to identify potential CYB561-related

functions and pathways (Yu *et al.*, 2012). Only enrichment in function or pathway with an adjusted *p*-value < 0.05 and a false discovery rate (FDR) < 0.25 were considered statistically significant.

### DNA methylation analysis
The methylation status of the CYB561 promoter in BRCA was identified using the UALCAN database (http://ualcan.path.uab.edu) (Chandrashekar *et al.*, 2017). Correlations between the expression of CYB561 and DNA methyltransferases were calculated using Spearman's correlation analysis. The heatmap of DNA methylation sites and the prognostic value of CYB561-methylation levels were obtained from a web-based MethSurv methylation analysis tool (https://biit.cs.ut.ee/methsurv/) (Modhukur *et al.*, 2017).

### Immune cell infiltration analysis
The relative infiltration of immune cells in the BRCA microenvironment was analyzed using single-sample gene set enrichment analysis (ssGSEA), which was implemented using the GSVA package. Spearman's correlation analysis was utilized to explore the correlation between various immune cell subsets and CYB561 expression. Next, the Wilcoxon rank sum test was used to compare immune cell infiltration between CYB561 high and low groups, and a *p*-value < 0.05 was set as the threshold. Additionally, the correlations between CYB561 expression and tumor-related immune checkpoint genes were evaluated using the R package IOBR (Zeng *et al.*, 2021).

### Survival analysis
The BRCA patients were divided into high and low CYB561 expression groups based on the median value. Overall survival (OS) and disease-specific survival (DSS) were calculated using the Kaplan–Meier method with the log-rank test. Subsequently, we used univariate and multivariate Cox regression analysis to evaluate the effect of CYB561 expression on OS and DSS in the context of other clinicopathological variables.

### Nomogram construction and validation
The results of the multivariate Cox analysis were used to construct a nomogram that predicts the OS probability. The bootstrap validation method was used to calculate the consistency index (C-index) to estimate the bias-corrected or overfitting-corrected prediction accuracy of the model. The nomogram and calibration plots were generated with the package RMS of R software. The prediction performance of the model was also evaluated by using R package timeROC through time-dependent receiver operating characteristic (ROC) curves.

### Statistical analysis
Statistical analysis was performed using code written in R programming language (version 3.6.3) (https://www.r-project.org/). The Wilcoxon rank sum test and paired sample t-test were used to evaluate CYB561 expression in unpaired and paired samples, respectively. Wilcoxon rank-sum test and logistic regression were applied to calculate the

associations between CYB561 expression and clinical variables. In all tests, *p*-value < 0.05 was considered statistically significant.

## Results

### Patient characteristics

In our study, a total of 1065 BRCA cases with RNA-sequencing data and matched clinical information were included. Among them, 110 cases had paired tumor and adjacent normal tissue samples. The clinicopathological characteristics of the cases are shown in Suppl. Table S1.

### Overexpression of CYB561 in BRCA

We first compared the expression levels of CYB561 in 33 tumor tissues and corresponding normal tissues. As shown in Fig. 1A, the expression of CYB561 was significantly high in most types of cancers, such as bladder urothelial carcinoma, cholangiocarcinoma, and colon adenocarcinoma (Fig. 1A). Next, we evaluated the expression of CYB561 in BRCA samples. The results showed that CYB561 was highly expressed in BRCA compared to normal breast tissues in both unpaired and paired tests ($p < 0.001$; Figs. 1B and 1C). Additionally, the ROC curve showed that CYB561 expression had great diagnostic accuracy to distinguish BRCA tissues from normal tissues, with an area under the curve (AUC) of 0.919 (95% confidence interval [CI] = 0.902–0.935) (Fig. 1D).

### Association between CYB561 expression and clinicopathologic features

To clarify the significance of CYB561 expression, we then evaluated the relationship between CYB561 expression and clinicopathological features of the patients. As shown in Table 1 and Figs. 2A–2I, elevated expression of CYB561 was significantly correlated with histological type ($p < 0.001$), estrogen receptor (ER) status ($p < 0.001$), progesterone receptor (PR) status ($p = 0.012$), human epidermal growth factor receptor-2 (HER-2) status ($p < 0.001$), and PAM50 ($p < 0.001$). Moreover, the results of a univariate logistic regression analysis revealed that overexpression of CYB561 was remarkably associated with age (odds ratio [OR] = 1.362, 95% CI = 1.069–1.736, $p = 0.013$), ER status (OR = 2.188, 95% CI = 1.622–2.968, $p < 0.001$), PR status (OR = 1.420, 95% CI = 1.093–1.848, $p = 0.009$), histological type (OR = 0.461, 95% CI = 0.333–0.635, $p < 0.001$), and menopausal status (OR = 1.644, 95% CI = 1.235–2.195, $p < 0.001$) (Table 2).

### Identification of DEGs in BRCA

We used the DEseq2 package to identify DEGs between CYB561 high- and low-expression groups and identified a total of 267 DEGs, of which 69 were upregulated and 198 were downregulated (adjusted $p < 0.05$, |log2FC | > 1.5; Fig. 3A). Fig. 3B showed correlations between CYB561 expression and the top 10 DEGs, which included CSN2, LALBA, CPLX2, FTHL17, CHGA, MUC2, CARTPT, XAGE2, SYT4, and NCAN.
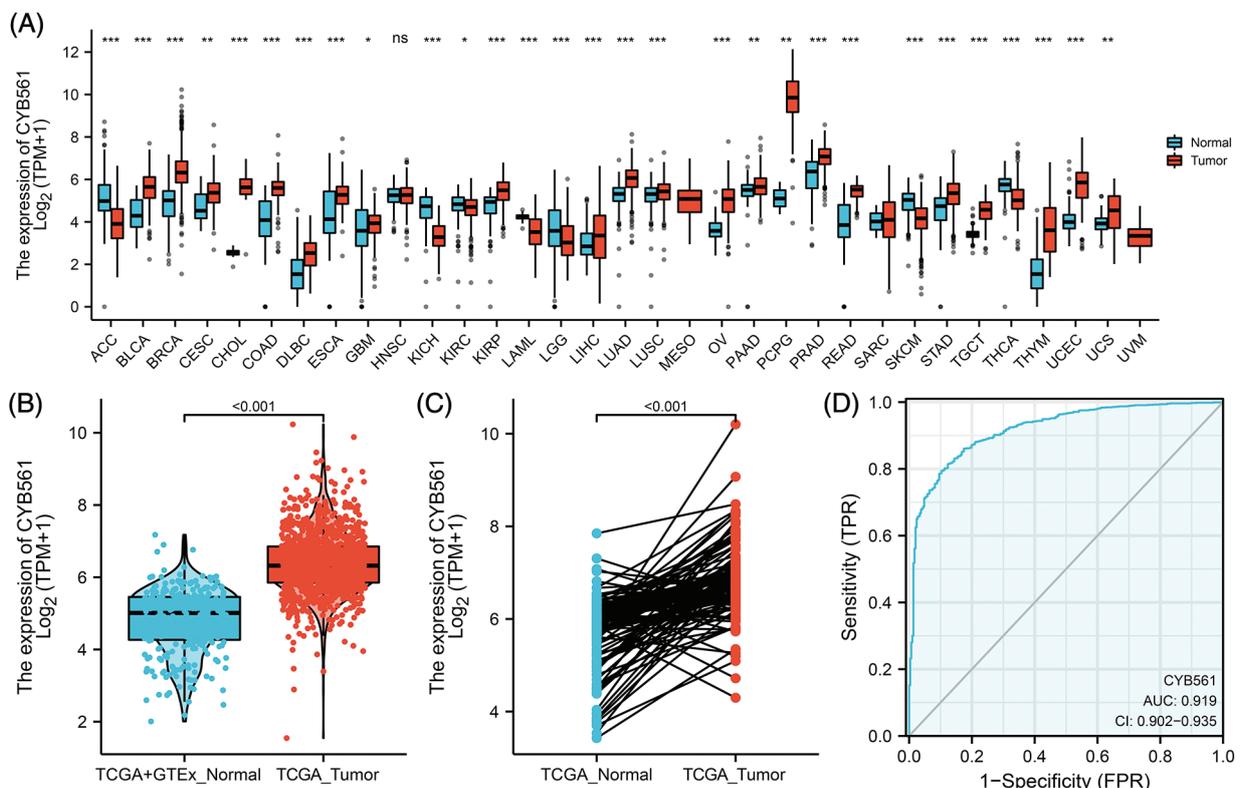


**FIGURE 1.** Expression of CYB561 in BRCA and normal tissues. (A) Expression of CYB561 in 33 types of cancer and normal tissues. (B) Expression of CYB561 in BRCA and normal tissues. (C) Expression of CYB561 in BRCA and matched normal tissues. (D) ROC curve shows that CYB561 has good discrimination ability between cancer and normal tissues. BRCA, breast cancer; TCGA, The Cancer Genome Atlas; GTEx, Genotype Tissue Expression Project; ROC, receiver operating characteristic. *$p < 0.05$, ** $p < 0.01$, and ***$p < 0.001$.

## TABLE 1

### Clinicopathological characteristics of BRCA patients

| Characteristic | Low expression of CYB561 | High expression of CYB561 | p value |
|---|---|---|---|
| n | 532 | 533 | |
| Age, median (IQR) | 56 (48, 65.25) | 59 (50, 69) | **0.003** |
| Age, n (%) | | | **0.015** |
| ≤60 | 314 (29.5%) | 274 (25.7%) | |
| >60 | 218 (20.5%) | 259 (24.3%) | |
| T stage, n (%) | | | 0.060 |
| T1 | 132 (12.4%) | 143 (13.5%) | |
| T2 | 310 (29.2%) | 305 (28.7%) | |
| T3 | 77 (7.3%) | 60 (5.6%) | |
| T4 | 11 (1%) | 24 (2.3%) | |
| N stage, n (%) | | | 0.902 |
| N0 | 259 (24.8%) | 248 (23.7%) | |
| N1 | 170 (16.3%) | 179 (17.1%) | |
| N2 | 60 (5.7%) | 56 (5.4%) | |
| N3 | 37 (3.5%) | 37 (3.5%) | |
| M stage, n (%) | | | 0.833 |
| M0 | 444 (48.8%) | 445 (49%) | |
| M1 | 9 (1%) | 11 (1.2%) | |
| Pathologic stage, n (%) | | | 0.641 |
| Stage I | 84 (8.1%) | 96 (9.2%) | |
| Stage II | 313 (30%) | 293 (28.1%) | |
| Stage III | 118 (11.3%) | 120 (11.5%) | |
| Stage IV | 8 (0.8%) | 10 (1%) | |
| Race, n (%) | | | 0.345 |
| Asian | 26 (2.7%) | 34 (3.5%) | |
| Black or African American | 97 (9.9%) | 82 (8.4%) | |
| White | 382 (39.1%) | 355 (36.4%) | |
| Histological type, n (%) | | | **<0.001** |
| Infiltrating ductal carcinoma | 348 (36.3%) | 409 (42.6%) | |
| Infiltrating lobular carcinoma | 131 (13.7%) | 71 (7.4%) | |
| PR status, n (%) | | | **0.012** |
| Negative | 190 (18.7%) | 148 (14.6%) | |
| Indeterminate | 1 (0.1%) | 3 (0.3%) | |
| Positive | 320 (31.5%) | 354 (34.8%) | |
| ER status, n (%) | | | **<0.001** |
| Negative | 154 (15.1%) | 83 (8.2%) | |
| Indeterminate | 0 (0%) | 2 (0.2%) | |
| Positive | 357 (35.1%) | 421 (41.4%) | |
| HER2 status, n (%) | | | **<0.001** |
| Negative | 302 (42.1%) | 246 (34.3%) | |
| Indeterminate | 6 (0.8%) | 6 (0.8%) | |

(Continued)

### Table 1 (continued)

| Characteristic | Low expression of CYB561 | High expression of CYB561 | p value |
|---|---|---|---|
| Positive | 56 (7.8%) | 101 (14.1%) | |
| PAM50, n (%) | | | **<0.001** |
| Normal | 33 (3.1%) | 7 (0.7%) | |
| Luminal A | 272 (25.5%) | 279 (26.2%) | |
| Luminal B | 61 (5.7%) | 141 (13.2%) | |
| Her2 | 20 (1.9%) | 62 (5.8%) | |
| Basal | 146 (13.7%) | 44 (4.1%) | |
| Menopausal status, n (%) | | | **<0.001** |
| Pre | 126 (13.2%) | 98 (10.3%) | |
| Peri | 29 (3%) | 10 (1%) | |
| Post | 323 (33.8%) | 370 (38.7%) | |
| Anatomic neoplasm subdivisions, n (%) | | | 0.975 |
| Left | 277 (26%) | 276 (25.9%) | |
| Right | 255 (23.9%) | 257 (24.1%) | |
| Radiation therapy, n (%) | | | 0.591 |
| No | 214 (22%) | 218 (22.4%) | |
| Yes | 278 (28.6%) | 262 (27%) | |
| Overall survival event, n (%) | | | **0.022** |
| Alive | 472 (44.3%) | 446 (41.9%) | |
| Dead | 60 (5.6%) | 87 (8.2%) | |
| Disease-specific survival event, n (%) | | | 0.226 |
| Alive | 491 (46.9%) | 474 (45.3%) | |
| Dead | 35 (3.3%) | 46 (4.4%) | |

Note: Abbreviations: BRCA, breast cancer; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; Values in bold are statistically significant ($p < 0.05$).

### Functional enrichment analysis

To explore the role of CYB561 dysregulation in the progenesis of BRCA, functional enrichment analysis including GO, KEGG and GSEA were performed. The results of GO term enrichment analysis of DEGs indicated that significantly enriched GO terms included epidermal cell differentiation, keratinocyte differentiation, intermediate filament cytoskeleton, intermediate filament, endopeptidase regulator activity, peptidase inhibitor activity, endopeptidase inhibitor activity, and structural constituents of the cytoskeleton. KEGG pathway analysis suggested that DEGs may take part in the estrogen signaling pathway, *Staphylococcus aureus* infection, and synaptic vesicle cycle (Fig. 3C, Suppl. Tables S2 and S3).

Furthermore, the results of GSEA indicated that the inflammatory response, KRAS signaling pathway, epithelial-mesenchymal transition (EMT), and interferon gamma response were strongly enriched in the low CYB561 expression group (Figs. 4A–4D). Besides, in the low CYB561 expression group, the biological process terms associated
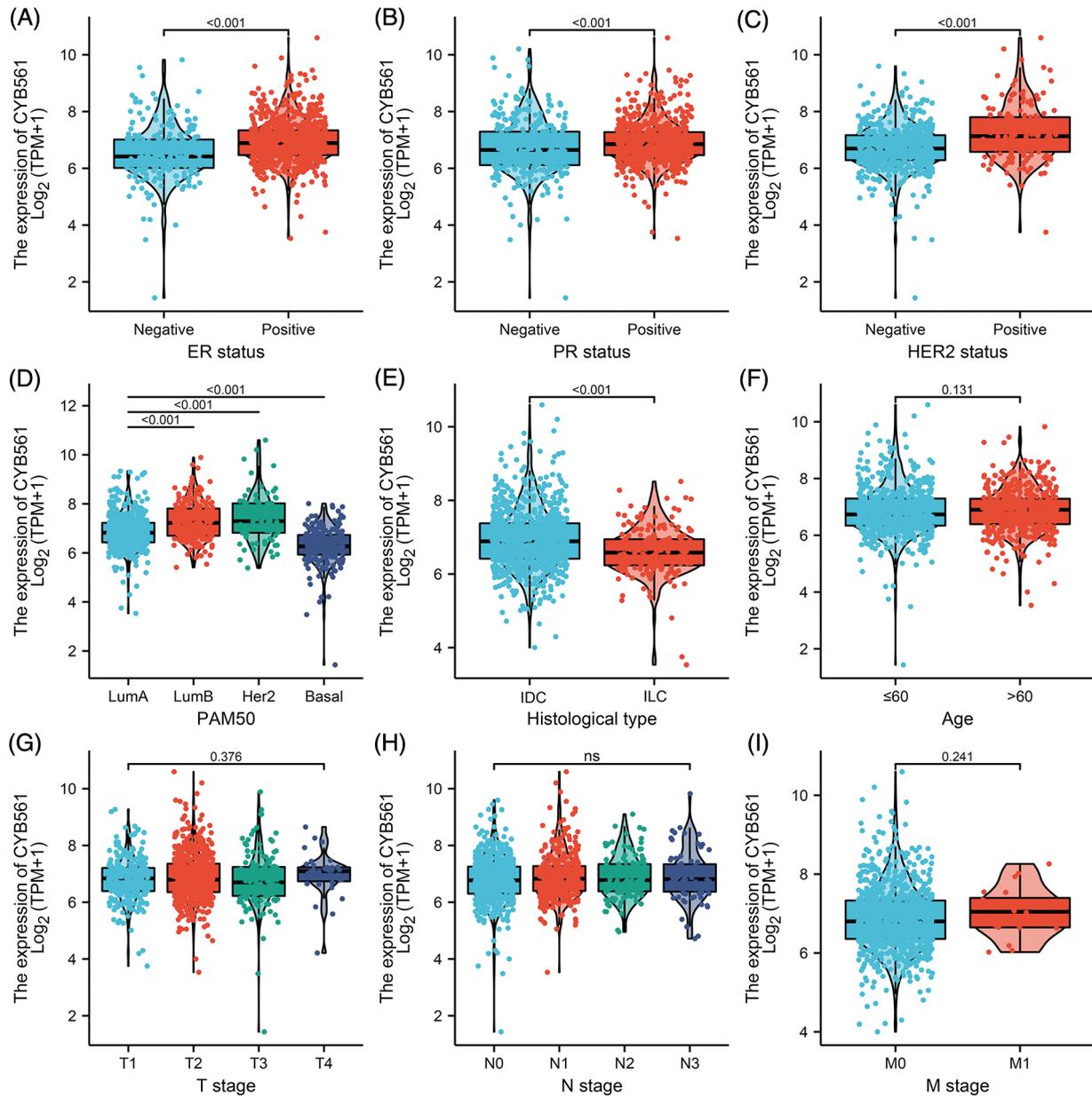
**FIGURE 2.** Association of CYB561 expression with clinicopathologic characteristics. Data are shown for (A) ER status; (B) PR status; (C) HER2 status; (D) PAM50; (E) histological type; (F) age; (G) T stage; (H) N stage; (I) M stage. LumA, Luminal A; LumB, Luminal B; IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

TABLE 2

**Relationship between CYB561 expression and clinicopathological characteristics of BRCA patients (n = 1065)**

| Characteristics | Total (N) | Odds Ratio (OR) | $p$ value |
|---|---|---|---|
| T stage (T3&T4 *vs.* T1&T2) | 1,062 | 0.942 (0.679–1.306) | 0.719 |
| N stage (N2&N3 *vs.* N0&N1) | 1,046 | 0.963 (0.703–1.319) | 0.815 |
| M stage (M1 *vs.* M0) | 909 | 1.219 (0.500–3.053) | 0.662 |
| Pathologic stage (Stage III & Stage IV *vs.* Stage I & Stage II) | 1,042 | 1.053 (0.794–1.397) | 0.720 |
| Age (>60 *vs.* ≤60) | 1,065 | 1.362 (1.069–1.736) | **0.013** |
| ER status (Positive *vs.* Negative) | 1,015 | 2.188 (1.622–2.968) | **<0.001** |
| PR status (Positive *vs.* Negative) | 1,012 | 1.420 (1.093–1.848) | **0.009** |
| HER2 status (Positive *vs.* Negative) | 705 | 2.214 (1.539–3.212) | **<0.001** |
| Race (White *vs.* Asian &Black or African American) | 976 | 0.985 (0.736–1.320) | 0.921 |

(Continued)

**Table 2  (continued)**

| Characteristics | Total (N) | Odds Ratio (OR) | *p* value |
|---|---|---|---|
| Histological type (Infiltrating Lobular Carcinoma *vs.* Infiltrating Ductal Carcinoma) | 959 | 0.461 (0.333–0.635) | **<0.001** |
| PAM50 (Luminal B & Her2 & Basal *vs.* Luminal A) | 1,025 | 1.061 (0.830–1.356) | 0.638 |
| Menopausal status (Post *vs.* Pre &Peri) | 956 | 1.644 (1.235–2.195) | **<0.001** |
| Anatomic neoplasm subdivisions (Right *vs.* Left) | 1,065 | 1.011 (0.795–1.287) | 0.926 |
| Radiation therapy (Yes *vs.* No) | 972 | 0.925 (0.718–1.192) | 0.547 |

Note: Abbreviations: BRCA, breast cancer; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2. Values in bold are statistically significant ($p < 0.05$).



**FIGURE 3.** CYB561-related DEGs and functional enrichment analysis of these DEGs. (A) Volcano plot of DEGs between the high and low CYB561 expression groups. Blue and red dots represent downregulated and upregulated DEGs, respectively. (B) Heatmap of the top 10 DEGs. (C) Representative results of GO and KEGG analysis. DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.
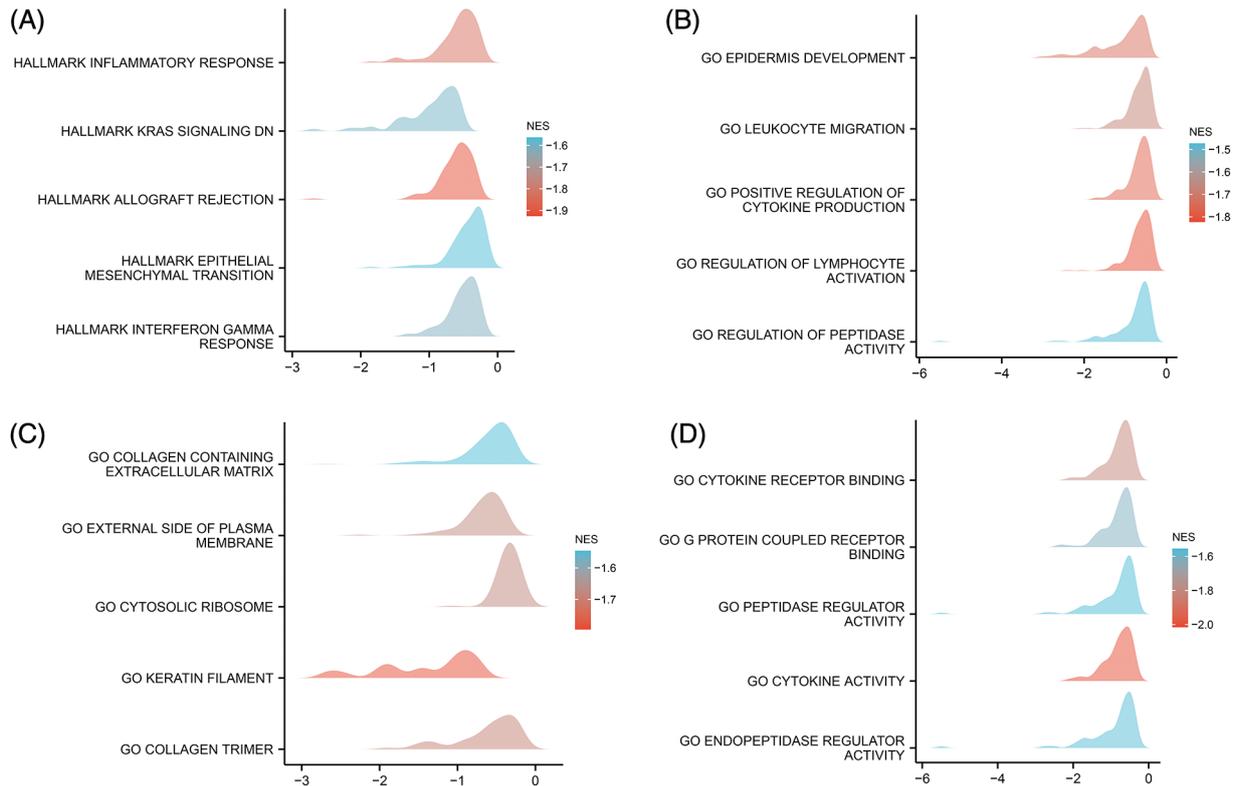
**FIGURE 4.** Pathway and functional enrichment were assessed by GSEA. (A) GSEA was performed using the MSigDB Hallmark gene sets. (B) GSEA was performed using the MSigDB bp of GO gene sets. (C) GSEA was performed using the MSigDB cc of GO gene sets. (D) GSEA was performed using the MSigDB mf of GO gene sets. GSEA, Gene Set enrichment analysis; MSigDB, Molecular Signatures Database; NES, normalized enrichment score; GO, Gene Ontology; bp, biological process; cc, cellular component; mf, molecular function.

immunity, such as leukocyte migration and regulation of lymphocyte activation, were notably enriched.

*DNA methylation status of CYB561*
To determine the mechanism of CYB561 dysregulation, we attempted to evaluate the methylation status of the CYB561 promoter. The expression level of CYB561 showed a significant positive correlation with the expression levels of DNMT1 and DNMT3A (all $p < 0.05$) (Figs. 5A–5C). Heatmap showed methylation levels of individual methylation sites on CYB561 DNA sequence in breast cancer tissues (Fig. 5D). Furthermore, Kaplan–Meier survival curves revealed unfavorable survival of patients with lower methylation levels at cg14204735 in TSS1500 region, cg04987499 in TSS1500 region, cg22207733 in TSS200 and TSS1500 region, cg27279583 in the body, cg26300322 in the body, and cg18499750 in the 3′UTR of CYB561 (Figs. 6A–6F).

*Correlation of CYB561 expression with immune infiltration level in BRCA*
Given the interesting results we found, we further investigated the correlation of CYB561 expression with infiltrating immune cells in BRCA. CYB561 expression was notably correlated with 20 out of 24 types of immune infiltrating cells (Fig. 7A). B cells, plasmacytoid dendritic cells (pDCs), dendritic cells (DCs), and neutrophils were more infiltrated

in the CYB561 low expression group (all $p < 0.001$) (Figs. 7B–7E). Moreover, CYB561 expression had a significant negative correlation with the immune infiltration of B cells, pDCs, DCs, and neutrophils (all $p < 0.001$) (Figs. 7F–7I). Furthermore, multiple immune checkpoint-related molecules, such as CTLA4, IDO1, LAG3, PDCD1, PDCD1LG2 (also known as PD-L2), TIGIT, and VTCN1 were observed to be more highly expressed in the CYB561 high expression group (Fig. 7J).

*Prognostic value of CYB561 in BRCA patients*
Subsequently, we examined the prognostic significance of CYB561 expression in BRCA. In the TCGA cohort, BRCA patients with high CYB561 expression had significantly worse OS and DSS compared to those with low CYB561 expression [OS: hazard ratio (HR) = 1.79, 95% CI = 1.29–2.48, $p < 0.001$; DSS: HR = 1.65, 95% CI = 1.06–2.58, $p = 0.026$] (Figs. 8A and 8B). Similarly, in the METABRIC cohort, patients with increased CYB561 expression were remarkably associated with the decline in OS (HR = 1.32, 95% CI = 1.17–1.48, $p < 0.001$) (Fig. 8C). Additionally, regardless of the OS or DSS, BRCA patients with increased CYB561 expression had notably unfavorable survival probabilities in several subgroups, including ER positive, PR negative, T stage 1–3, N stage 0–2, M stage 0, and pathologic stages II–III Subgroups (all $p < 0.05$) (Figs. 8D–8O).
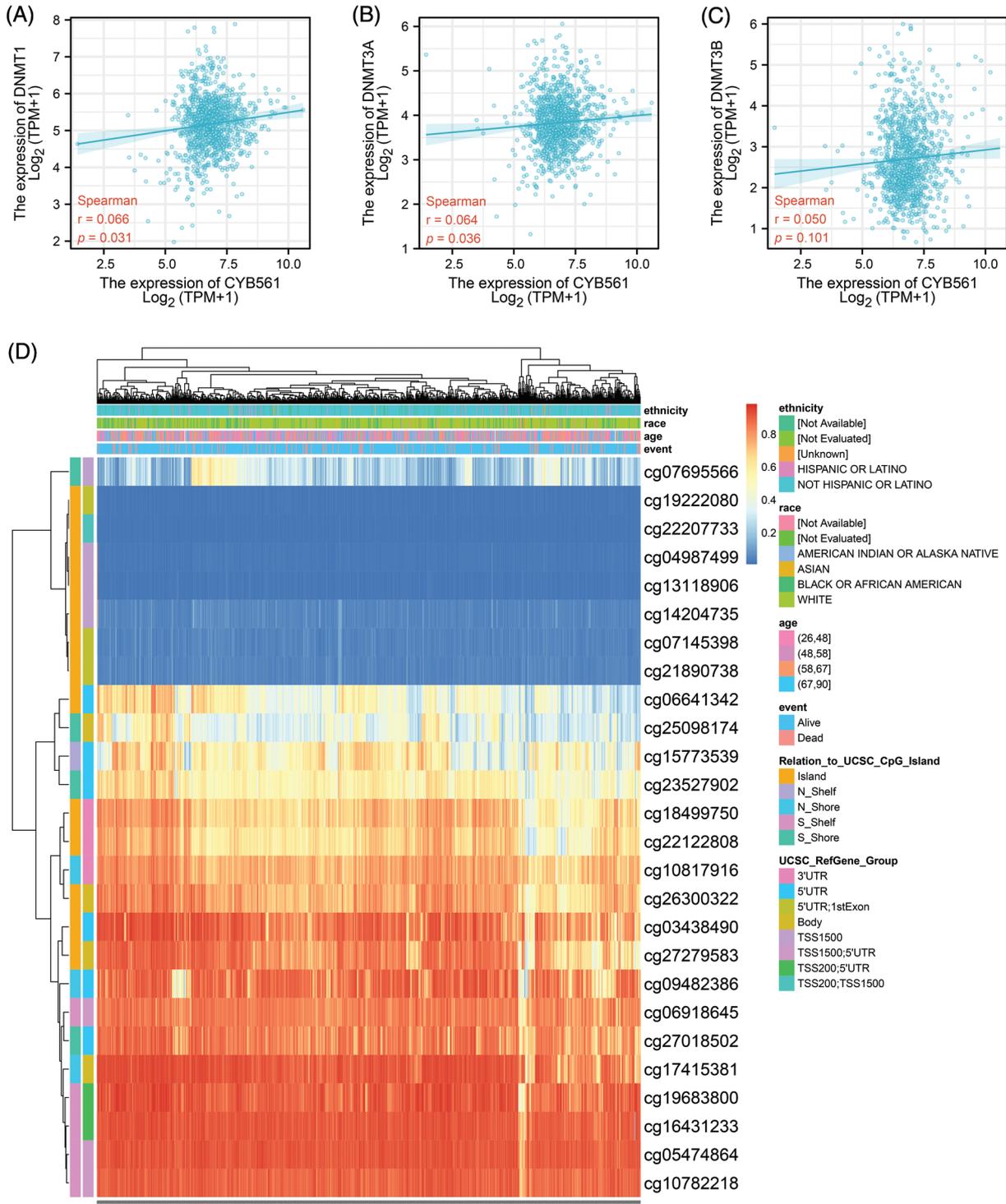
FIGURE 5. Methylation status of CYB561 in BRCA patients. (A) Analysis of correlation between CYB561 and DNTM1expression levels. (B) Analysis of correlation between CYB561 and DNTM3A expression levels. (C) Analysis of correlation between CYB561 and DNTM3B expression levels. (D) Heatmap of methylation status of serval methylation sites of CYB561. BRCA, breast cancer.

Moreover, multivariate Cox regression analysis indicated that CYB561 expression (adjusted HR = 1.818, 95% CI = 1.007–3.283, $p$ = 0.48), pathological stage (adjusted HR = 3.508, 95% CI = 1.242–9.910, $p$ = 0.018), age (adjusted HR = 2.917, 95% CI = 1.613–5.272, $p$ < 0.001), and radiation therapy (adjusted HR = 0.488, 95% CI = 0.241–0.830, $p$ = 0.011) were independent prognostic markers for OS in BRCA patients (Table 3). Likewise, for DSS, age (adjusted HR = 1.731, 95% CI = 1.033–2.901, $p$ =

0.037), and histological type (adjusted HR = 0.381, 95% CI = 0.156–0.929, $p$ = 0.034) were identified as independent prognostic indicators (Suppl. Table S4).

*Construction and evaluation of a nomogram*
To further predict prognostic risk in BRCA patients, a nomogram was developed using the results of multivariate COX analysis (Fig. 9A). In the multivariate analysis, pathologic stage, age, radiation therapy, and *CYB561*
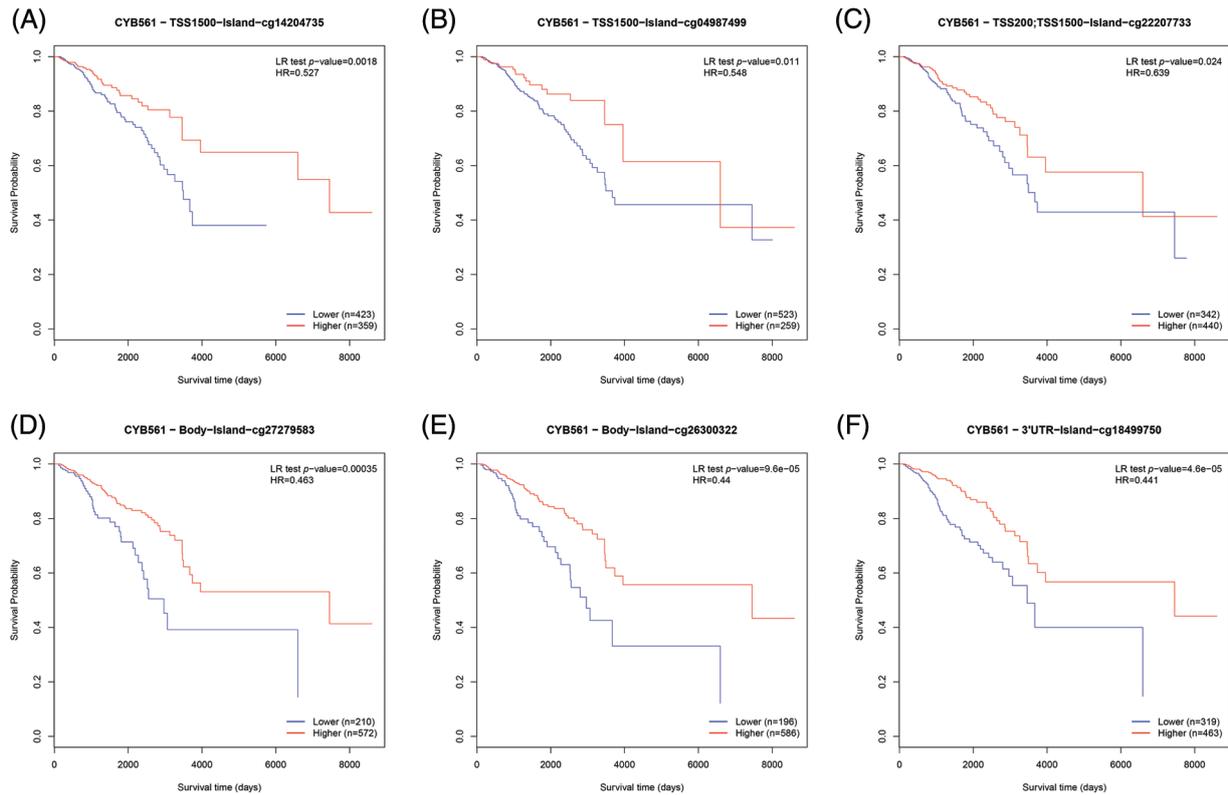
**FIGURE 6.** Effect of DNA methylation status of CYB561 on prognosis of BRCA patients. (A–F) Kaplan–Meier survival curves of CYB561 methylation sites. BRCA, breast cancer.

expression were independent predictors of OS, and these variables were included in the nomogram. According to the nomogram, these factors were used to calculate a weighted total score for each patient and to estimate the patient's 1-, 3-, and 5-year OS. The obtained model was verified internally using the bootstrap resampling method (1000 repetitions), with a C-index of 0.771 (95% CI: 0.745–0.797). The bias-corrected lines shown in the calibration plots were very close to the ideal curve, indicating that the predicted values agreed well with the observed values (Figs. 9B–9D). Additionally, the time-dependent ROC curve was generated, and the AUC of the nomogram model for predicting OS probability at 1 year, 3 years and 5 years was 0.861, 0.748, and 0.757, respectively (Fig. 9E).

## Discussion

As a transmembrane electron transfer protein, human CYB561 has a physiological function that is closely related to ASC. The ASC-dependent β-hydroxylation of dopamine is an important process in the synthesis of norepinephrine (Zhang et al., 2014; van den Berg et al., 2018). CYB561 exhibits ASC-mediated $Fe^{3+}$ reductase activity, which provides reduced iron for transmembrane transport (Su and Asard, 2006; Berczi et al., 2007). Currently, few studies have described the relationship between CYB561 and malignant tumors, especially BRCA, and CYB561 expression status, potential function, and ability of predicting prognosis in BRCA remain unclear.

In our study, bioinformatics analysis was performed using high-throughput RNA-seq data obtained from TCGA database to evaluate the methylation status, immune infiltration score, and prognostic value of CYB561 in BRCA patients. Our results showed that CYB561 is highly expressed in BRCA and may be a potential negative prognostic biomarker, which is consistent with previous studies on CYB561 expression in BRCA (Yang et al., 2021; Zhou et al., 2022). In contrast, overexpression of CYB561 has been reported to be a marker for good prognosis in ovarian serous carcinoma (Willis et al., 2016).

Since the biological function of CYB561 in BRCA is still unclear, functional enrichment analysis was conducted. Our findings revealed that CYB561 may act on BRCA through EMT, KRAS signaling pathway, and estrogen signaling pathway. EMT plays an important role in the development and progression of cancer, including carcinogenesis, metastasis, and drug resistance (Nieto et al., 2016; Zhang and Weinberg, 2018). As a proto-oncogene, previous studies demonstrated that elevated KRAS expression was associated with BRCA progression and can be used as a prognostic factor of BRCA (Banys-Paluchowski et al., 2020; Liang et al., 2021). Moreover, KRAS can promote the proliferation, apoptosis, and invasion of BRCA via EMT (Jung et al., 2019; Zhang et al., 2020). Additionally, it has been reported that estrogen contributes to regulate the proliferation and differentiation of normal mammary epithelial cells (Saha et al., 2019). Persistent estrogen exposure, or dysregulated estrogen signaling, has been implicated in many types of cancers, including breast, endometrial, ovarian, and prostate
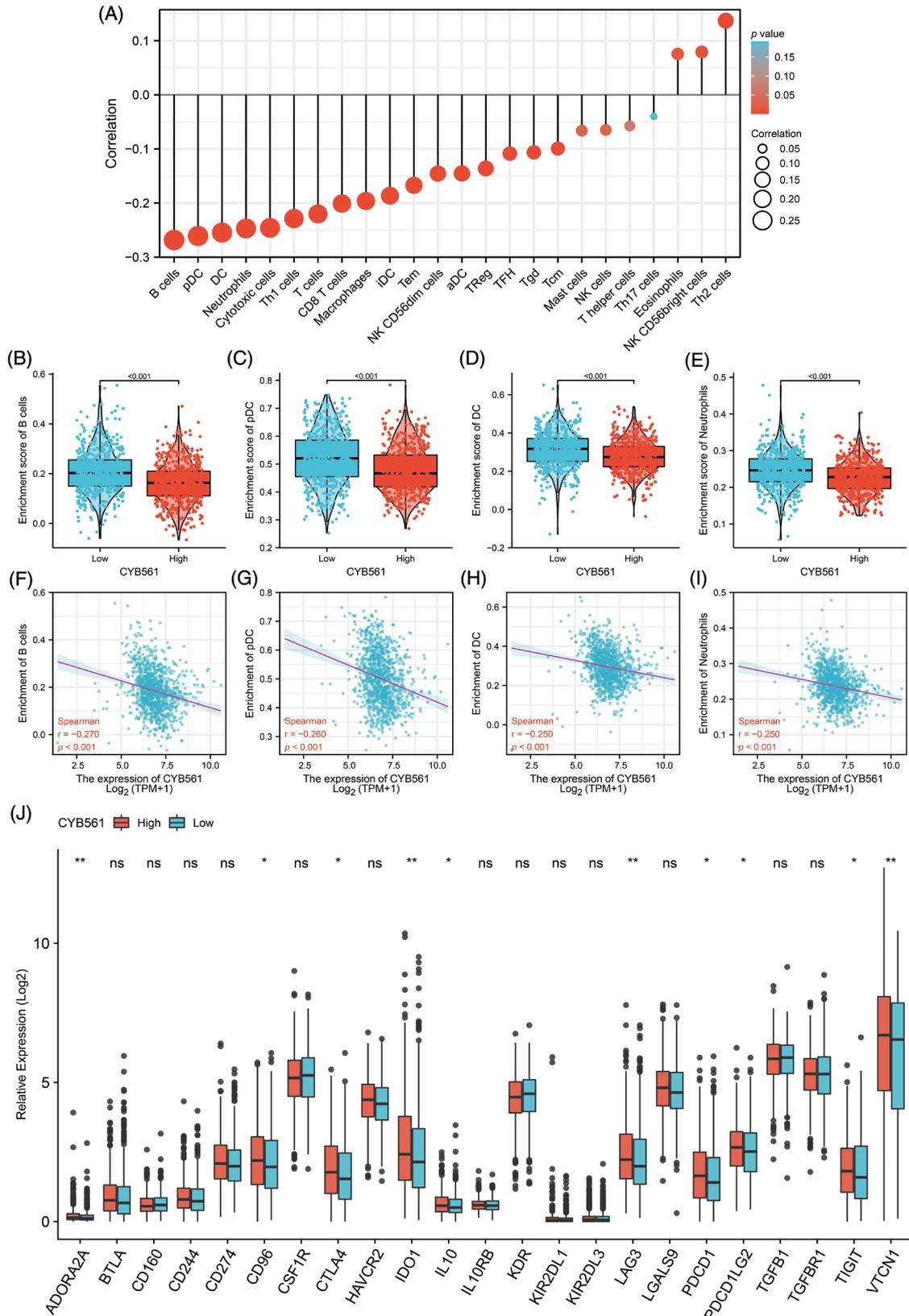
**FIGURE 7.** Correlation between CYB561 expression and immune cell infiltration in BRCA. (A) Correlation between CYB561 expression and immune cell infiltration. The size of the black circles corresponds to the absolute values of the Spearman's correlation coefficient. (B–E) Comparison of the relative enrichment scores of immune cells (including B cells, pDCs, DCs, and Neutrophils) between the high- and low-CYB561 expression groups. (F–I) Analysis of the correlation between the relative enrichment scores of immune cells (including B cells, pDCs, DCs, and Neutrophils) and CYB561 expression levels. (J) Comparison of the expression of immune checkpoint-related genes between the high- and low-CYB561 expression groups. BRCA, breast cancer; pDCs, plasmacytoid dendritic cells; DCs, dendritic cells. $^*p <$ 0.05, and $^{**}p < 0.01$.

cancers (Liang and Shang, 2013). As a mammary gland carcinogen, estrogen participates in the initiation, promotion, and progression of breast cancer (Yager and Davidson, 2006). Furthermore, our results also suggested that CYB561 may be involved in inflammation-related pathways, such as inflammatory response, interferon gamma response, *Staphylococcus aureus* infection, and leukocyte migration. Numerous studies have confirmed that chronic inflammation plays a crucial role in different stages of tumorigenesis, including initiation, proliferation,
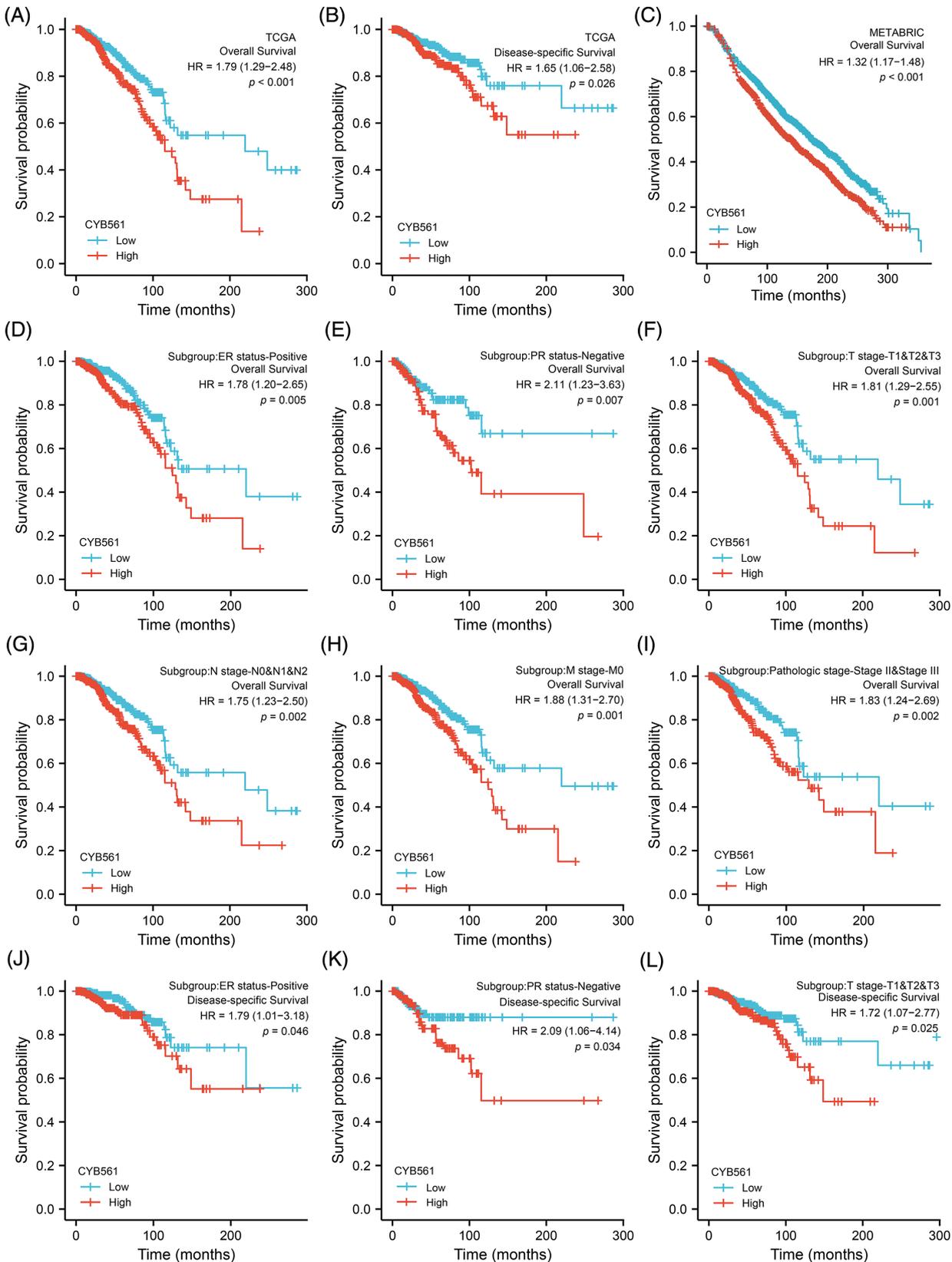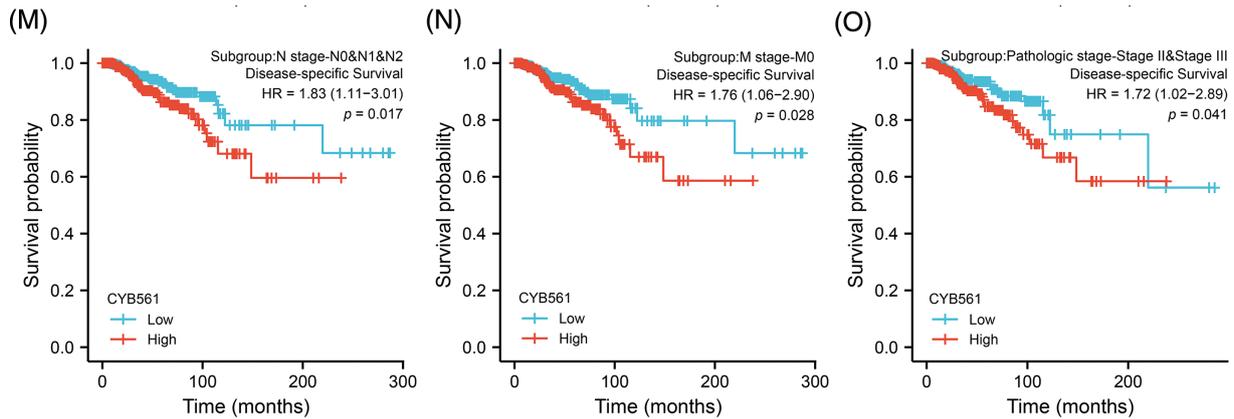


FIGURE 8. (continued)

**FIGURE 8.** Prognostic values of CYB561 expression in BRCA patients. Effect of CYB561 expression on overall survival (A) and disease-specific survival (B) of BRCA patients in the TCGA database. Effect of CYB561 expression on overall survival (C) of BRCA patients in the METABRIC database. (D–I) Overall survival curves of ER positive; PR positive; T1, T2 and T3; N0, N1, and N2; M0; stages II and III subgroups of BRCA patients with high and low CYB561 expression. (J–O) Disease-specific survival curves of ER positive; PR positive; T1, T2 and T3; N0, N1, and N2; M0; stages II and III subgroups of BRCA patients with high and low CYB561 expression. BRCA, breast cancer; TCGA, The Cancer Genome Atlas; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium; ER, estrogen receptor; PR, progesterone receptor.

**TABLE 3**

**Univariate and multivariate analyses of overall survival in BRCA patients**

| Characteristics | Total (N) | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|---|
| | | Hazard ratio (95% CI) | *p* value | Hazard ratio (95% CI) | *p* value |
| T stage (T3&T4 *vs.* T1&T2) | 1,079 | 1.608 (1.110–2.329) | **0.012** | 1.847 (0.813–4.197) | 0.143 |
| N stage (N2&N3 *vs.* N0&N1) | 1,063 | 2.163 (1.472–3.180) | **<0.001** | 1.242 (0.463–3.327) | 0.667 |
| Pathologic stage (Stage III & Stage IV *vs.* Stage I & Stage II) | 1,059 | 2.391 (1.703–3.355) | **<0.001** | 3.508 (1.242–9.910) | **0.018** |
| Age (>60 *vs.* ≤60) | 1,082 | 2.020 (1.465–2.784) | **<0.001** | 2.917 (1.613–5.276) | **<0.001** |
| ER status (Positive *vs.* Negative) | 1,032 | 0.712 (0.495–1.023) | 0.066 | 0.597 (0.240–1.483) | 0.266 |
| PR status (Positive *vs.* Negative) | 1,029 | 0.732 (0.523–1.024) | 0.068 | 0.688 (0.297–1.593) | 0.383 |
| HER2 status (Positive *vs.* Negative) | 715 | 1.593 (0.973–2.609) | 0.064 | 0.816 (0.403–1.649) | 0.570 |
| Radiation therapy (Yes *vs.* No) | 986 | 0.576 (0.394–0.841) | **0.004** | 0.448 (0.241–0.830) | **0.011** |
| Histological type (Infiltrating Lobular Carcinoma *vs.* Infiltrating Ductal Carcinoma) | 977 | 0.827 (0.526–1.299) | 0.410 | – | – |
| CYB561 (High *vs.* Low) | 1,082 | 1.788 (1.291–2.477) | **<0.001** | 1.818 (1.007–3.283) | **0.048** |

Note: Abbreviations: BRCA, breast cancer; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2. Values in bold are statistically significant ($p < 0.05$).

angiogenesis, and metastasis (Diakos *et al.*, 2014; Hanahan, 2022). Inflammation is a threat to the long-term survival of BRCA patients (Mahmoud *et al.*, 2012). A recent clinical study showed that the use of nonsteroidal anti-inflammatory drugs can prevent the progression of biopsy-proven benign breast tumor to breast cancer (Sherman *et al.*, 2020). Interestingly, it has been shown that there is an association between *Staphylococcus aureus* infection at the surgical site and breast cancer recurrence (O'Connor *et al.*, 2020). Leukocyte migration is critical for the maintenance and regulation of immune responses and is involved in inflammatory responses and the composition of the tumor microenvironment (Kameritsch and Renkawitz, 2020).

As one of the most common epigenetic mechanisms, DNA methylation regulates gene expression (Moore *et al.*, 2013). To our knowledge, there are no studies available to date on the methylation status of CYB561 in tumors. Thus, we attempted to study the underlying mechanism of overexpression of CYB561 in BRCA. Our findings showed that several methylated sites on the DNA sequence of the CYB561 gene are hypomethylated, which were strongly associated with poor prognosis in BRCA patients. This suggests that the methylation of CYB561 may be introduced by DNTM1 and DNMT3A.

The tumor microenvironment (TME) is thought to play an important regulatory role in tumorigenesis and progression
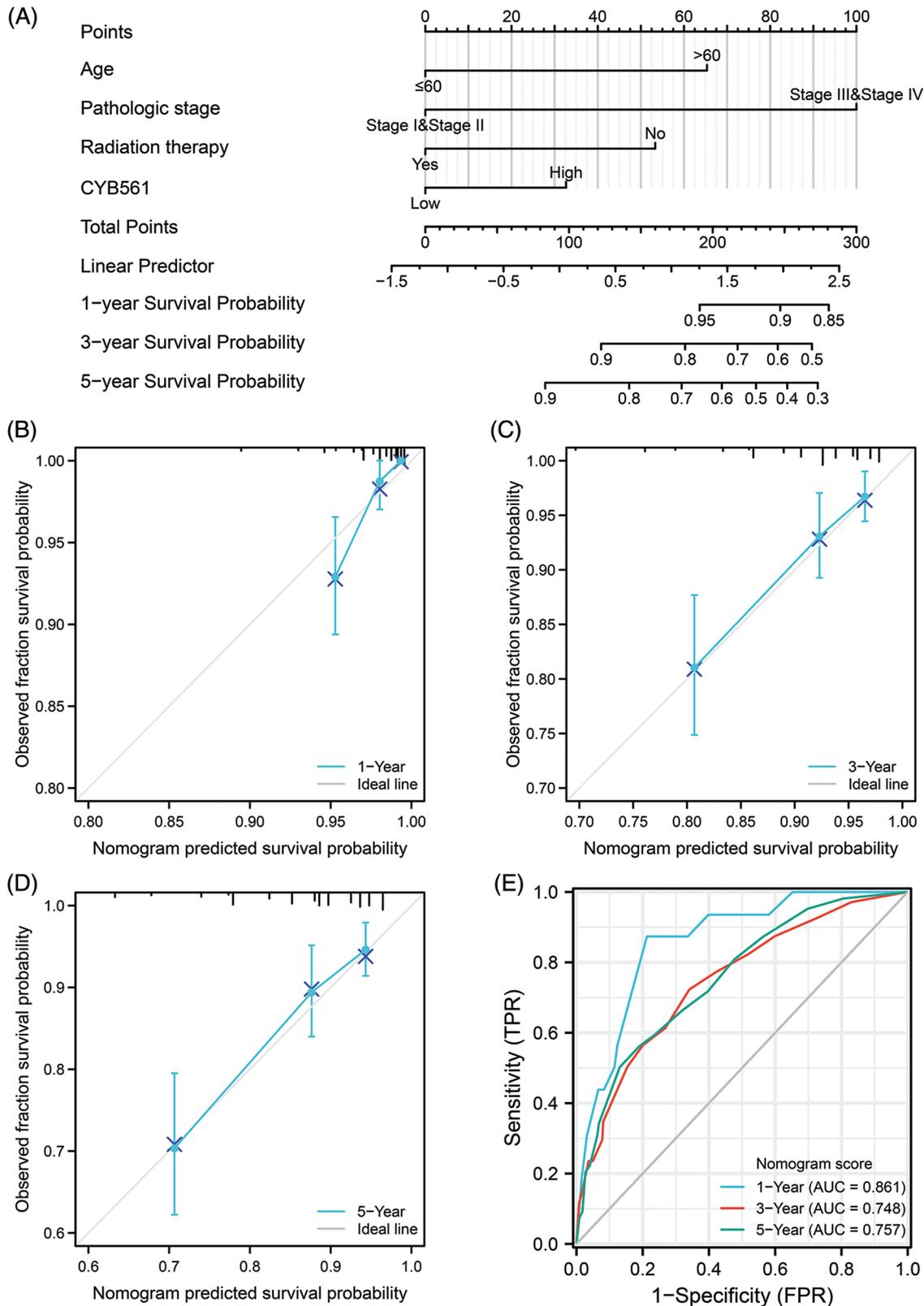
**FIGURE 9.** A nomogram for predicting overall survival of BRCA patients and its predictive performance. (A) A nomogram for predicting overall survival of BRCA patients. (B–D) Calibration plots of nomogram for 1-, 3-, and 5-year overall survival, respectively. (E) Time-dependent ROC curve of nomogram. BRCA, breast cancer.

(Quail and Joyce, 2013). Tumor-infiltrating immune cells are an integral part of the TME and can recognize, inhibit, or promote tumor progression (Ostrand-Rosenberg, 2008). The number of tumor-infiltrating lymphocytes (TILs) often indicates the prognosis of BRCA patients. Increased TILs predict a response to neoadjuvant chemotherapy in most molecular subtypes of BRCA and are particularly associated with survival benefits in HER2-positive and triple-negative

breast cancer (TNBC) (Denkert et al., 2018; Loi et al., 2019; Park et al., 2019). TILs comprise a group of heterogeneous immune cells, usually dominated by T cells, but also include B cells, NK cells, and myeloid-lineage cells such as macrophages, neutrophils, and mast cells. In our study, we found that B cells infiltrated more in the CYB561 low expression group. A previous study showed that the density of tumor-infiltrating B cells was increased in untreated BRCA compared to normal breast tissues and was strongly related to higher tumor grade, higher proliferation, and hormone receptor negativity (Garaud et al., 2019). Besides, B cells have been reported to be associated with poor prognosis in BRCA (Shariati et al., 2020). In contrast, a higher total number of infiltrating B cells in BRCA have previously been related to improved clinical prognosis (Mahmoud et al., 2012). One possible explanation for these divergent results may be the differences in the samples and assays used. pDCs and DCs, as important components of innate immunity system, have been observed to have inhibitory effects on BRCA cell proliferation (Wu et al., 2016). Neutrophils are the most abundant circulating leukocytes in the human body, which can regulate not only the adaptive immune system, but also the innate immune system (Rosales, 2020). Growing evidence suggests that neutrophils may play an important role in the progression and metastasis of BRCA (Coffelt et al., 2015; Park et al., 2016). In a previous meta-analysis, elevated neutrophil-to-lymphocyte ratio in the peripheral blood of BRCA patients was associated with an adverse OS and DFS (Ethier et al., 2017).

Immune evasion is a key factor in tumor progression. Recently, immunotherapy, especially immune checkpoint inhibitors (ICIs), has emerged as an important treatment for many malignant tumors (Moujaess et al., 2019). The programmed cell death receptor-1/ligand 1 (PD-1/L1) and cytotoxic T-lymphocyte associated protein-4 (CTLA-4) are the most important targets of ICIs. PD-L1 is highly expressed in a variety of tumors and can predict the clinical response to PD-1-targeted immunotherapy (Zou and Chen, 2008). Since BRCA has not traditionally been considered a highly immunogenic disease, the efficacy of ICIs is largely limited by low response rates (Tokumaru et al., 2020). TNBC is by far the most promising molecular subtype of BRCA for immune checkpoint blockade. A previous study demonstrated that patients with TNBC who were PD-L1-positive had longer median OS than those who were PD-L1-negative (AiErken et al., 2017). Although TNBC has a higher response rate to ICIs than other subtypes of BRCA, the efficacy of ICIs remains low, with response rates ranging from approximately 5% to 23% (Keenan and Tolaney, 2020). Therefore, we must explore novel and effective targets for immune checkpoint blockade in BRCA. In our study, we found that elevated expression of CYB561 was closely related to the increased expression of CTLA4, IDO1, LAG3, PDCD1, PDCD1LG2, and TIGIT, which exhibited different immunomodulatory effects in different tissue sites. These results suggest that patients with high expression of CYB561 may be more likely to benefit from immunotherapy.

Although our study provides novel insights into the relationship between CYB561 expression and BRCA from multiple perspectives and levels by mining public databases, there are still several limitations that warrant consideration. First, given that our study was mainly based on public databases, selection bias may affect our findings. Second, some important clinical features, such as surgical approach and adjuvant chemotherapy regimens, could not be collected due to limitations in the public databases; hence, we need to validate the results using a patient cohort in our center. Third, considering that all the results were obtained using bioinformatics, further systematic experimental studies are required to unravel the underlying biological mechanisms of CYB561 in BRCA.

In summary, all the data in our study demonstrate that elevated CYB561 is strongly linked with aggressive clinicopathologic characteristics, unfavorable immune infiltration, and poor patient outcomes, which may be a novel prognostic biomarker for BRCA patients. However, more studies are needed to explore the function of CYB561 in BRCA and more importantly, how other molecules contribute to this function in tumorigenesis and progression.

**Author Contributions:** The authors confirm contribution to the paper as follows: wrote the manuscript: XY; data analysis: H-XW and CX; data collection: BZ and M-LL; the technical support: BZ and JQ; conceived the idea, designed the study, and revised the manuscript: MD. All authors reviewed the results and approved the final version of the manuscript.

**Availability of Data and Materials:** The datasets analyzed during the current study are available in the following repositories: The Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/), Genotype Tissue Expression Project (GTEx) database (https://commonfund.nih.gov/gtex), cBioPortal database (http://www.Cbioportal.org/), UCSC Xena database (https://xenabrowser.net/datapages/), MethSurv methylation analysis tool (https://biit.cs.ut.ee/methsurv/).

**Ethics Approval:** Not applicable.

**Conflicts of Interest:** The authors declare that they have no conflicts of interest to report regarding the present study.

**Supplementary Materials:** The supplementary material is available online at DOI: 10.32604/biocell.2023.027103.

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**Supplementary Materials**

**Table S1.** Baseline characteristics of patients (n = 1065)
**Table S2.** DEGs enriched Gene Ontology (GO) terms
**Table S3.** DEGs enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) terms
**Table S4.** Univariate and multivariate analysis of disease-specific survival in patients with breast cancer