Original Article

BUB1 as a novel marker for predicting the immunotherapy efficacy and prognosis of breast cancer

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Background: Budding uninhibited by benzimidazole 1 (BUB1) is a highly conserved serine/threonine kinase, showing prominent importance for proper function during mitosis. However, little is known about *BUB1* mRNA expression in breast cancer (BRCA) and its correlation with prognosis and immune infiltration. Hence, we aimed to unveil its potential as groundbreaking biomarkers for immunotherapy efficacy and the prognosis of BRCA.

Methods: Database for Annotation, Visualization, and Integrated Discovery (DAVID) is a potent tool for identifying significant clusters of genes and pathways in the resulting dataset. In this study, gene set enrichment analysis of BUB1 was conducted using DAVID. The clinical characteristics of patients with or without altered *BUB1* mRNA expression were compared using cBioPortal. Tumor Immune Estimation Resource (TIMER) is a known as database for comprehensive analysis of tumor-infiltrating immune cells in various cancers. In the present study, the relationship between BUB1 expression and the abundance of immune infiltrates was explored using TIMER in BRCA. Immunohistochemistry staining was performed to analyze the protein expression of BUB1 in tumor tissue specimens. We used PrognoScan and Kaplan-Meier Plotter to evaluate the prognosis of patients with different BUB1 expression levels.

Results: The expression of BUB1 in various tumor tissues was higher than that in adjacent normal tissues. BUB1 was mainly localized to the nucleoplasm and additionally localized to the cytosol. Functional enrichment analyses revealed that the cell cycle was the most significant pathway. Abnormal *BUB1* mRNA expression was more frequently detected in invasive ductal carcinoma with higher histological grades and BRCAs with estrogen receptor (ER)-negative, human epidermal growth receptor 2 (HER2)-negative, and basal-like phenotypes. The BUB1 expression was correlated positively with tumor purity, B cells, $CD8^+$ T cells, $CD4^+$ T cells, neutrophils, and dendritic cells, while BUB1 had no significant correlation with macrophages. The results of immunohistochemical staining from clinical samples further confirmed that BUB1 was overexpressed in BRCA compared to benign tumor (fibroadenoma of breast) ($P<0.01$). BRCA patients with lower BUB1 expression had a better prognosis than those with higher BUB1 expression in overall survival (OS) curves, distant metastasis-free survival (DMFS) curves, and relapse-free survival (RFS) curves ($P<0.05$).

Conclusions: Our results suggest that BUB1 is a potential molecular biomarker for evaluating the prognosis and predicting the effectiveness of immunotherapy for BRCA.

Keywords: Breast cancer (BRCA); BUB1; biomarker; prognosis; immunotherapy

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Introduction

Breast cancer (BRCA) is a highly heterogeneous malignancy with different molecular subtypes and biological characteristics, which has become the most frequently diagnosed cancer among women worldwide (1). The incidence rate of BRCA is rising year by year and the age of onset also shows a younger trend in China (2-4). According to the expression of estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), and Ki-67 detected by immunohistochemical staining, BRCA is clinically divided into four subtypes: luminal A, luminal B, HER2-enriched subtype, and basal-like subtype (triple-negative breast cancer) (5). Despite the advancement of various treatments for BRCA including surgical resection, radiotherapy, targeted endocrine, molecular therapy, chemotherapy, and neoadjuvant chemotherapy (6), the survival outcomes of some patients are still dismal due to disease recurrence and drug resistance (7). For example, metastatic ER-positive BRCA may be resistant to tamoxifen, and HER2-positive BRCA may be resistant to trastuzumab (8,9). Therefore, there is an urgent need to find effective biomarkers for early BRCA screening or molecular targets for personalized therapy.

BUB1 gene (budding uninhibited by benzimidazole 1) is located in chromosome 2q13 (GRCh38/hg38) and encodes a serine/threonine-protein kinase. *BUB1* performs multiple tasks in mitosis and its encoded protein functions in part by phosphorylating members of the mitotic checkpoint complex and activating the spindle checkpoint. *BUB1* is one of the important components of spindle assembly checkpoint (*SAC*). When sister chromatid is not assembled correctly, *SAC* is activated and *BUB1* recruits *MAD1-MAD2*, *CDC20* (cell division cycle 20), *BUBR1* (BUB1-related)/*MAD3*, and other related component proteins (10,11). The phosphorylation of CDC20 by BUB1 leads to the inhibition of *APC/C* (anaphase-promoting complex/cyclosome), while the phosphorylated *CDC20* can delay the mitotic M-phase and finally prevent the mitotic process (12). *BUB1* inhibition can lead to kinetochore assembly error and eventually chromosomal instability (13). Also, the protein may play an active role in DNA damage response. *BUB1* mutation is associated with several forms of cancer (14-18). Despite the low frequency of *BUB1* mutation, many tumor cells do have spindle checkpoint function defects. In recent years, many scholars have confirmed that the expressions of *BUB1* and *MAD2* are related to colorectal cancer, gastric cancer, BRCA, leukemia, nasopharyngeal carcinoma, and prostate cancer by the use of immunohistochemistry, polymerase chain reaction (PCR), and various hybridization techniques (19-26). *BUB1* is considered an important tumor promoter in various malignant tumors and drives tumorigenesis and development by facilitating tumor cell proliferation, invasion and migration, but the functional role and prognostic value of *BUB1* in BRCA is rarely reported.

This study aimed to explore the correlation of BUB1 expression with the prognosis and immune infiltration of BRCA. We used publicly available website to construct BUB1-related gene regulatory networks. Genomic data and other freely available databases were used to analyze the significance of BUB1 in the prognosis and tumor immune infiltration of BRCA. The study of immune infiltration related to BUB1 will provide new directions for BRCA immunotherapy. We suggest that BUB1 may be a promising therapeutic target for BRCA in the future. We present this article in accordance with the REMARK reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-24-704/rc).

Highlight box

Key findings

- BUB1 is a potential molecular biomarker for evaluating the prognosis and predicting the effectiveness of immunotherapy for breast cancer (BRCA).

What is known and what is new?

- BRCA is a highly heterogeneous malignancy with different molecular subtypes and biological characteristics.
- The expression of BUB1 in various tumor tissues was higher than that in adjacent normal tissues.

What is the implication, and what should change now?

- BUB1 could be a potential molecular biomarker for evacuating prognosis and predicting effectiveness of immunotherapy for BRCA, hence providing new preventative and therapeutic options for BRCA.

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Methods

TIMER analysis

Tumor Immune Estimation Resource (TIMER, https://cistrome.shinyapps.io/timer) is a database for comprehensive analysis of tumor-infiltrating immune cells in more than 30 cancer types (27), which can provide a comprehensive analysis of the molecular characterization of tumor-immune interactions. We used this database to compare the differential expression pattern of BUB1 between tumor tissues and adjacent normal tissues. The Wilcoxon test was to evaluate the statistical significance of differential expression, with a value of $P<0.05$ indicating a statistically significant difference. Moreover, the correlation between the expression of BUB1 and the abundance of immune infiltrates in BRCA was explored.

Human Protein Atlas (HPA)

HPA (www.proteinatlas.org) is a freely available database, containing more than 13 million immunohistochemistry images and providing exploration of drug targets and disease biomarkers (28). We used the map to get a subcellular localization map of the BUB1 human proteome.

Functional and pathway enrichment analysis

We identified genes co-expressed with *BUB1* using the cBioPortal database. Database for Annotation, Visualization, and Integrated Discovery (DAVID, https://david.ncifcrf.gov) was used to identify significant clusters of genes and pathways in resulting dataset (29). We used an online tool (https://www.bioinformatics.com.cn) to analyze Gene Ontology (GO) covering cellular component (CC), biological process (BP), molecular function (MF), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. LinkedOmics (http://www.linkedomics.org) database contains multi-omics data and clinical data for 32 cancer types and more than 11,000 patients from The Cancer Genome Atlas (TCGA, www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga) (30). We used the website to construct a heat map showing the genes and miRNAs correlated with *BUB1* in BRCA. Further, Linkedomics was utilized to perform Pearson correlation analysis of miRNAs and Cox proportional hazard (CoxPH) test of *BUB1*.

STRING and cytoscape

STRING (https://cn.string-db.org/) contains protein interaction data from over 2,000 species, and provides known and predicted functional associations between proteins for a large number of organisms (31,32). Cytoscape (http://apps.cytoscape.org/apps/stringapp) is a software for network visualization and analysis. Cytoscape app can easily import STRING networks into Cytoscape and perform complex network analysis and visualization tasks. We applied STRING website and Cytoscape app to construct the protein-protein interaction network of BUB1 and its significantly associated genes.

cBioPortal

All cases were from the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) database. Data are listed in the cBioCancer Genomics Portal (cBioPortal, http://www.cbioportal.org), a database for analyzing large-scale genomics (33). We compared the clinical characteristics of patients between the *BUB1* mRNA expression altered group and the non-altered group in the METABRIC study.

Tumor-Immune System Interactions and Drug Bank (TISIDB)

We used the TTISIDB (http://cis.hku.hk/TISIDB) to analyze the associations between BUB1 and immune features, such as lymphocytes, immunoinhibitors, immunostimulators, major histocompatibility complex (MHC) molecules, chemokines, receptors, and immune subtypes in BRCA. TISIDB database is commonly used to pre-calculate the associations between genes and immune features in 30 TCGA cancer types (34).

Immunohistochemistry

A total of 48 cancer tissues, along with the corresponding benign tissue samples were collected in the Department of Clinical Pathology at the First Affiliated Hospital of Jinan University. The enrolled cases were categorized into two groups: invasive non-special-type ductal carcinoma cases who were surgically treated and diagnosed with invasive ductal carcinoma (24 cases) and benign cases (fibroadenoma of breast, 24 cases). The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). All samples and clinical information used in this study were approved by the Institutional Review Board (IRB) of the First Affiliated Hospital of

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Jinan University (approval No. KY-2023-116). This study does not involve human trials, personal privacy or commercial interests, so this study obtains a waiver of informed consent. Immunohistochemistry staining was performed to analyze the protein expression of BUB1 in tumor tissue specimens. BUB1 (#2978S, 1:500 dilution) was purchased from Beyotime Biotechnology. All specimens were analyzed by using the EnVision two-step method, and immunohistochemical sections were evaluated by two pathologists independently. The scoring criteria for the percentage of positive range were as follows: no positive expression in the tumor region (0 points), positive expression region ≤10% (1 point), positive expression region 11–50% (2 points), and positive expression region >50% (3 points). The scoring system of staining intensity: uncolored (0 points), light yellow (1 point), yellow (2 points), and brown (3 points). A cumulative score ≤3 was considered low expression of BUB1, whereas >3 was considered high expression of BUB1.

PrognoScan and Kaplan-Meier plotter

PrognoScan (http://gibk21.bse.kyutech.ac.jp/PrognoScan/index.html) is an emerging database for the meta-analysis of the prognostic value of genes), the database can evaluate potential tumor markers and therapeutic targets (35). We used PrognoScan to evaluate the prognosis of BRCA patients with different expression levels of BUB1.

Kaplan-Meier Plotter (www.kmplot.com) (36) is an available website and analyzes the survival of BRCA patients based on the *BUB1* mRNA expression. The cutoff points of each amplified gene sample and non-amplified sample were set in the quartile with the higher amplification value.

DrugBank analysis

The DrugBank (https://go.drugbank.com/) database contains comprehensive molecular information, mechanisms, interactions, and targets of drugs (37). We used Drugbank to analyze the pharmaco-transcriptomics of BUB1.

Statistical analysis

The experimental data were analyzed by SPSS 28.0 statistical software (IBM). The correlation analysis between the expression levels of BUB1 and the benign and malignant breast tumors of breast was conducted using the Chi-square test. P<0.05 was considered statistically significant.

Results

The overall workflow diagram of this study is summarized in *Figure 1*.

BUB1 expression in pan-cancer and BUB1 localization in cells

To explore the expression levels of BUB1 in various types of normal human tissues, we evaluated the mRNA expression of *BUB1* using the HPA database. We also used the TIMER2.0 database to compare the differential expressions of BUB1 between tumor tissues and adjacent normal tissues. As shown in *Figure 2A*, the tissues with the highest BUB1 expression were the testes, thymus, tonsils, and lymph nodes. *Figure 2B* shows that BUB1 was highly expressed in various tumor types including bladder urothelial carcinoma and breast invasive carcinoma. Notably, the expression of BUB1 in basal-like subtype was higher than that in luminal subtypes or HER2-enriched subtype in BRCA (*Figure 2*). Moreover, through the HPA platform, we confirmed that BUB1 was mainly located in the nucleoplasm and additionally in the cytosol (*Figure 3*).

Functional enrichment analysis of BUB1

The top 200 genes co-expressed with *BUB1* obtained from the cBioPortal had an average Spearman's correlation coefficient of 0.778. We then performed functional and pathway enrichment analyses based on these genes using the DAVID database. For BP, these genes were mainly enriched in processes such as cell division, mitotic sister chromatid segregation, and chromosome segregation (*Figure 4A*). For CC, these genes were mainly enriched in components such as nucleoplasm, kinetochore, and nucleus (*Figure 4B*). For MF, these genes were mainly enriched in ATP binding, protein binding, and microtubule binding (*Figure 4C*). KEGG pathway analysis suggested that they were mainly enriched in cell cycle, oocyte meiosis, and DNA replication (*Figure 4D*).

Enrichment analysis of genes and miRNAs correlated with BUB1 in BRCA

We employed the LinkedOmics database to determine the co-expressed genes correlated with *BUB1* in BRAC. The volcano plot (*Figure 5A*) illustrated genes with a positive or negative correlation with *BUB1*, where the red dots

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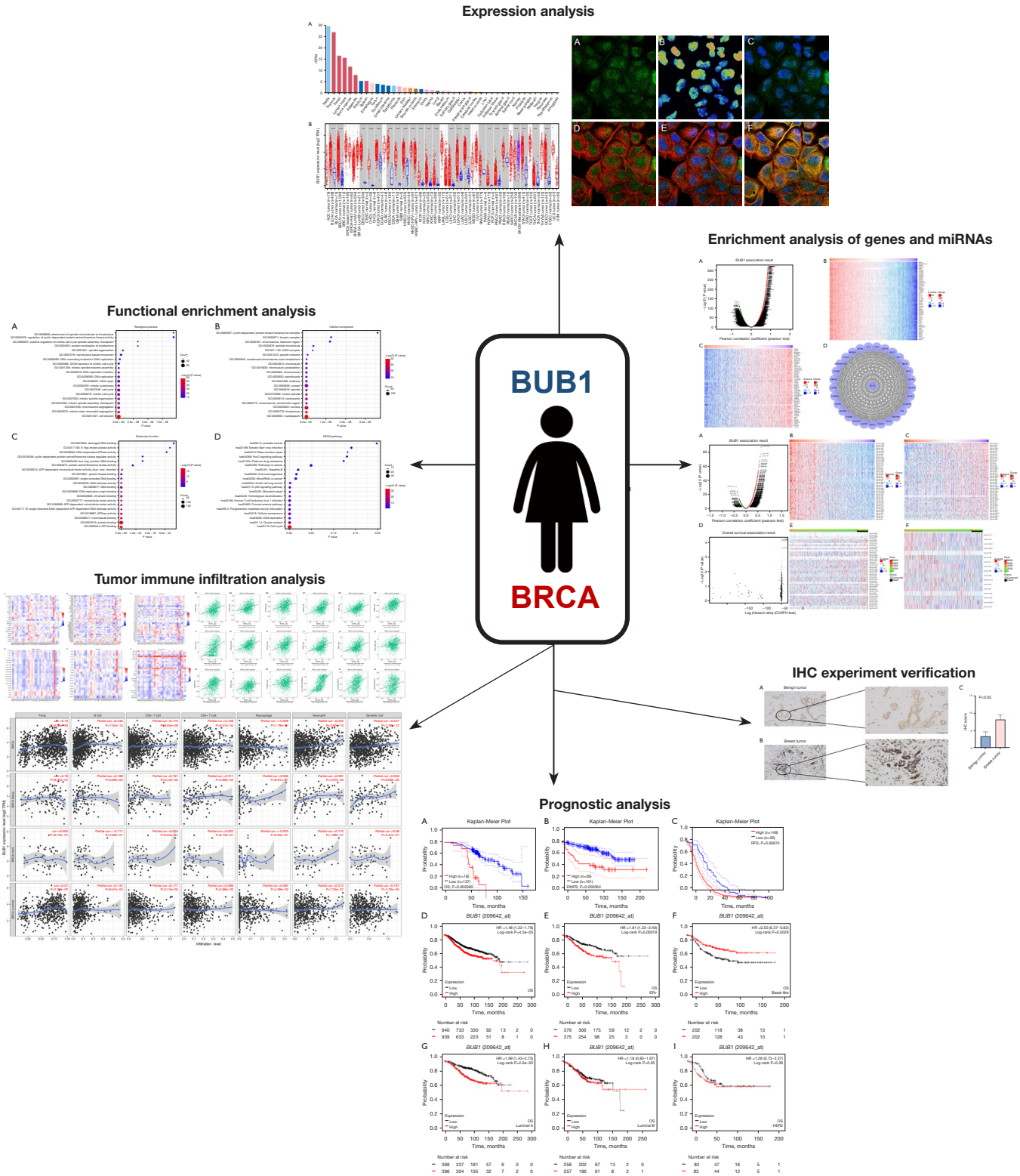

Figure 1 The workflow chart of the study. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BRCA, breast cancer; IHC, immunohistochemistry; MHC, major histocompatibility complex; TPM, transcript per million; OS, overall survival; DMFS, distant metastasis free survival; RFS, relapse free survival; HR, hazard ratio; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2.

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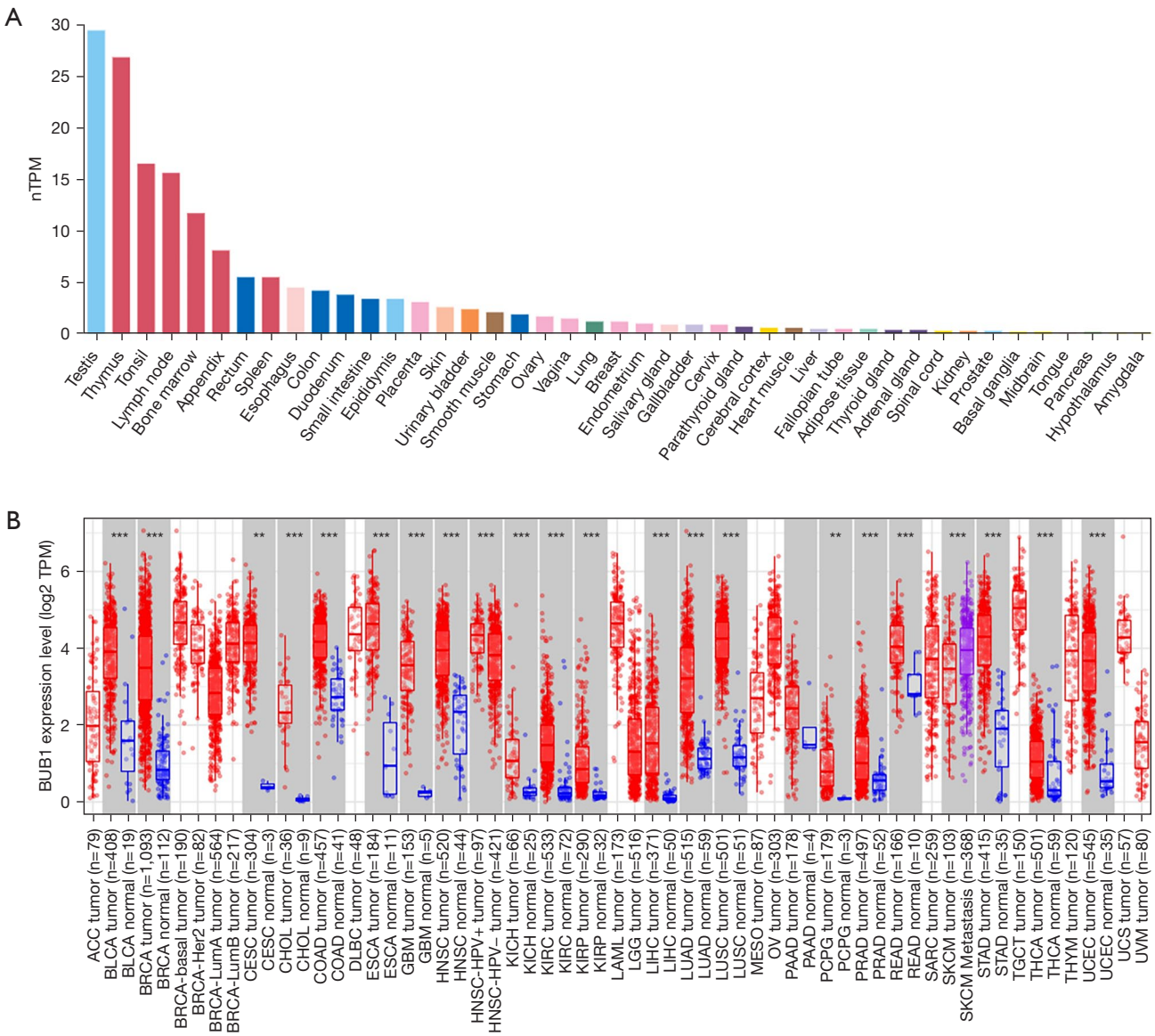

Figure 2 BUB1 expression in various human normal tissues. (A) *BUB1* mRNA expression profiles in normal human tissues. (B) The expression differences of BUB1 between normal and tumor tissues. P value significant codes: **, P<0.01; ***, P<0.001. TPM, transcripts per million; ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, invasive breast carcinoma; CESC, cervical and endocervical cancer; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck cancer; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; SKCM-metastasis, skin cutaneous melanoma metastasis; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumor; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.

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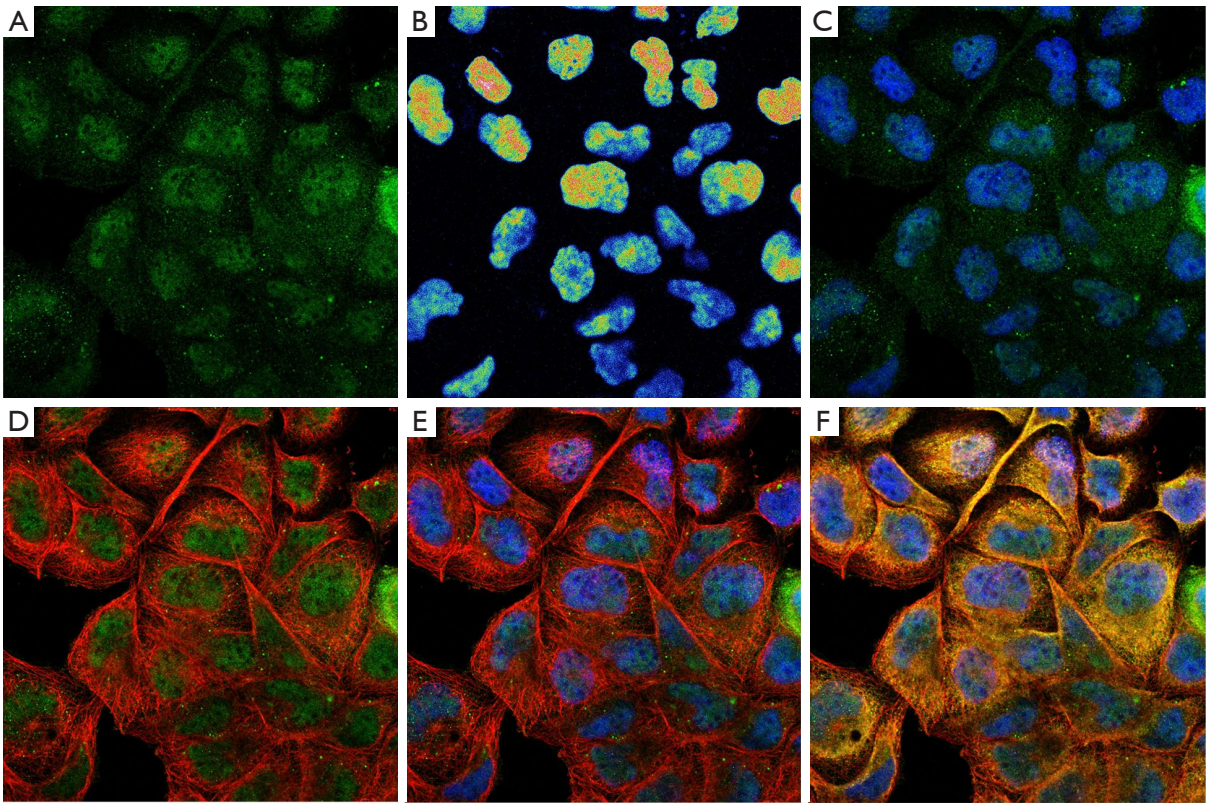

Figure 3 Subcellular mapping of the BUB1 human proteome. (A-F) Subcellular mapping of BUB1 human proteome A-431, HPA006123). (A) Target protein (https://images.proteinatlas.org/6123/80_C7_1_green_medium.jpg). (B) Intensity and nucleus (https://images.proteinatlas.org/6123/80_C7_1_blue_lut_medium.jpg). (C) Target protein and nucleus (https://images.proteinatlas.org/6123/80_C7_1_blue_green_medium.jpg). (D) Target protein and microtubule (https://images.proteinatlas.org/6123/80_C7_1_red_green_medium.jpg). (E) Target protein, nucleus, and microtubule (https://images.proteinatlas.org/6123/80_C7_1_blue_red_green_medium.jpg). (F) Target protein, nucleus, endoplasmic reticulum, and microtubule (https://images.proteinatlas.org/6123/80_C7_1_blue_red_green_yellow_medium.jpg).

indicated positive correlations with *BUB1* and the green dots indicated negative correlations. The top 50 significant genes positively or negatively correlated with BUB1 were presented by a heat map (*Figure 5B,5C*). The top 5 genes having a positive correlation (Cor) with *BUB1* in BRCA were *CKAP2L* (Cor =0.9375, P=1.000e−316), *NCAPH* (Cor =0.9226, P=1.000e−316), *TPX2* (Cor =0.9159, P<1.000e−316), *DLGAP5* (Cor =0.9140, P=1.000e−316), and *SGOL1* (Cor =0.9109, P=1.000e−316). The top 5 genes having a negative correlation with *BUB1* in BRCA were *CIRBP* (Cor =−0.6482, P=2.803e−131), *TENC1* (Cor =−0.6258, P=7.089e−120), *RAPGEF3* (Cor =−0.685, P=1.096e−111), *CYB5D2* (Cor =−0.6069, P=5.683e−111), and *SLC27A1* (Cor =−0.6024, P=6.621e−109) (*Table 1*). *Figure 5D* shows that BUB1 interacted strongly with 29 proteins/genes.

Next, we also performed Pearson correlation analysis on miRNAs and CoxPH test of *BUB1* (*Figure 6*). *Figure 6A* shows the volcano plot of *BUB1*-related miRNAs. The miRNAs hsa-mir-106b, hsa-mir-130b, hsa-mir-1307, hsa-mir-942, and hsa-mir-1301 were correlated positively with *BUB1*, while the miRNAs hsa-mir-101-2, hsa-mir-30a, hsa-mir-29c, hsa-mir-190b, and hsa-mir-10b were negatively correlated with BUB1. *Figure 6B,6C* shows the top 50 miRNAs with positive or negative correlations with *BUB1*. We constructed a volcano plot of *BUB1*-correlated miRNAs for the prognosis of BRCA patients (*Figure 6D*). In addition, LinkedOmics was used to analyze the correlations between the top 50 important miRNAs and the BRCA survival quality (*Figure 6E,6F*). The 5 miRNAs including hsa-mir-302a, hsa-mir-586, hsa-mir-3923, hsa-mir-621, and hsa-mir-549 had the most significant positive correlations

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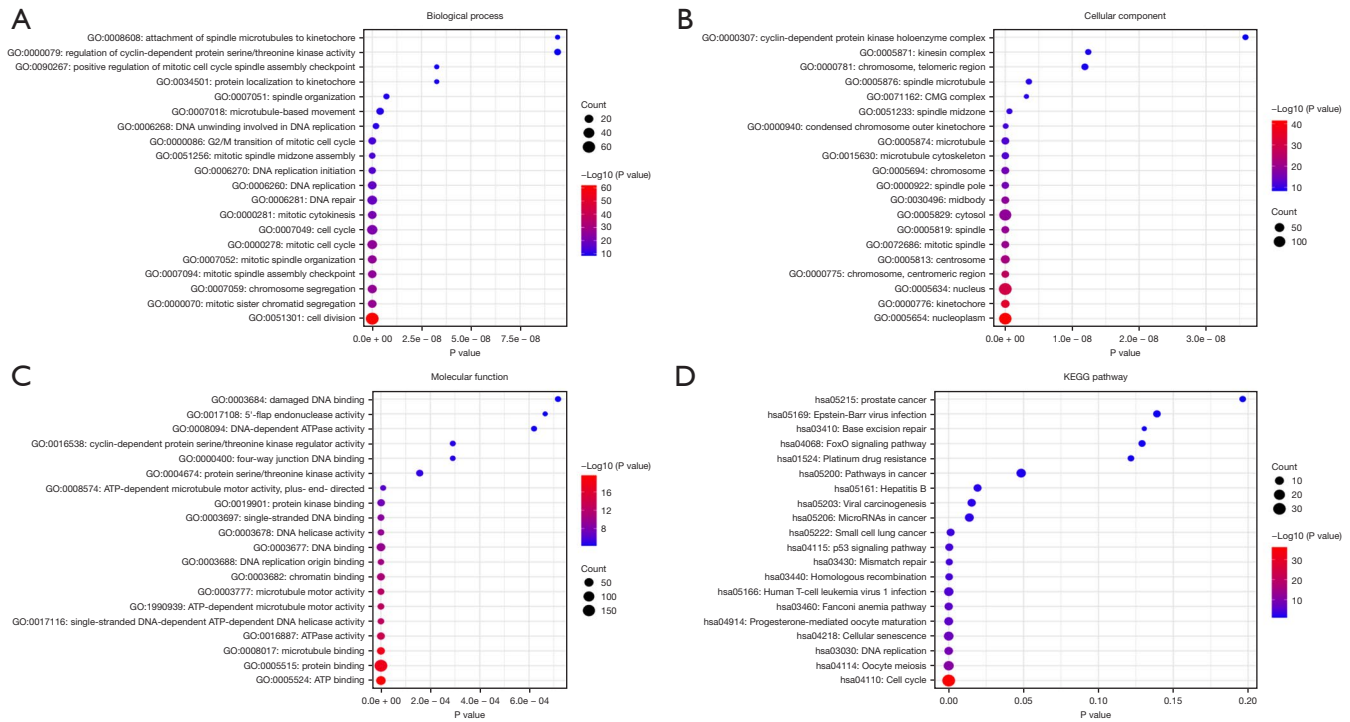

Figure 4 GO functional and KEGG pathway enrichment analyses of genes co-expressed with *BUB1* (Database for Annotation, Visualization, and Integrated Discovery, DAVID) (A-D). GO functional and KEGG pathway enrichment analyses based on the co-expressed genes using the DAVID database. (A) BP; (B) CC; (C) MF; (D) KEGG pathway enrichment analysis. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function.

with the survival quality of patients. Importantly, hsa-mir-3923 (Cor =9.621e−02, P=8.243e−03), hsa-mir-621 (Cor =9.408e−02, P=9.793e−03), and hsa-mir-549 (Cor =1.506e−01, P=3.327e−05) were all positively correlated with BUB1.

Association of BUB1 mRNA expression with clinical significance in BRCA

BUB1 mRNA expression was analyzed in the METABRIC study (n=1,904), and there were 88 samples in the BUB1 mRNA expression altered group (n=88), including 86 high expression samples and 2 low expression samples. The patients in the *BUB1* mRNA expression altered group were frequently diagnosed invasive ductal carcinoma with higher histological grades (*Table 2*). More interestingly, ER-negative, HER2-negative, and basal-like phenotype in the PAM50 classification were more commonly seen in the *BUB1* mRNA expression altered group. Besides, the age at diagnosis in the altered group was younger than that in the non-altered group.

Association of BUB1 expression with immune cell infiltration in BRCA

By using the TISIDB database, we constructed the heat maps showing the correlation between the expression of BUB1 and different tumor immunoinhibitors, immunostimulators, chemokines, MHC molecules, receptors, and lymphocytes in multiple cancers (*Figure 7A-7F*). The colors and their intensity on the bar scale denoted the nature of the correlation, with the darker shade of blue indicating more positive correlations (close to 1) and the darker shade of red indicating more negative correlations (closer to −1). We found that the top three immunoinhibitors positively correlated with BUB1 in BRCA were IDO1 (Cor =0.336, P<2.2e−16), LAG3 (Cor =0.297, P=4.31e−24), and CTLA4 (Cor =0.275, P=1.73e−20) (*Figure 7*, G1-G3); the top three immunostimulators positively correlated with BUB1 in BRCA were ULBP1 (Cor =0.433, P<2.2e−16), PVR (Cor =0.431, P<2.2e−16), and CD80 (Cor =0.364, P<2.2e−16) (*Figure 7*, H1-H3); the top three chemokines positively correlated with BUB1 in BRCA were CCL7 (Cor =0.435,

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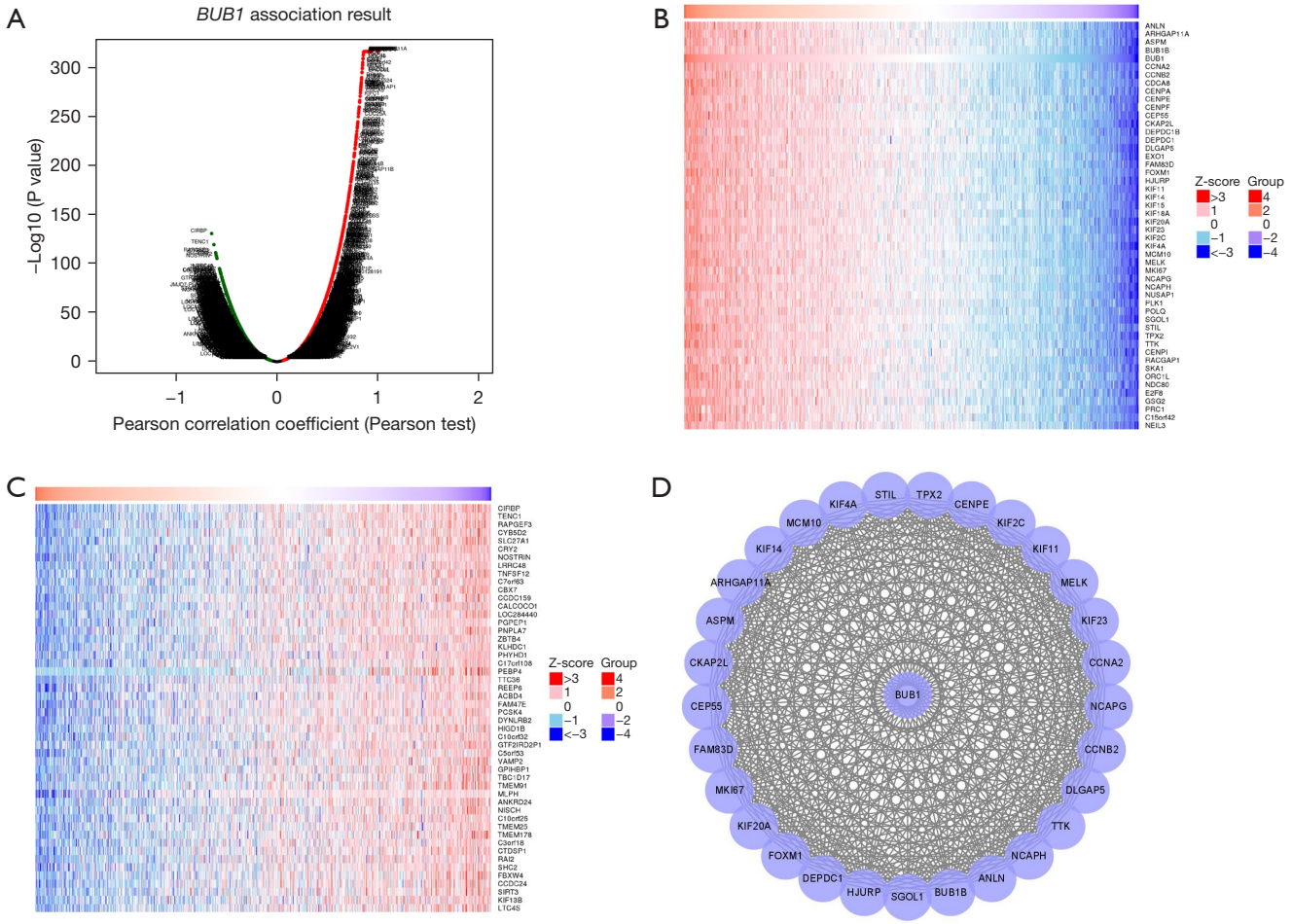

Figure 5 Genes highly correlated with *BUB1*. (A) Volcano plot showing genes correlated with *BUB1* through Pearson's test in BRCA. One dot represents a gene, red dots represent genes that are positively correlated with BUB1, and green dots represent genes that are negatively correlated with *BUB1*. (B,C) Heat maps showing the top 50 genes positively and negatively correlated with *BUB1* in BRCA; red (positive), blue (negative). (D) Protein-protein network of *BUB1* and its significantly associated genes. BRCA, breast cancer.

P<2.2e−16), CXCL10 (Cor =0.399, P<2.2e−16), and CCL18 (Cor =0.368, P<2.2e−16) (*Figure* 7, I1-I3); the top three MHC molecules positively correlated with BUB1 in BRCA were TAP2 (Cor =0.385, P<2.2e−16), TAP1 (Cor =0.355, P<2.2e−16), and HLA-DOB (Cor =0.171, P=1.2e−08) (*Figure* 7, J1-J3); the top three receptors positively correlated with BUB1 in BRCA were CCR8 (Cor =0.343, P<2.2e−16), CCR1 (Cor =0.283, P=1.23e−21), and CXCR6 (Cor =0.155, P<2.49e−07) (*Figure* 7, K1-K3); the top three lymphocytes positively correlated with BUB1 in BRCA were Act CD4 (Cor =0.66, P<2.2e−16), Th2 (Cor =0.388, P<2.2e−16), and Tgd (Cor =0.202, P=1.35e−11) (*Figure* 7, L1-L3). Furthermore, we investigated the correlation between the transcriptional level of BUB1 and immune infiltration in BRCA through the TIMER database. The expression of BUB1 was correlated positively with tumor purity (Cor =0.13, P=4.02e−05), B cells (Cor =0.246, P=7.04e−15), $CD8^+$ T cells (Cor =0.175, P=3.85e−08), $CD4^+$ T cells (Cor =0.108, P=8.27e−04), neutrophils (Cor =0.252, P=2.90e−15), and dendritic cells (Cor =0.227, P=1.38e−12), while BUB1 expression had no significant association with macrophages (Cor =−0.009, P=7.78e−01). We also analyzed the correlation between the transcriptional level of BUB1 and immune infiltration in BRCA molecular subtypes. In basal-like BRCA, the expression of BUB1 was correlated positively with B cells (Cor =0.189, P=3.59e−02), $CD8^+$ T cell (Cor= 0.191, P=3.42e−02), $CD4^+$ T cells (Cor =0.311, P=4.88e−04), neutrophils (Cor =0.381, P=4.07e−05), and

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Table 1 The top 10 genes with highly positive and negative correlations with BUB1 in BRCA

Gene	Correlation	P value
BUB1	1.0000	1.000e–316
CKAP2L	0.9375	1.000e–316
NCAPH	0.9226	1.000e–316
TPX2	0.9159	1.000e–316
DLGAP5	0.9140	1.000e–316
SGOL1	0.9109	1.000e–316
BUB1B	0.9072	1.000e–316
KIF4A	0.9057	1.000e–316
CEP55	0.9044	1.000e–316
CCNA2	0.8999	1.000e–316
CIRBP	–0.6482	2.803e–131
TENC1	–0.6258	7.089e–120
RAPGEF3	–0.6085	1.096e–111
CYB5D2	–0.6069	5.683e–111
SLC27A1	–0.6024	6.621e–109
CRY2	–0.5996	1.153e–107
NOSTRIN	–0.5954	8.086e–106
LRRC48	–0.5706	2.072e–95
TNFSF12	–0.5688	1.080e–94
C7orf63	–0.5652	2.736e–93

BRCA, breast cancer.

dendritic cells (Cor =0.359, P=8.69e–05), while BUB1 had no significant association with tumor purity (Cor =0.019, P=8.35e–01) and macrophages (Cor =0.036, P=6.91e–01). In HER2-positive BRCA, BUB1 expression was not significantly correlated with tumor purity, B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells (P>0.05), while in luminal BRCA subtypes, BUB1 expression was correlated positively with tumor purity (Cor =0.21, P=7.38e–07), B cells (Cor =0.142, P=9.41e–04), $CD8^+$ T cells (Cor =0.177, P=3.74e–05), $CD4^+$ T cells (Cor =0.096, P=2.68e–02), macrophages (Cor =0.084, P=4.98e–02), neutrophils (Cor =0.212, P=7.34e–07), and dendritic cells (Cor =0.187, P=1.33e–05) (*Figure 8*).

Validation of the protein levels of BUB1 in clinical samples

To verify the results derived from the published databases, 24 BRCA tissue samples and 24 fibroadenoma tissue samples with complete clinical data were collected. Immunohistochemistry (IHC) was conducted following a standard operation procedure in our clinical laboratory. The BUB1 protein level was verified by IHC in BRCA tissues and fibroadenoma tissues (*Figure 9*). According to relative protein level of BUB1, all BRCA patients were categorized to the high expression group (n=24), and breast fibroadenoma patients were divided into the low (n=18) and high (n=6) expression groups. BUB1 was overexpressed in BRCA tissues (*Figure 9B*) compared to benign tumor tissues (fibroadenoma of breast) (P<0.01) (*Figure 9A*). *Figure 9C* confirms that the protein level of BUB1 was higher in BRCA tissues than that in the breast fibroadenoma tissues (P<0.01).

Prognostic value of BUB1 mRNA level in BRCA patients

To determine the role of BUB1 in BRCA prognosis, we further studied the correlation between BUB1 expression and BRCA survival through the PrognoScan and Kaplan-Meier Plotter databases. *Figure 10A-10C* show the overall survival (OS) curves, distant metastasis-free survival (DMFS) curves, and relapse-free survival (RFS) curves of BRCA patients. We discovered that BRCA patients with low BUB1 expression had a longer survival time than those with high BUB1 expression in the OS, DMFS, and RFS curves (P<0.05). *Figure 10D-10I* showed the OS curves across BRCAs, ER+, luminal A, luminal B, HER2+, and basal-like BRCAs from the Kaplan-Meier Plotter database. We observed that low expression of BUB1 was associated with favorable prognosis in BRCA patients (*Figure 10*). *Figure 10A-10D* showed that BRCA patients with higher BUB1 expression had worse prognosis than those with high BUB1 expression in the OS curves, DMFS curves, and RFS curves (P<0.05). Furthermore, we found that ER+ and luminal A BRCAs with low BUB1 expression had a longer survival time than those with high BUB1 expression in the OS curves (P<0.05), but basal-like BRCAs with high BUB1 expression had a longer survival time than those with low BUB1 expression in the OS curves (P<0.05) (*Figure 10E-10G*). However, there was no statistical difference between low BUB1 expression and high BUB1 expression in luminal B and HER2+ BRCAs (*Figure 10H-10I*). These results revealed that BUB1 expression was significantly associated with the prognosis of BRCA patients.

Through the DrugBank database, we found that acetaminophen, estradiol, and silver nitrate could result in increased *BUB1* mRNA expression. Similarly, cyclosporine,

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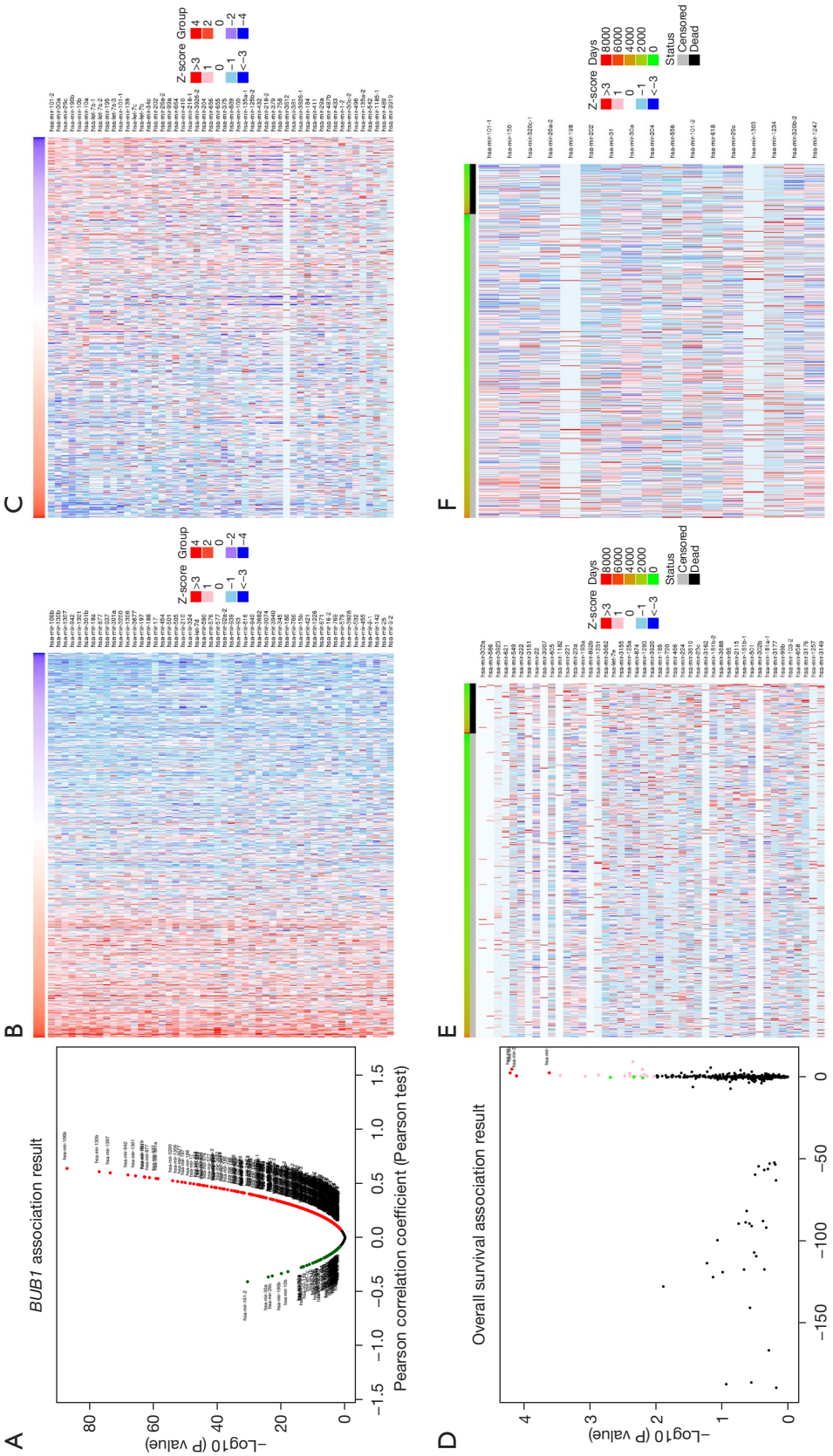

Figure 6 miRNAs highly correlated with BUB1. (A) Volcano plot showing BUB1-related miRNAs; one dot represents a miRNA, red dots represent miRNAs that are positively correlated with BUB1, and green dots represent miRNAs that are negatively correlated with BUB1. (B,C) Heat maps showing the significant miRNAs positively and negatively correlated with BUB1. (D) Volcano plot showing the miRNAs related to the overall survival of BRCA, one dot represents one miRNA. (E,F) miRNAs positively and negatively related to the overall survival of BRCA. BRCA, breast cancer.

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Table 2 Comparison of *BUB1* mRNA expression between the altered group (n=88) and the non-altered (n=1,816) group in the METABRIC study

Characteristics	Altered (%)	Non-altered (%)	P
Histological type			
IDC	77 (88.51)	1,377 (76.42)	
ILC	4 (4.60)	138 (7.66)	
Medullary	4 (4.60)	21 (1.17)	5.82e−3
IMixed	1 (1.15)	206 (11.43)	
Mucinous	1 (1.15)	21 (1.17)	
Other	0	39 (2.17)	
Three-gene classifier			
ER+/HER2−/proliferation high	3 (4.23)	600 (36.83)	
ER+/HER2−/proliferation low	2 (2.82)	617 (37.88)	<1.00e−10
HER2+	8 (11.27)	180 (11.05)	
ER−/HER2−	58 (81.69)	232 (14.24)	
Neoplasm histological grade			
Grade 1	0	165 (9.46)	
Grade 2	6 (6.82)	734 (42.09)	<1.00e−10
Grade 3	82 (93.18)	845 (48.45)	
PAM50 type			
Basal	60 (68.18)	139 (7.65)	
HER2	7 (7.95)	213 (11.73)	
Luminal A	1 (1.14)	678 (37.33)	
Luminal B	4 (4.55)	457 (25.17)	<1.00e−10
NA	0	6 (0.33)	
Normal	1 (1.14)	139 (7.65)	
Claudin-low	15 (17.05)	184 (10.13)	
Age at diagnosis (median)	52.77 years	62.10 years	1.53e−8

χ^2 test P values in the last column refer to comparisons between the amplified and non-amplified groups, and the cases with no available information for characteristic comparisons are excluded. IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; IMixed, invasive mixed ductal and lobular carcinoma; Mucinous, mucinous carcinoma; NA, not available.

dasatinib, calcitriol, dinitrochlorobenzene, etoposide, eugenol, fluorouracil, formaldehyde, fulvestrant, and irinotecan could result in decreased *BUB1* mRNA expression (*Table 3*).

Discussion

In our study, we found that the expression of BUB1 in BRCA tissues was significantly higher than that in adjacent normal tissues. Furthermore, the high expression of BUB1 was positively correlated with poor prognosis in BRCA. Therefore, *BUB1* might be an important gene that affected tumor activity and BRCA patient survival. We also inferred that BUB1 was a potential biomarker for predicting the effect of BRCA immunotherapy.

Nowadays, the diagnosis of BRCA has achieved considerable progress, such as pathological tissue biopsy and imaging examination. However, tumor recurrence and drug resistance still lead to unsatisfactory survival of BRCA patients. In addition, accurately predicting the prognosis of BRCA remains a major challenge. Even though many clinical, pathological, and molecular markers have been identified as prognostic markers of BRCA, including tumor staging, histological grading, lymph node metastasis, molecular subtypes, and Ki67 proliferation index, they lack accuracy in predicting prognosis and treatment response (38). High expression of BUB1 has been observed in hepatocellular carcinoma, prostate cancer, gastric cancer, and pancreatic ductal adenocarcinoma (39-42). Especially in hepatocellular carcinoma, BUB1 expression is significantly elevated and participates in tumor immune infiltration, affecting the tumor microenvironment and angiogenesis (43). Similarly, our study indicated that BUB1 expression was increased in bladder urothelial carcinoma, invasive breast carcinoma, cholangiocarcinoma, colon adenocarcinoma, esophageal carcinoma, and other tumors. Particularly, BUB1 expression was significantly increased in BRCA. There is currently limited research on the relationship between BUB1 expression and immune infiltration and prognosis of BRCA. Our study suggested that BUB1 might be a novel prognostic biomarker and potential therapeutic target for BRCA.

Accumulating studies have revealed that high expression of BUB1 is related to poor prognosis of many malignancies, including ovarian cancer, sarcomas, pancreatic ductal adenocarcinoma, hepatocellular carcinoma, and non-small cell lung cancer (40,44-47). The expression of BUB1 is correlated with the tumor size of pancreatic ductal adenocarcinoma (40). In our study, we also confirmed that high *BUB1* mRNA expression was indicative of poor prognosis of BRCA patients, especially in basal-like BRCA and luminal A BRCA patients.

Furthermore, BUB1 exhibited the potential to predict

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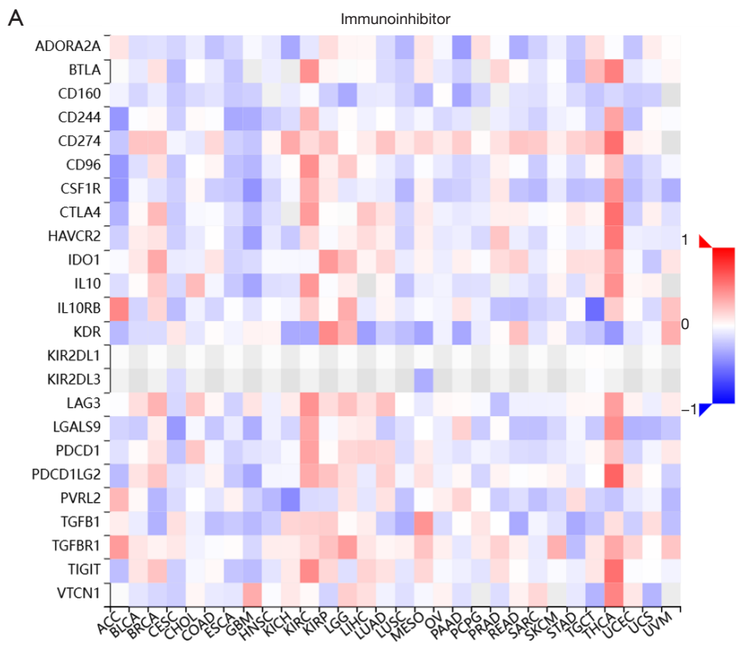

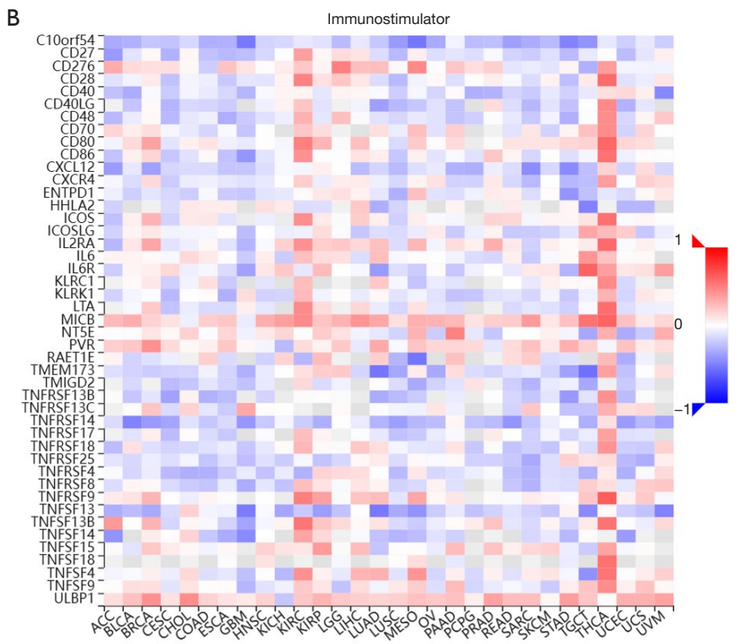

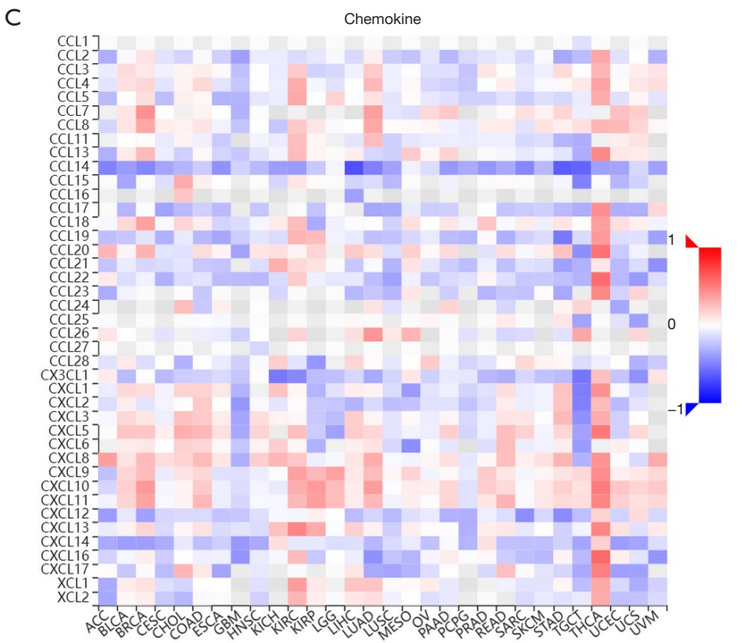

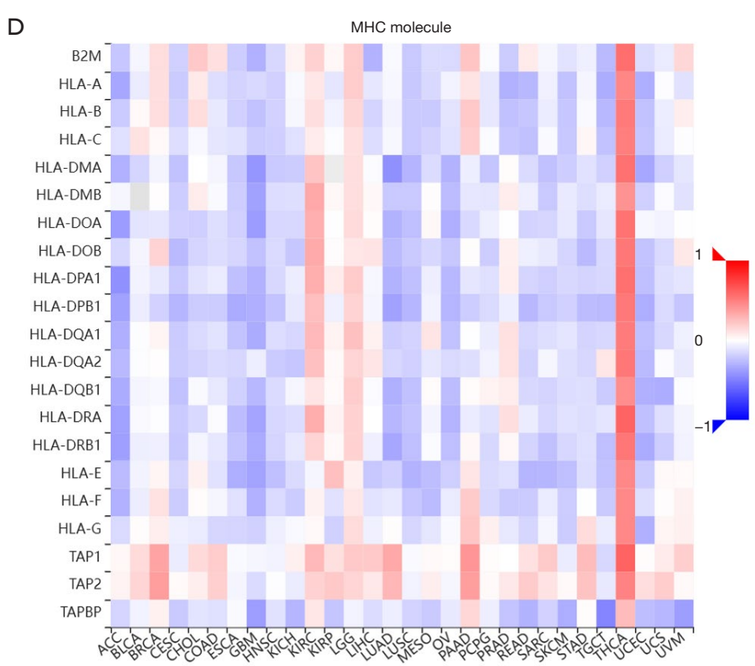

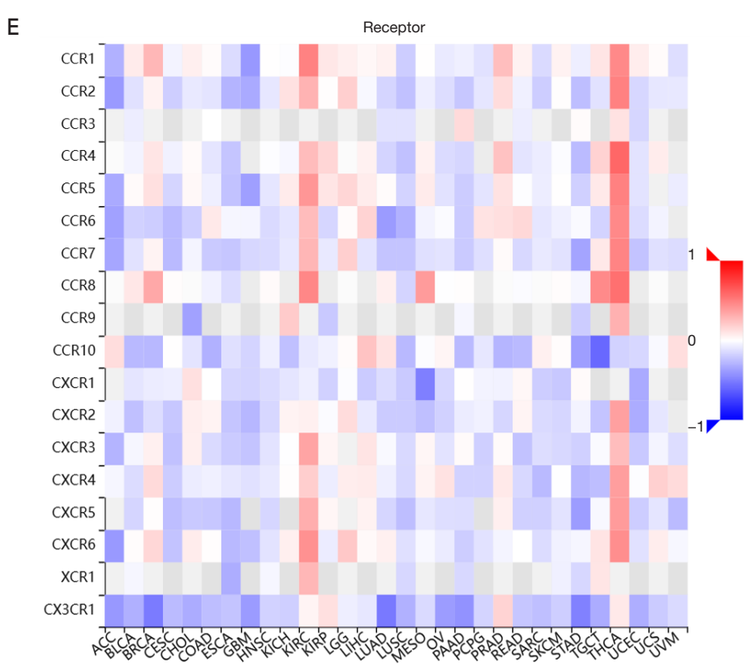

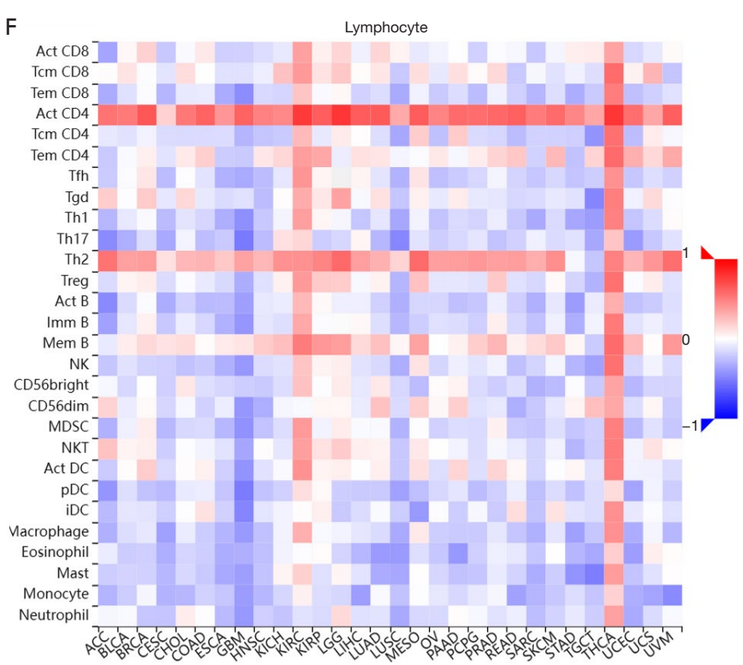

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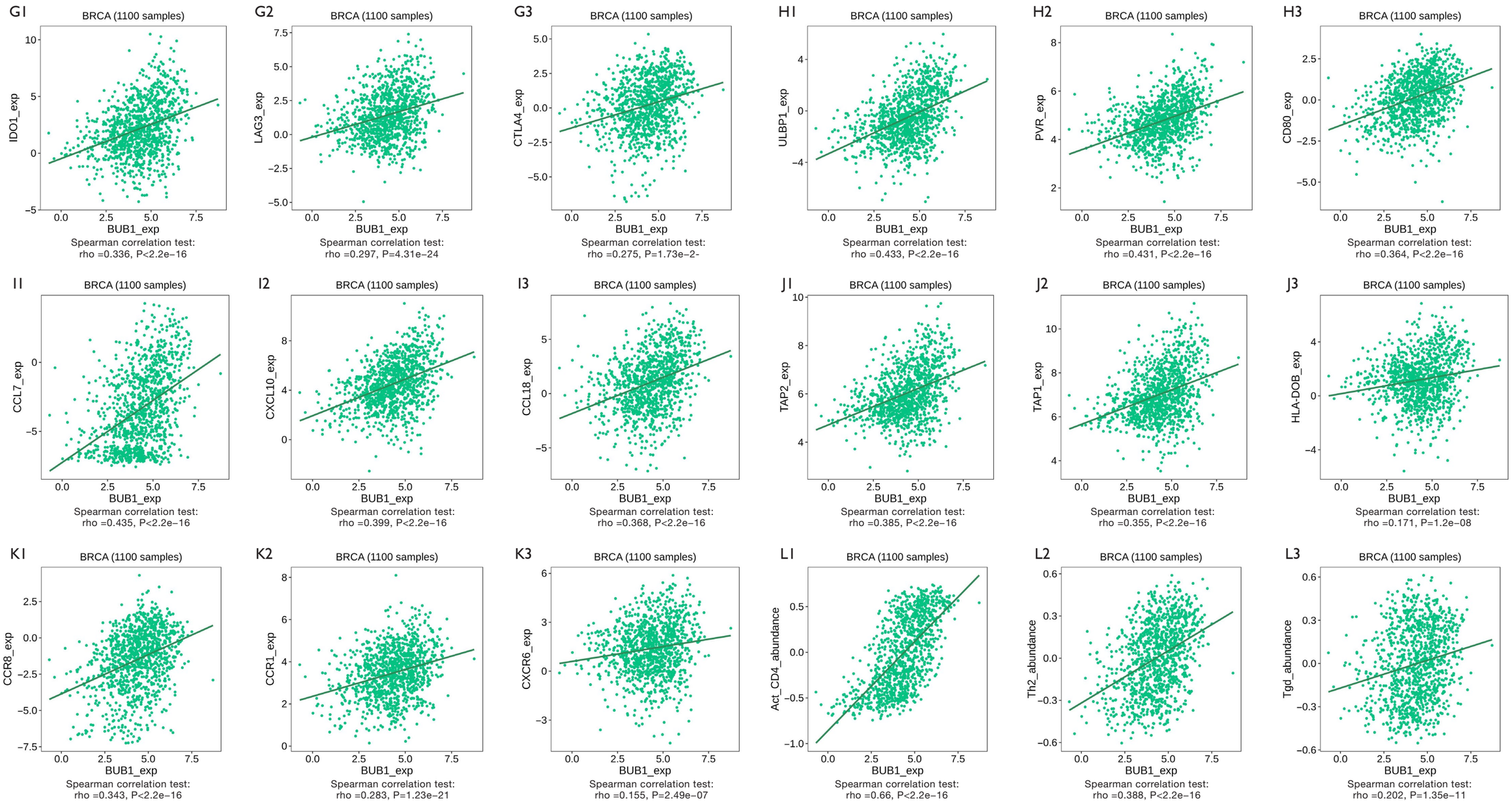

Figure 7 The correlation between the expression of BUB1 and immunoinhibitors, immunostimulators, chemokines, MHC molecules, receptors, and lymphocytes in different cancers (A-F). (G1-G3) Scatter plots of the top three immunoinhibitors positively correlated with BUB1 expression in BRCA; (H1-H3) scatter plots of the top three immunostimulators positively correlated with BUB1 expression in BRCA; (I1-I3) scatter plots of the top three chemokines positively correlated with BUB1 expression in BRCA; (J1-J3) scatter plots of the top three MHC molecules positively correlated with BUB1 expression in BRCA; (K1-K3) Scatter plots of the top three receptors positively correlated with BUB1 expression in BRCA; (L1-L3) scatter plots of the top three lymphocytes positively correlated with BUB1 expression in BRCA. MHC, major histocompatibility complex; BRCA, breast cancer.

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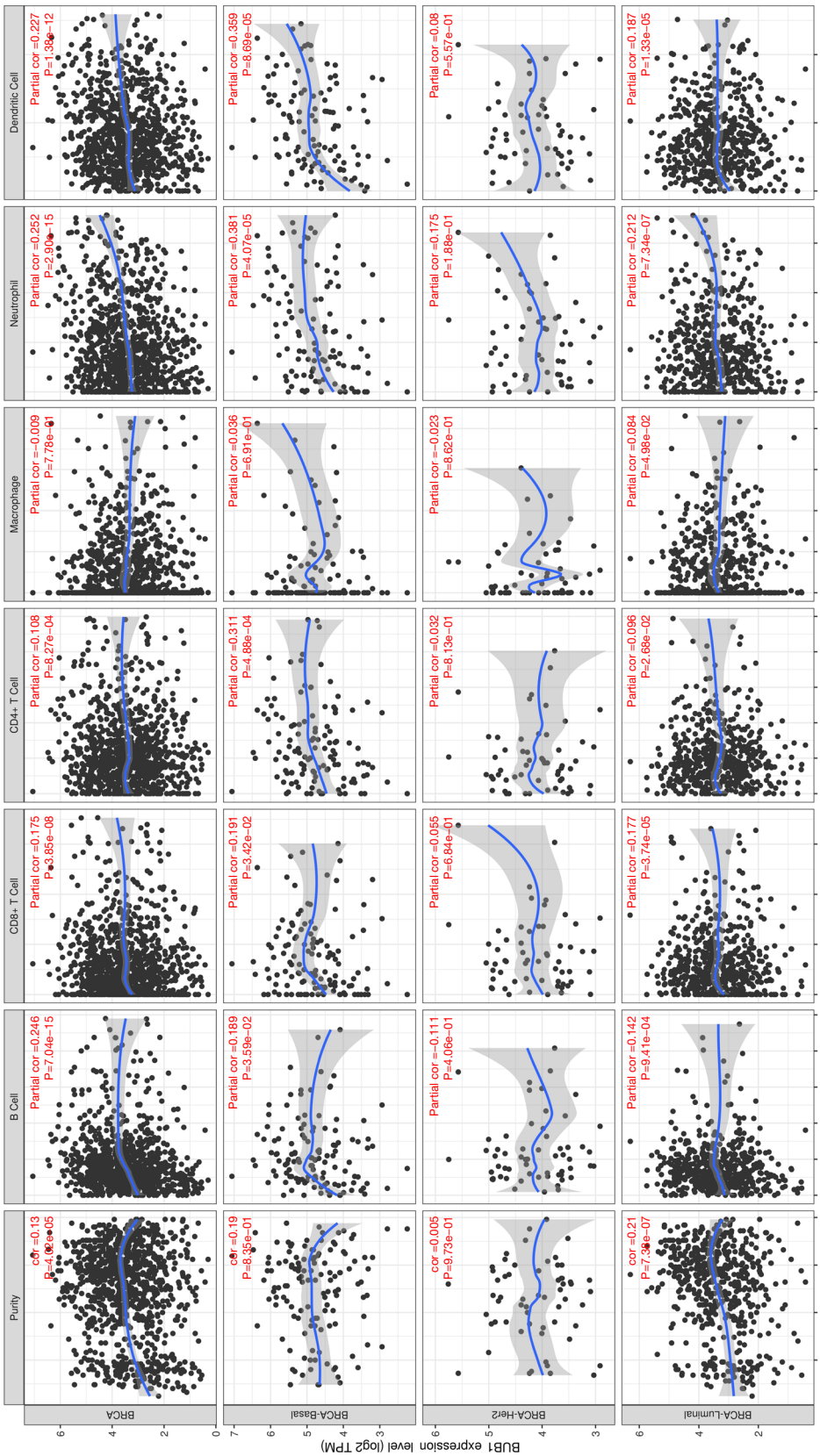

Figure 8 The correlation between BUB1 and immune infiltrates. TPM, transcripts per million; BRCA, breast cancer.

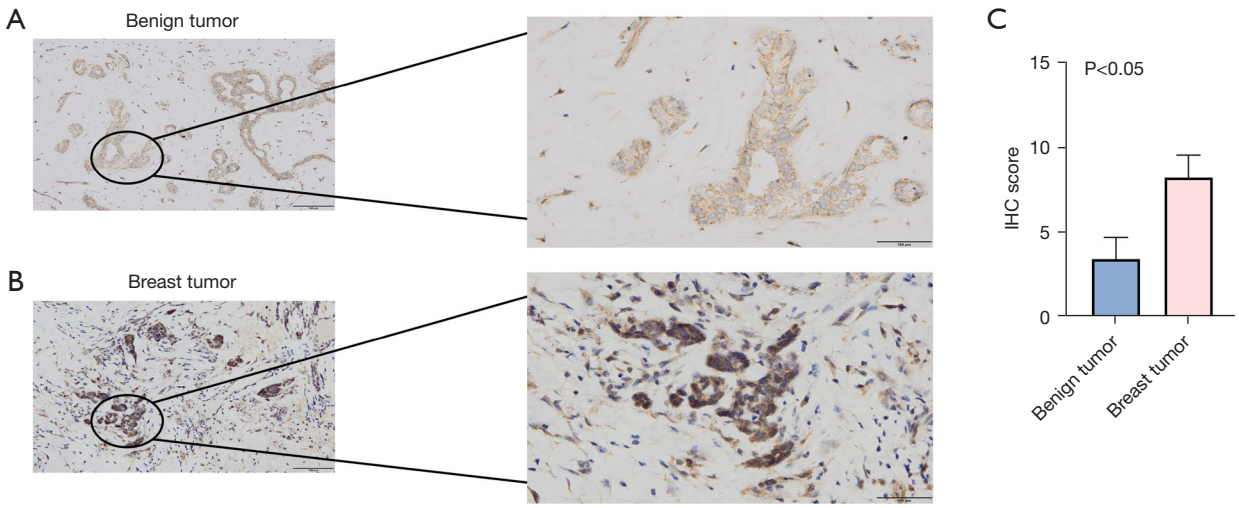

Figure 9 IHC staining validating the expression level of BUB1 in benign tumor and breast cancer. Representative IHC images of BUB1 in benign tumors and tumor breast tissues (A,B; ×400, scale bar =100 μm). The IHC score of BUB1 is presented as the mean ± SD (C). IHC, immunohistochemistry; SD, standard deviation.

the effectiveness of immunotherapy. A previous study has found that ten genes related to cell proliferation, including BUB1, can predict the therapeutic response to immune checkpoint inhibitors in negative renal cell carcinoma (48). Another study has shown that although *BUB1*, *CCNB2*, *CDC6*, *CDC20*, *CDK1*, and *MCM2* have negative impacts on the prognosis of cancer patients receiving chemotherapy and surgery, they are positively associated with the drug response of immunotherapy (49). Our study also discovered that the expression of BUB1 was correlated positively with tumor purity, B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells in luminal BRCAs; the expression of BUB1 had no significant correlation with tumor purity, B cells, $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and dendritic cells in HER2-positive BRCA, and the expression of BUB1 was correlated positively with B cells, $CD8^+$ T cell, $CD4^+$ T cells, neutrophils, and dendritic cells in basal-like BRCA. Based on these results, we inferred that BUB1 might provide a new direction for BRCA immunotherapy.

At present, some studies believe that BUB1 is related to tumorigenesis, invasion, and metastasis, and meanwhile, BUB1 participates in many important BPs such as cell proliferation, invasion, and migration. In hepatocellular carcinoma, abnormal expression of BUB1 activates the transforming growth factor-β (TGF-β) pathway and Smad protein to promote TGF-β-mediated epithelial mesenchymal transformation, resulting in tumor invasion and metastasis (50). BUB1 inhibition can reduce tumor cell proliferation (51). In addition, some researchers have revealed that BUB1 overexpression is associated with tumor cell proliferation (19). For example, BUB1 overexpression in transgenic mice yields a variety of tumors and accelerate myc-induced lymphomagenesis (52). It is probably because the cell cycle involves the process of cell growth and division, and uncontrolled cell proliferation is a hallmark of cancer progress (53,54). Song *et al.* (55) have previously demonstrated the role of the tumor-associated gene BUB1 in neuroblastoma. BUB1 may act as an oncogene via regulating the expression of various pathogenicity-related proteins, such as proteins involved in epithelial-mesenchymal transition (EMT), apoptosis, and the Wnt signaling pathway. A study identifies BUB1 regulates G2/M transition to promote the proliferation of bladder cancer cells, suggesting that it can serve as a prognostic marker for non-muscle-invasive bladder cancer (56). Zhang *et al.* (57) found that the mitotic spindle-related signature (KIF15, BUB1, CCNB2, CDK1, KIF4A, DLGAP5, ECT2, and ANLN) can more accurately predict the prognosis of patients. Hence, it is not surprising that BUB1 can predict the prognosis of the disease.

Conclusions

In our study, we found that *BUB1* mRNA expression dysregulation was more frequently detected in invasive

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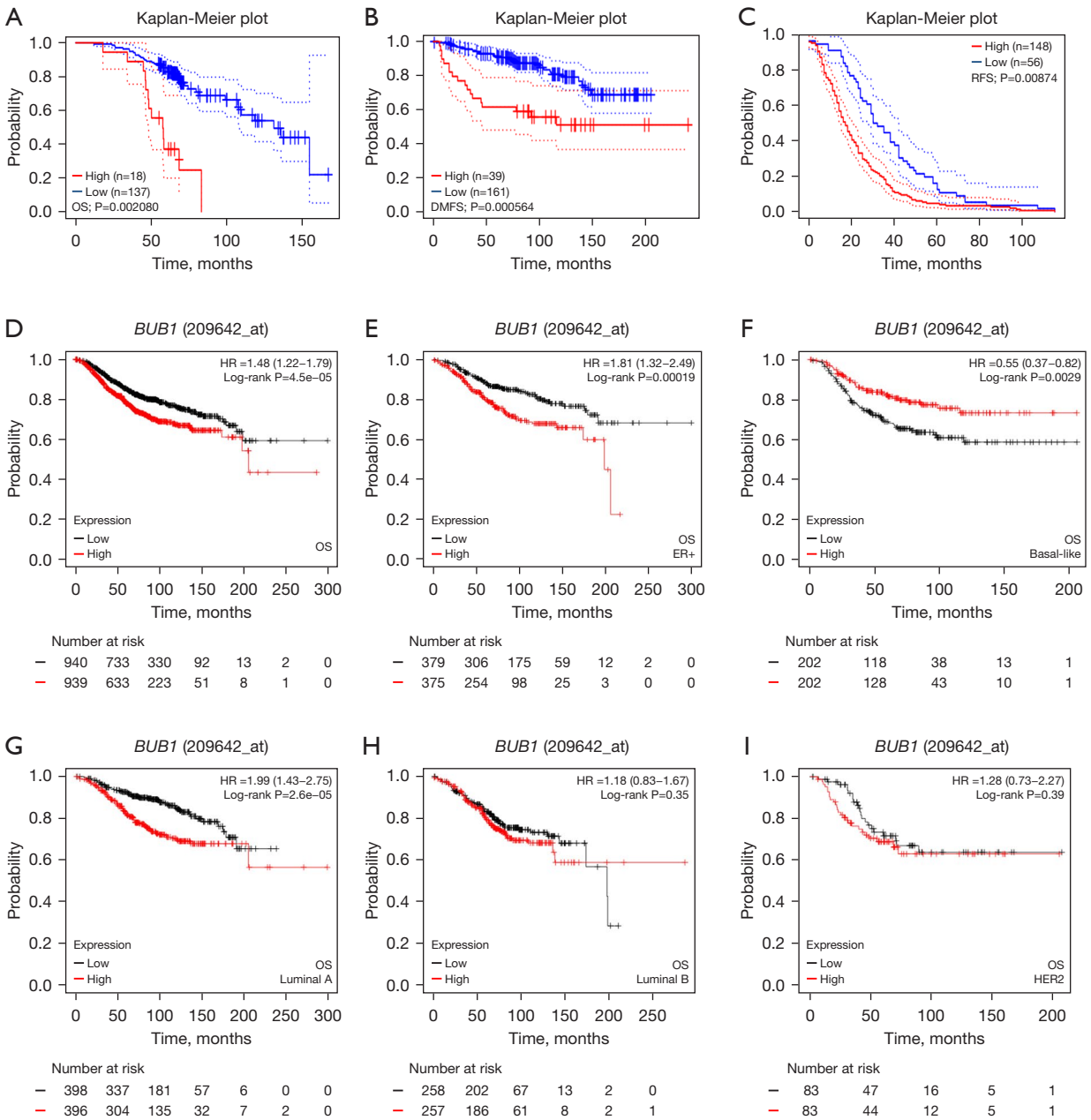

Figure 10 OS curves, RFS curves, and DMFS curves of BUB1 in BRCA. The blue or black curves represent BRCA patients with low expression of BUB1, and the red curves represent BRCA patients with high expression of BUB1 (the data in A-C are from the PrognoScan, the data in D-I are from the Kaplan-Meier Plotter database). OS, overall survival; DMFS, distant metastasis-free survival; RFS, relapse-free survival; HR, hazard ratio; ER+, estrogen receptor positive; HER2, human epidermal growth receptor 2; BRCA, breast cancer.

ductal carcinoma with higher histological grades and BRCAs with ER-negative, HER2-negative, and basal like phenotype. BUB1 might become a new immunotherapy target for BRCA. BUB1 was overexpressed in BRCA compared to benign tumor (fibroadenoma of breast). Through survival analyses, we revealed that BUB1 high expression was related to poor prognosis in BRCA. In summary, we indicated that BUB1 could be a potential molecular biomarker for evacuating prognosis and predicting effectiveness of immunotherapy for BRCA.

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Table 3 The pharmaco-transcriptomics of BUB1 (up or downregulation of genes caused by the metabolism of pharmaceutical compounds)

Drug	Change	Interaction	PubMed ID
Acetaminophen	Upregulated	Acetaminophen results in increased expression of BUB1 mRNA	22230336
Estradiol	Upregulated	Estradiol results in increased expression of BUB1 mRNA	19167446
Cyclosporine	Downregulated	Cyclosporine results in decreased expression of BUB1 mRNA	20106945
			21632981
			25562108
Dasatinib	Downregulated	Dasatinib results in decreased expression of BUB1 mRNA	20579391
Calcitriol	Downregulated	Calcitriol results in decreased expression of BUB1 mRNA	21592394
Dinitrochlorobenzene	Downregulated	Dinitrochlorobenzene results in decreased expression of BUB1 mRNA	17374397
Etoposide	Downregulated	Etoposide results in decreased expression of BUB1 mRNA	16120219
Eugenol	Downregulated	Eugenol results in decreased expression of BUB1 mRNA	17374397
Fluorouracil	Downregulated	Fluorouracil results in decreased expression of BUB1 mRNA	16709241
Formaldehyde	Downregulated	Formaldehyde results in decreased expression of BUB1 mRNA	23649840
Fulvestrant	Downregulated	Fulvestrant results in decreased expression of BUB1 mRNA	19016759
Irinotecan	Downregulated	Irinotecan metabolite results in decreased expression of BUB1 mRNA	15956246
Silver nitrate	Upregulated	Silver nitrate analog results in increased expression of BUB1 mRNA	22831968
Silver nitrate	Upregulated	Silver nitrate results in increased expression of BUB1 mRNA	22831968

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Footnote

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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). The study was approved by the Institutional Review Board (IRB) of the First Affiliated Hospital of Jinan University (approval No. KY-2023-116). This study does

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not involve human trials, personal privacy or commercial interests, so this study obtains IRB approval and waiver of informed consent. Confidentiality and anonymity were assured as no personal identifiers were used.

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