International Journal of General Medicine

Dovepress

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ORIGINAL RESEARCH

The Potential of FOXP3 in Predicting Survival and Treatment Response in Breast Cancer

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Background: Breast cancer (BC) continues to pose a substantial challenge to global health, necessitating an enhanced understanding of its fundamental mechanisms. Among its various pathological classifications, breast invasive carcinoma (BRCA) is the most prevalent. The role of the transcription factor forkhead box P3 (*FOXP3*), associated with regulatory T cells, in BRCA's diagnosis and prognosis remains insufficiently explored, despite its recognized importance.

Methods: We examined the mRNA expression profile of *FOXP3* in BRCA patients, assessing its correlation with disease detection, patient survival, immune checkpoint alterations, and response to anticancer drugs.

Results: Our analysis revealed significantly elevated *FOXP3* mRNA levels in BRCA patients, with a 95.7% accuracy for BRCA detection based on the area under the curve. High *FOXP3* mRNA levels were positively correlated with overall survival and showed significant associations with *CTLA4, CD274, PDCD1*, TMB, and immune cell infiltration status. Furthermore, *FOXP3* mRNA expression was linked to the efficacy of anticancer drugs and the tumor inflammation signature.

Discussion: These findings suggest that *FOXP3* serves as a promising biomarker for BRCA, offering valuable insights into its diagnosis and prognosis. The correlation between *FOXP3* expression and immune checkpoint alterations, along with its predictive value for treatment response, underscores its potential in guiding therapeutic strategies.

Conclusion: *FOXP3* stands out as an influential factor in BRCA, highlighting its diagnostic accuracy and prognostic value. Its association with immune responses and treatment efficacy opens new avenues for research and clinical applications, positioning *FOXP3* as a vital target for further investigation in BRCA management.

Keywords: breast cancer, *FOXP3*, regulatory T cells, immune checkpoint alterations, anticancer drugs response, immune cell infiltration

Introduction

BRCA burden is rising rapidly and varies widely from country to country. The incidence and mortality of BRCA in China and South Korea have increased rapidly, whereas those in the United States have declined.[1] In 2020, new cases of BRCA exceeded cases of lung malignancy (2.2 million), reaching 2.26 million and making it the most prevalent cancer in the world.[2]

Implementing strategies such as improving healthcare access, increasing health awareness, and employing efficient prevention measures are critical in containing the escalating burden of BRCA. In recent years, BRCA treatment has made great progress through the unremitting efforts of medical and pharmaceutical researchers.[3,4] However, the pathogenesis of BRCA, especially metastatic BRCA, still needs to be unraveled, and the lack of biomarkers for efficient treatment and diagnosis must be addressed.[5,6]

The tumor microenvironment (TME) is an intricate system, consisting of stromal, immune, and cancer cells. It has a critical function in the onset and advancement of tumors. Cells linked with the immune system can impact the progression of tumors by altering the TME. Regulatory T cells (Treg cells), which are suppressive cells tied to the

Received: 11 December 2023
Accepted: 25 March 2024
Published: 28 March 2024

immune system, are minimally present in healthy individuals, primarily to prevent the overactivity of immune cells leading to autoimmune diseases. Studies have shown an inverse relationship between the count of Treg cells and tumor prognosis. Their existence is favorable to the expansion, proliferation, encroachment, and metastasis of tumor cells.[7]

FOXP3, a transcription factor specifically associated with CD4+ CD25+ Treg cells, participates in Treg cell differentiation. *FOXP3* controls the regulation of gene transcription related to cancer occurrence, invasion, metastasis, differentiation, and angiogenesis.[8–11] Beyond its direct implications, the expression of *FOXP3* is regulated by tumor suppressor genes and epigenetic mechanisms. Epigenetics refers to a mechanism that regulates gene expression by altering chromatin structure without changing the DNA sequence, and has garnered significant interest for its potential role in modulating *FOXP3* expression.[12] Consequently, a strong link between *FOXP3* and BRCA exists. Research has also detected increased levels of *FOXP3* and other regulatory T cell-related molecules in BRCA tissues compared to normal tissues, which are linked to BRCA patients' prognoses.[13,14] Merlo et al reported that about 53% of 187 BRCA subjects expressed *FOXP3*, and suggested that *FOXP3* could be used as a predictor of poor prognosis in BRCA.[15] Understanding the diverse cis-regulatory elements at the *FOXP3* locus and how they undergo epigenetic modifications across different CD4+ T cell subsets is crucial. This not only enhances our comprehension of Treg cell biology but also offers hope for therapeutic interventions exploiting the suppressive capabilities of Tregs.[7,16] Therefore, the goal of this study was to scrutinize the mutation and expression of *FOXP3* in subjects with BRCA, utilizing the gene expression or alteration data accessible online. This is to ascertain the prognostic and diagnostic significance of *FOXP3* in BRCA.

Materials and Methods

Utilization of Tumour Immune Estimation Resource (TIMER) Database

The TIMER2 v2.0 database (http://timer.cistrome.org/)[17] was harnessed to explore the expression pattern of *FOXP3* in tumor tissues and their neighboring normal tissues. The correlation between the infiltration status of immune cells and the expression of *FOXP3* was also analyzed.

Acquisition of Ribonucleic Acid (RNA)-Sequencing (Seq) Data of *FOXP3* in Human BRCA

We procured expression data related to 1226 tumor tissues and 113 adjacent non-tumor tissues from The Cancer Genome Atlas (TCGA) (https://portal.gdc.cancer.gov/), focusing on the RNA-Seq data of *FOXP3* in BRCA. The selected samples encompassed mRNA data of *FOXP3* and relevant clinical details, such as T stage, progesterone receptor (PR) status.

Immunohistochemistry (IHC)

A total of 10 samples of paraffin-embedded of BRCA patients who received surgical intervention were provided by Hunan Provincial People's Hospital/The First Affiliated Hospital of Hunan Normal University. Tumor and adjacent non-tumor tissue samples were prepared into paraffin sections (4μm) and incubated at 4°C overnight with primary rabbit monoclonal antibodies targeting *FOXP3* at a dilution ratio of 1:200 (Santa Cruz Biotechnology, Santa Cruz, USA). Following this, sections were introduced to horseradish peroxidase-conjugated goat anti-rabbit antibody (1:400, Abcam, USA) for a period of 60 minutes at ambient temperature. Subsequently, the sections were stained with the 3,3-diaminobenzidine reagent, followed by a mild counterstain application with hematoxylin.

The Kaplan–Meier Plotter Analysis

The prognostic implications of *FOXP3* gene expression in patients with breast cancer (BRCA) were evaluated utilizing the Kaplan-Meier plotter (www.kmplot.com).[18,19] We examined survival outcomes for patients, encompassing progression-free survival (PFS), post-progression survival (PPS), distant metastasis-free survival (DMFS), and overall survival (OS). Patients were stratified into two groups based on *FOXP3* expression data: those with high expression and those with low expression.

Nomogram Construction for BRCA Survival Prediction

A nomogram was established using clinicopathological prognostic indices to predict the 1-, 3-, and 5-year overall survival (OS) probabilities of BRCA patients. The accuracy of the nomogram's predicted OS probabilities was evaluated against the observed actual probabilities using a calibration curve.

Alterations in *FOXP3* Genetics and Immune Checkpoints in Patients with BRCA

FOXP3 and immune checkpoint expression levels were analyzed using the cBioPortal (www.cbioportal.org) with a breast-invasive carcinoma dataset.[18,19] The Cancer Genome Atlas (TCGA)-related tumor data, including gene amplification, missense mutations, truncating mutations, and deep deletions, were extracted for BRCA. The "Mutations" module in cBioPortal provided information on the mutated sites of *FOXP3*, displayed as either a 3D structure or a schematic representation of the protein's structure.

STRINGS Analysis

The STRING database (www.string-db.org), an online platform for protein-protein interaction (PPI) analysis, was utilized in this study to examine the potential role of *FOXP3* in breast cancer.[20] By investigating the PPI network associated with *FOXP3*, we aimed to gain insights into its functional implications in BRCA.

GeneMANIA Analysis Implementation

GeneMANIA (www.genemania.org), an internet-based tool devised for gene function examination,[21] was utilized in this study. We employed GeneMANIA to discern the top 50 genes having the most significant interactions with *FOXP3*. This data was subsequently used to establish a gene-gene interaction (GGI) network, which further elucidated the functional correlations of *FOXP3* in the context of breast cancer.

Chemical-Gene Interaction

To explore the chemical-gene interactions involving *FOXP3*, we used the Comparative Toxicogenomics Database (CTD). By constructing an interaction network between *FOXP3* and various chemicals, we sought to identify specific chemicals that could potentially modulate *FOXP3* gene expression levels, either by increasing or decreasing its expression.

Utilization of Receiver Operating Characteristic (ROC) Plotter for Evaluating Drug Sensitivity

To gauge the effect of *FOXP3* expression on possible treatment options with anticancer drugs, we made use of the ROC plotter (http://www.rocplot.org/). This open-access database holds transcriptomic information that assists in predicting drug responses and validating biomarkers. Analyses were performed on samples demonstrating both a pathological complete response and a pathological response to *FOXP3*.

Analysis of Functional Enrichment

The 50 most relevant genes discerned from GeneMANIA were subjected to additional analysis to ascertain their positive or negative correlation with *FOXP3*. Classification of these genes was achieved through Gene Ontology (GO) enrichment analysis, based on cellular components, biological processes, and molecular functions. The R package ggplot2 was utilized to execute the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Moreover, using the R package with method="ssGSEA" as the parameter, gene set variation analysis was performed. The connection between pathway scores and specific genes was ascertained using Spearman correlation.

Statistical Analyses

The data were statistically analyzed utilizing R software (www.r-project.org). Visualization of clinical and gene expression data was achieved using the ggplot2 package in R.[22] To establish the cut-off values for *FOXP3, BRCA1, BRCA2*, and *ERBB2*, ROC curve analysis was deployed. Furthermore, the interrelation between 24 immune cells was ascertained

using Spearman correlation, and this was represented as a lollipop chart.[23,24] In instances where quantitative variables did not follow a normal distribution, Spearman correlation analysis was employed. A p-value of less than 0.05 was deemed to carry statistical significance.

Results

Analysis of Gene Expression Data

We examined the levels of *FOXP3* expression in different cancer types from TCGA utilizing TIMER2. The findings indicated a surge in *FOXP3* transcription within BRCA (Figure 1A). A summary of the relationship between *FOXP3* expression and clinical characteristics in BRCA patients is presented in Table 1. When comparing *FOXP3* expression in BRCA tissues (n = 1093) and neighboring normal tissues (n = 112), a significantly elevated level was observed in BRCA tissues, as revealed by unpaired data analysis (Figure 1B). However, BRCA patients with T4 stage showed lower *FOXP3* expression than those with T1/T2 stage (p-adjusted <0.05) (Figure 1C). Estrogen receptor (ER) or PR positive values were associated with lower *FOXP3* expression, while negative values demonstrated the opposite trend (p-adjusted <0.001) (Figure 1D and E). Interestingly, Basal and HER2 subtypes displayed higher *FOXP3* expression levels compared to Luminal A/B subtypes (p-adjusted <0.01) (Figure 1F).

IHC was utilized to probe the expression of *FOXP3* protein in BRCA tissues and their corresponding adjacent normal tissues. The findings suggested an escalation in *FOXP3* protein levels within BRCA tissues when juxtaposed with adjacent normal tissues (Figure 1G).

FOXP3 Kaplan-Meier Survival Analysis

The correlation between *FOXP3* mRNA expression and survival rates in BRCA patients was evaluated using Kaplan-Meier plots (Figure 2A). It was found that an elevation in *FOXP3* mRNA expression significantly corresponded to better OS in patients with breast cancer. Nonetheless, no significant correlation was discerned between *FOXP3* mRNA expression and DMFS. In the context of BRCA patients, an augmented expression of *FOXP3* was linked to extended recurrence-free survival (RFS), while a diminished expression of the gene was associated with PPS.

Co-Expression of *FOXP3*

The TCGA-BRCA cohort was employed to scrutinize the potential correlation and mutual exclusivity among *FOXP3* genes. The analysis unveiled significant co-expression associations between *FOXP3* and *ERBB2/BRCA2, ERBB2* and *BRCA1/BRCA2*, and *BRCA1* and *BRCA2* ($p < 0.05$). Conversely, the data did not demonstrate a significant co-expression relationship between *FOXP3* and *BRCA1* ($p > 0.05$) (Figure 2B).

FOXP3 as a Prospective Biomarker

An analysis of the ROC curve was conducted to evaluate the potential of *FOXP3* to differentiate between normal samples and breast-invasive malignant samples, juxtaposing it with control markers *BRCA2, BRCA1*, and *ERBB2*. The resulting data revealed the following values: *BRCA1* (AUC: 0.861, Accuracy: 0.973), *ERBB2* (AUC: 0.801, Accuracy: 0.900), *BRCA2* (AUC: 0.849, Accuracy: 0.957), and *FOXP3* (AUC: 0.932, Accuracy: 0.957) (Figure 2C). This suggests that *FOXP3* may function as a reliable biomarker to distinguish between normal and breast-invasive cancerous tissues.

FOXP3 in Prognostic Modeling

A nomogram, derived from the data gathered via Cox regression analysis, was constructed utilizing the "Regression modeling strategies" package in R software to evaluate the prognosis of subjects with BRCA. The model integrated five variables related to prognosis, which included *FOXP3* expression, race, along with the T, N, and M stages. The aggregate of individual points assigned to these variables was considered as the total points. The probability of survival over 1, 3, and 5 years for BRCA subjects was calculated by drawing a direct line from the "total points" axis to the "outcome" axis. The prediction outcomes of the OS nomogram-related calibration curve were consistent with the observed results for all subjects (Figure 2D and E).

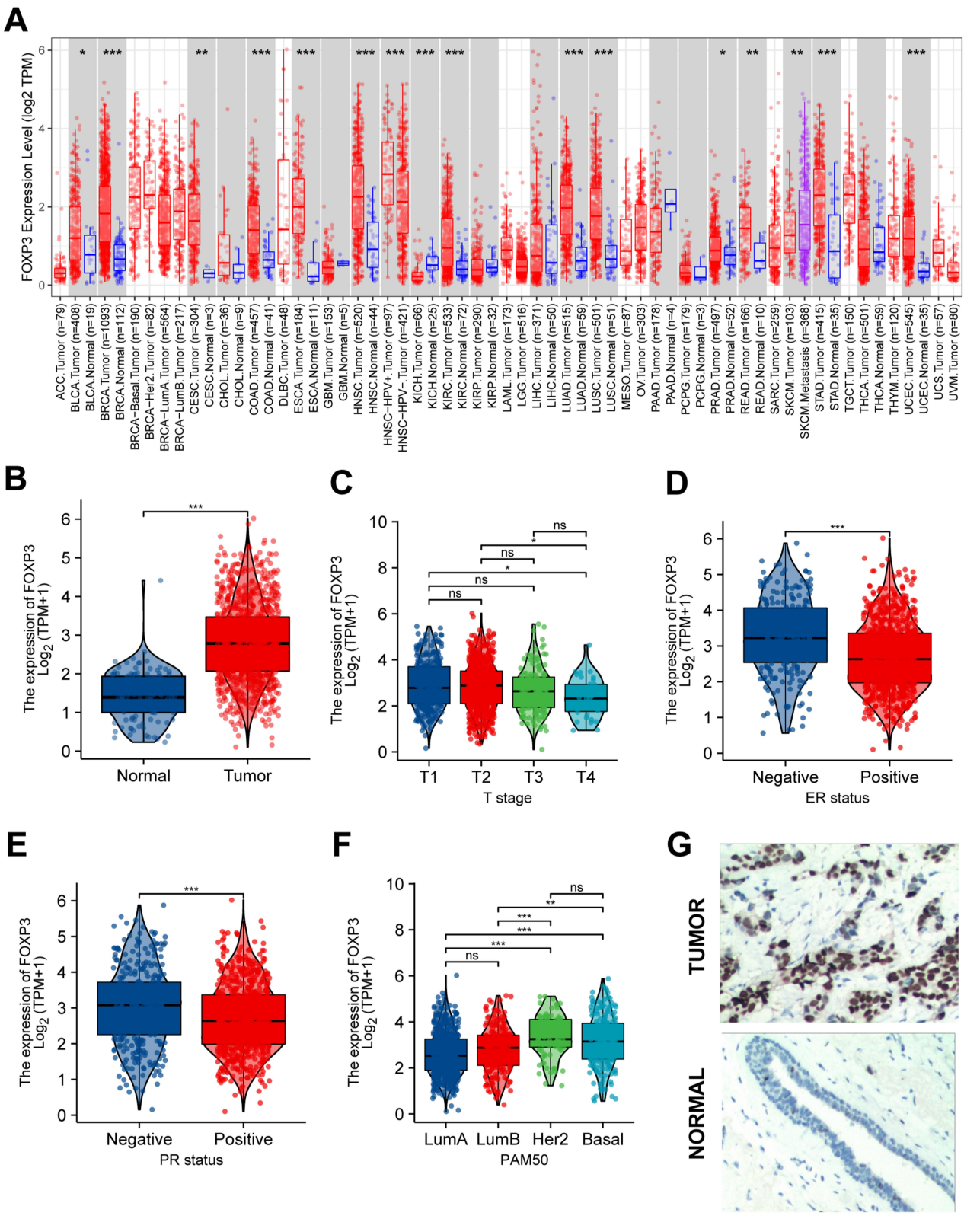

Figure 1 *FOXP3* Expression Levels, Clinical Characteristics and Immunohistochemistry. (**A**) The transcription levels of FOXP3 in different types of cancers (TIME). (**B**) The mRNA expression levels of *FOXP3* BRCA and normal samples from TCGA. (**C**) *FOXP3* mRNA expression was significantly lower in BRCA patients with T4 than BRCA patients with T1/T2. (**D** and **E**) Compared with that in BRCA patients with ER or PR negative, *FOXP3* mRNA expression was significantly lower in BRCA patients with ER or PR positive. (**F**) The expression level of *FOXP3* is significantly higher in Basal, Her2 than in LumA/B. (**G**) The Expression of FOXP3 in BRCA (IHC). (ns, no significance, *P < 0.05, **P < 0.01, ***P < 0.001).

https://doi.org/10.2147/IJGM.S454421 DovePress

Table 1 Correlation Between FOXP3 Expression and the Clinicopathological Features of the BRCA Cases

Characteristic	Low Expression of FOXP3	High Expression of FOXP3	p	Statistic	Method
n	532	533			
T stage, n (%)			0.007	12.12	Chisq. test
T1	138 (13%)	137 (12.9%)			
T2	288 (27.1%)	327 (30.8%)			
T3	79 (7.4%)	58 (5.5%)			
T4	25 (2.4%)	10 (0.9%)			
N stage, n (%)			0.910	0.54	Chisq. test
N0	255 (24.4%)	252 (24.1%)			
N1	174 (16.6%)	175 (16.7%)			
N2	54 (5.2%)	62 (5.9%)			
N3	37 (3.5%)	37 (3.5%)			
M stage, n (%)			0.434	0.61	Chisq. test
M0	432 (47.5%)	457 (50.3%)			
M1	12 (1.3%)	8 (0.9%)			
Pathologic stage, n (%)			0.377	3.1	Chisq. test
Stage I	96 (9.2%)	84 (8.1%)			
Stage II	289 (27.7%)	317 (30.4%)			
Stage III	122 (11.7%)	116 (11.1%)			
Stage IV	11 (1.1%)	7 (0.7%)			
Race, n (%)			0.022	7.6	Chisq. test
Asian	24 (2.5%)	36 (3.7%)			
Black or African American	78 (8%)	101 (10.3%)			
White	389 (39.9%)	348 (35.7%)			
Age, n (%)			0.025	5.04	Chisq. test
<=60	275 (25.8%)	313 (29.4%)			
>60	257 (24.1%)	220 (20.7%)			
Histological type, n (%)			0.162	1.95	Chisq. test
Infiltrating Ductal Carcinoma	353 (36.8%)	404 (42.1%)			
Infiltrating Lobular Carcinoma	106 (11.1%)	96 (10%)			
PR status, n (%)			< 0.001		Fisher. test
Negative	131 (12.9%)	207 (20.4%)			
Indeterminate	3 (0.3%)	1 (0.1%)			
Positive	372 (36.6%)	302 (29.7%)			

(*Continued*)

Table 1 (Continued).

Characteristic	Low Expression of FOXP3	High Expression of FOXP3	p	Statistic	Method
ER status, n (%)			< 0.001		Fisher. test
Negative	76 (7.5%)	161 (15.8%)			
Indeterminate	1 (0.1%)	1 (0.1%)			
Positive	430 (42.3%)	348 (34.2%)			
HER2 status, n (%)			0.072	5.26	Chisq. test
Negative	269 (37.5%)	279 (38.9%)			
Indeterminate	5 (0.7%)	7 (1%)			
Positive	61 (8.5%)	96 (13.4%)			
Age, median (IQR)	60 (50, 69)	57 (48, 66)	0.017	153,707.5	Wilcoxon

Genetic Alteration of *FOXP3* and Immune Checkpoint

To analyze the extent of *FOXP3* mutations in breast invasive carcinoma, we investigated the genome and copy number variations. We analyzed the *FOXP3* gene-related OncoPrint map of BRCA subjects in the TCGA dataset (Figure 2F). The *FOXP3* domain was examined to identify genomic alterations and their locations. In a single BRCA subject, the R417W alteration was observed within the domain and further analyzed by visualizing its impact on the 3D structure of the *FOXP3* protein (Figure 2G). Genomic-level investigation suggested that immune checkpoint alterations could be influenced by *FOXP3* in breast invasive carcinoma. A comprehensive visualization of the *FOXP3* genomic landscape and immune checkpoint alterations in breast invasive carcinoma was conducted, encompassing amplification, mRNA expression, structural variants, truncations, deep deletions, missense, and splice mutations (Figure 3A). Representative immune checkpoints associated with *FOXP3* were analyzed separately in greater detail (Table 2). Utilizing a two-gene correlation map, it was found that *FOXP3* exhibited strong positive correlations with *CD274, PDCD1*, and *CTLA4* (Figure 3B). The resulting data supports the role of *FOXP3* as an immune checkpoint co-regulator in BRCA.

Immune Infiltration of *FOXP3*

To visualize and elucidate the infiltration status of immune cells associated with *FOXP3* in BRCA, we generated four Lollipop charts using 24 immune cells (Figure 3C). The charts showed increased infiltration levels of immune cells in relation to *FOXP3* expression. We further scrutinized this correlation using the TIMER2 database, which connected *FOXP3* expression with the extent of immune cell infiltration. Our conclusions propose that *FOXP3* is involved in mediating immune cell infiltration and the subsequent inflammatory response. The resulting data highlighted a positive association between *FOXP3* expression and the infiltration levels of numerous immune cells, such as CD4+ T cells, CD8+ T cells, macrophages, B cells, neutrophils, and dendritic cells (Figure 3D).

PPI and Neighbour Gene Network of *FOXP3*

We employed STRING to scrutinize potential interactions and establish the PPI network of *FOXP3* at different transcriptional stages. The PPI network includes *FOXP3* and ten proteins closely linked to it (Figure 4A). Using these *FOXP3*-associated proteins, we created a GGI network and referenced the GeneMANIA database to explore their specific roles (Figure 4B). The central hub of *FOXP3* is encircled by 50 nodes, each representing genes that exhibit robust links with *FOXP3* based on shared protein domains, physical associations, colocalization, co-expression, prediction, genetic interactions, and pathways. *NFATC2* is connected to *FOXP3* through physical and genetic interactions. Both *NFATC2* and *CTLA4* have a pathway relationship with *FOXP3*, while *CTLA4* shares colocalization with *FOXP3*.

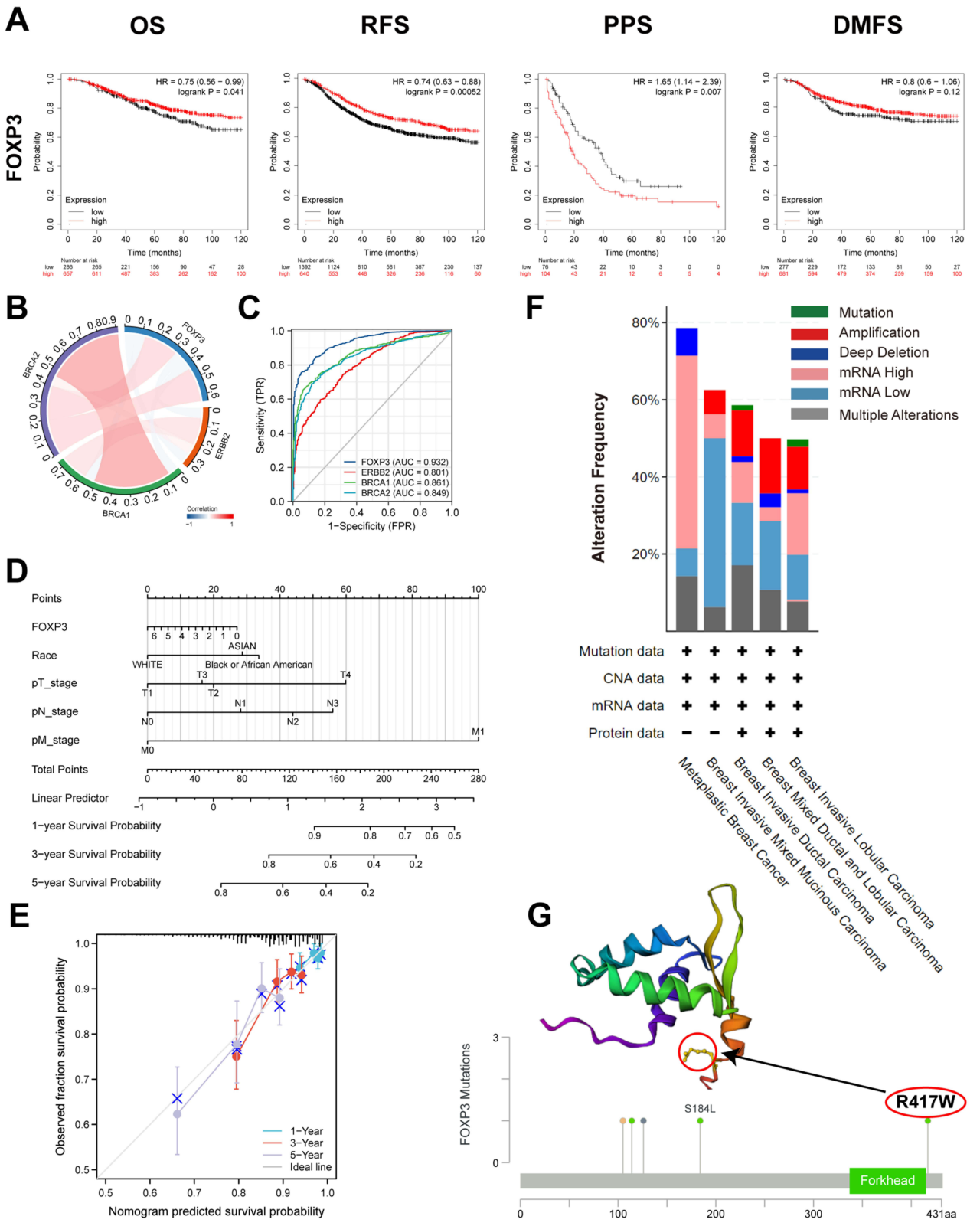

Figure 2 *FOXP3* Prognostic Value, Relevance of *FOXP3* and Important Genes, Diagnostic Value, Mutation Analysis and Prognostic Model in BRCA. (**A**) overall survival (OS), progression-free survival (PFS), post-progression survival (PPS) and distant metastasis-free survival (DMFS). (**B**) Correction between different *FOXP3* and Important Genes. (**C**) ROC curve analysis was performed to evaluate the diagnostic power of the *FOXP3* and Important Genes. (**D**) Nomogram for predicting the 1-, 3-, and 5-year overall survival rates. (**E**) Calibration for nomogram. (**F**) Summary of alterations in different expressed *FOXP3* in BRCA. (**G**) mutation site (R417W) in the 3D structure of *FOXP3* are displayed.

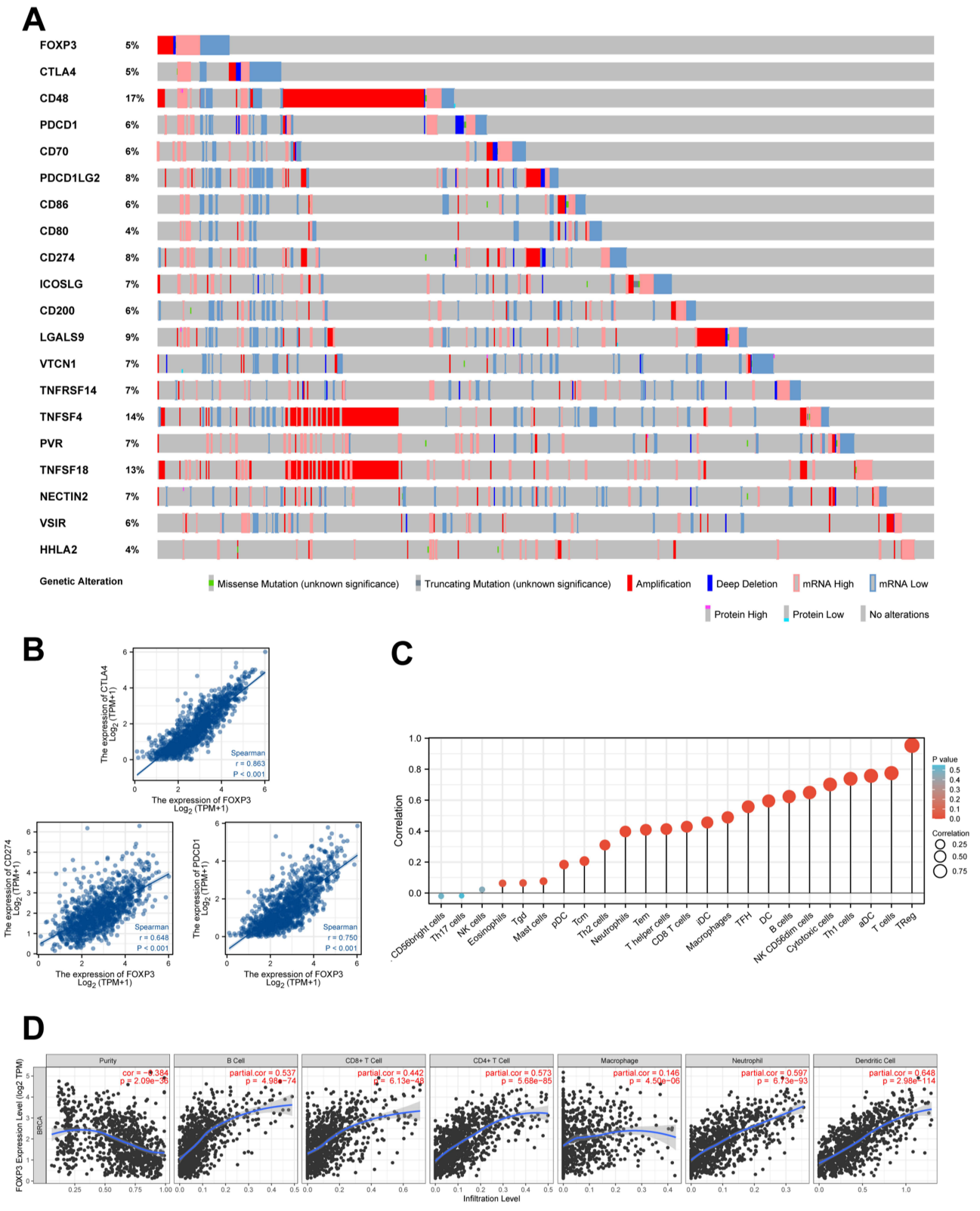

Figure 3 *FOXP3* and Immune Checkpoints Mutation Analysis, The Relationship Between the Expression of *FOXP3* and Immune Checkpoints Genes as well as Immune Infiltration in BRCA. (**A**) Landscape of *FOXP3* and immune checkpoint alteration in BRCA. (**B**) *FOXP3* has a significant positive correlation with *PDCD1, CD274, CTLA4*. (**C**) Lollipop chart about correlation between 24 immune cell and the expression of *FOXP3* in BRCA. (**D**) The correlation between the abundance of immune cell and the expression of *FOXP3* in BRCA.

Table 2 Mutual-Exclusivity Analysis Between FOXP3 and Multiple-Immune Checkpoints in BRCA

A	B	Neither	A Not B	B Not A	Both	Log2 Odds Ratio	p-value	q-value	Tendency	Significant
FOXP3	CTLA4	676	23	29	10	>3	<0.001	<0.001	Co-occurrence	Yes
FOXP3	CD48	600	17	105	16	2.427	<0.001	<0.001	Co-occurrence	Yes
FOXP3	PDCD1	666	23	39	10	2.892	<0.001	<0.001	Co-occurrence	Yes
FOXP3	CD70	671	24	34	9	2.888	<0.001	<0.001	Co-occurrence	Yes
FOXP3	PDCD1LG2	660	23	45	10	2.673	<0.001	<0.001	Co-occurrence	Yes
FOXP3	CD86	668	24	37	9	2.759	<0.001	<0.001	Co-occurrence	Yes
FOXP3	CD80	681	26	24	7	2.933	<0.001	0.001	Co-occurrence	Yes
FOXP3	CD274	655	24	50	9	2.296	<0.001	0.004	Co-occurrence	Yes
FOXP3	ICOSLG	655	24	50	9	2.296	<0.001	0.004	Co-occurrence	Yes
FOXP3	CD200	667	28	38	5	1.648	0.037	0.089	Co-occurrence	No
FOXP3	LGALS9	650	27	55	6	1.393	0.047	0.104	Co-occurrence	No
FOXP3	VTCN1	654	28	51	5	1.195	0.097	0.172	Co-occurrence	No
FOXP3	TNFRSF14	660	29	45	4	1.016	0.169	0.263	Co-occurrence	No
FOXP3	TNFSF4	618	27	87	6	0.659	0.227	0.32	Co-occurrence	No
FOXP3	PVR	662	30	43	3	0.622	0.339	0.425	Co-occurrence	No
FOXP3	TNFSF18	621	28	84	5	0.401	0.366	0.449	Co-occurrence	No
FOXP3	NECTIN2	656	30	49	3	0.421	0.415	0.494	Co-occurrence	No
FOXP3	VSIR	663	31	42	2	0.026	0.599	0.609	Co-occurrence	No
FOXP3	HHLA2	677	32	28	1	−0.404	0.624	0.627	Mutual exclusivity	No

Additionally, *GATAD2B, HDAC9, IKZF3, RORC, MKI67, SIVA1, SFTPC, TBR1*, and *FOXP3* demonstrate physical interaction. Comprehensive functional analysis suggests that these genes have a significant correlation with lymphocyte differentiation. Moreover, they are linked to regulatory roles concerning the differentiation of T cells, regulatory T cells, lymphocytes, leukocytes, and the modulation of hemopoiesis.

FOXP3 Functional Enrichment Analysis

We employed the "ClusterProfiler" package in R software to conduct pathway enrichment analysis and functional annotation for *FOXP3*, derived from the 50 nodes representing genes that exhibit a strong association with *FOXP3*. The top 12 items emerging from GO and KEGG enrichment analyses encompass cytokine receptor activity, interaction between cytokines, regulation of T cell and lymphocyte activation, activation of T cells, the plasma membrane's external side, MHC class I and II protein complex, binding of cytokines, MHC protein binding, rejection of allografts, and cell adhesion molecules (Figure 4C) (Table 3).

Chemical-Gene Interactions

To explore the interactions between *FOXP3* and chemicals used for cancer treatment, we utilized the CTD and visualized the results. A total of 21 chemicals affected *FOXP3* expression (Figure 4D). The chemical in the red circle decreased *FOXP3* expression levels, while the chemical in the blue circle increased *FOXP3* expression levels. We also examined drugs such as cyclophosphamide (Figure 4E), tamoxifen (Figure 4F), and fulvestrant (Figure 4G).

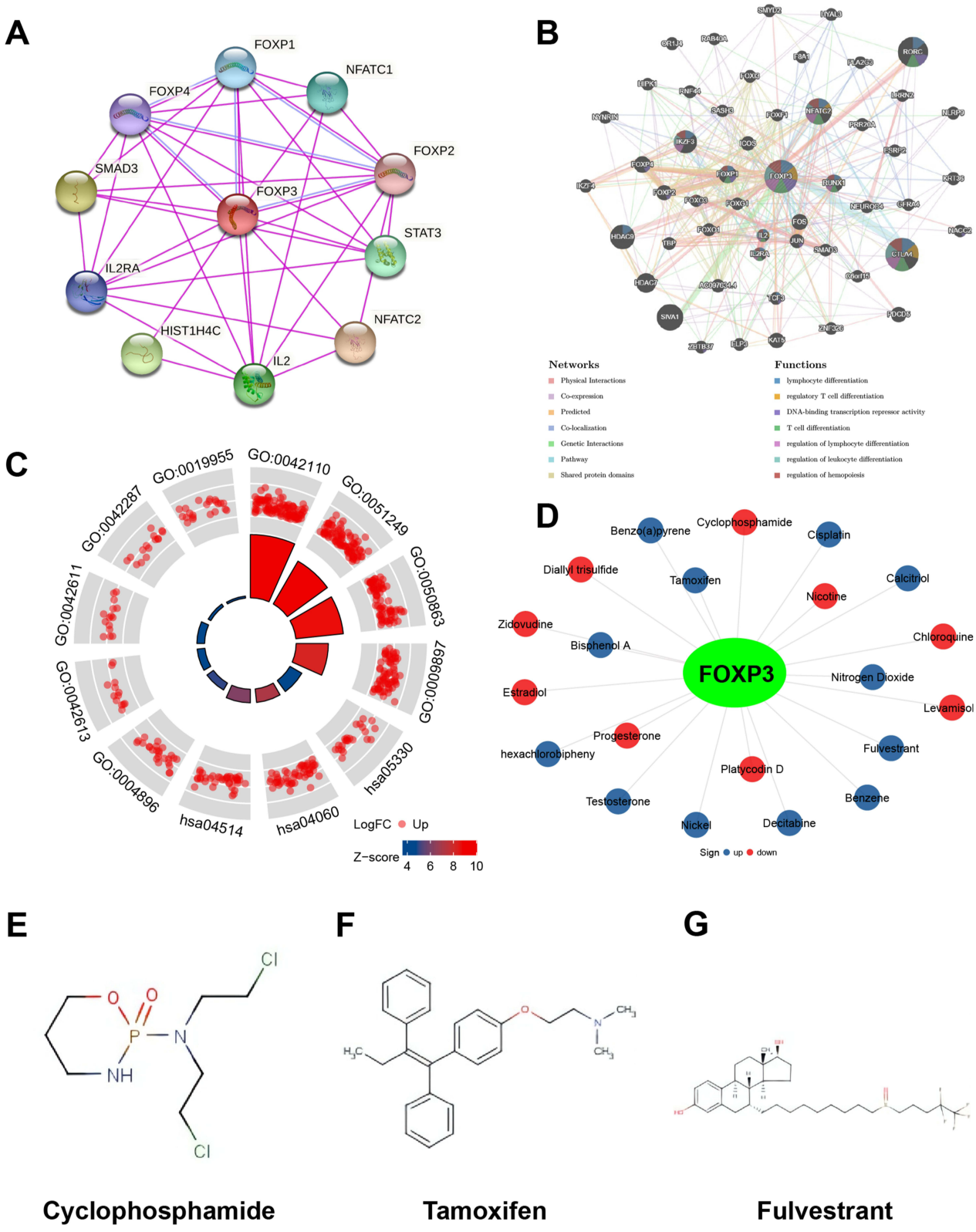

Figure 4 PPI Network, Neighbor Gene Network, The Functions of *FOXP3*, and Chemical-Gene Interaction. (**A**) Protein-protein interaction network of expressed *FOXP3*. (**B**) Gene-gene interaction network of expressed *FOXP3*. (**C**) The functions of *FOXP3* and genes significantly associated with *FOXP3* alterations were predicted by the analysis of GO and KEGG. (**D**) Chemical-gene interaction of *FOXP3*. Chemical structural (**E**) Cyclophosphamide, (**F**) Tamoxifen, (**G**) Fulvestrant.

Table 3 The GO and KEGG Function Enrichment Analysis of FOXP3 and Neighbor Genes in BRCA

Ontology	ID	Description	GeneRatio	BgRatio	p value	p. Adjust	q value	Count	z Score
BP	GO:0042110	T cell activation	101/373	464/18,670	7.76e-77	2.45e-73	1.78e-73	101	10.04987562
BP	GO:0051249	Regulation of lymphocyte activation	91/373	485/18,670	9.46e-63	1.50e-59	1.08e-59	91	9.539392014
BP	GO:0050863	Regulation of T cell activation	76/373	314/18,670	7.55e-61	7.96e-58	5.76e-58	76	8.717797887
CC	GO:0009897	External side of plasma membrane	68/381	393/19,717	6.87e-45	1.99e-42	1.55e-42	68	8.246211251
CC	GO:0042613	MHC class II protein complex	13/381	16/19,717	2.27e-20	3.29e-18	2.56e-18	13	3.605551275
CC	GO:0042611	MHC protein complex	15/381	25/19,717	4.08e-20	3.94e-18	3.06e-18	15	3.872983346
MF	GO:0004896	Cytokine receptor activity	25/364	96/17,697	5.92e-21	2.43e-18	1.97e-18	25	5
MF	GO:0042287	MHC protein binding	15/364	40/17,697	9.45e-16	1.94e-13	1.57e-13	15	3.872983346
MF	GO:0019955	Cytokine binding	22/364	128/17,697	1.70e-14	2.01e-12	1.63e-12	22	4.69041576
KEGG	hsa05330	Allograft rejection	23/238	38/8076	2.25e-26	4.91e-24	3.77e-24	23	4.795831523
KEGG	hsa04060	Cytokine-cytokine receptor interaction	51/238	295/8076	6.13e-26	6.39e-24	4.91e-24	51	7.141428429
KEGG	hsa04514	Cell adhesion molecules	38/238	149/8076	8.80e-26	6.39e-24	4.91e-24	38	6.164414003

Responsiveness to Chemotherapy and Targeted Therapy in Relation to *FOXP3* Expression: ROC Plotter Analysis

We conducted a ROC plotter analysis to evaluate the correlation between *FOXP3* expression and patient response to various therapies, as well as to measure the potential of *FOXP3* as a predictor for effective treatment in BRCA. We contrasted *FOXP3* expression in patients who responded and those who did not respond to treatments such as tamoxifen, aromatase inhibitors, trastuzumab, taxane, anthracycline, and the combination of fluorouracil, epirubicin, and cyclophosphamide (FEC) in BRCA samples. According to the 5-year RFS data, *FOXP3*'s sensitivity in predicting tamoxifen treatment efficacy for estrogen receptor (ER)-positive BRCA patients was not notably high (AUC = 0.552, p = 0.26) (Figure 5A). In contrast, based on the 5-year RFS data, *FOXP3* demonstrated sensitivity in predicting the treatment effect of aromatase inhibitors for ER-positive BRCA patients (AUC = 0.807, p = 2.6E-03) (Figure 5B). Regarding the pathological complete response (pCR) of HER2-positive BRCA, *FOXP3* could predict trastuzumab treatment efficacy (AUC = 0.638, p = 1.6E-02) (Figure 5C). For luminal-B BRCA pCR, *FOXP3* was sensitive in predicting the treatment effects of FEC (AUC = 0.832, p = 7.6E-07) (Figure 5D), taxane (AUC = 0.737, p = 1.2E-05) (Figure 5E), and anthracycline (AUC = 0.706, p = 2E-04) (Figure 5F). Furthermore, based on the pCR of luminal-A BRCA, *FOXP3* could predict the treatment effect of FEC (AUC = 0.836, p = 4.3E-05) (Figure 5G). In HER2-positive and ER-negative BRCA pCR, *FOXP3* could accurately predict the treatment effects of anthracycline (AUC = 0.733, p = 1.3E-06) (Figure 5H), FEC (AUC = 0.952, p < 0.001) (Figure 5I), and taxane (AUC = 0.682, p = 1.4E-03) (Figure 5J). Lastly, for triple-negative BRCA pCR, *FOXP3* could predict the treatment effect of anthracycline (AUC = 0.603, p = 3.4E-02) (Figure 5K), but not that of FEC (AUC = 0.603, p = 3.4E-02) (Figure 5L).

Correlation Between Gene Expression and Pathway Scores

To explore the connection between *FOXP3* and various biological pathways, we carried out an analysis correlating gene expression with pathway scores. Such information can assist in comprehending the potential involvement of *FOXP3* within these pathways. The pathways under examination encompassed the *PI3K/AKT/mTOR* pathway, genes up-regulated due to reactive oxygen species, anti-inflammatory signaling pathway of interleukin-10, cellular reaction to hypoxia, markers for epithelial-mesenchymal transition, extracellular matrix (ECM)-related genes, DNA replication, P53 pathway, tumor inflammation signature, tumor proliferation signature, G2M checkpoint, MYC targets, formation of collagen, degradation of ECM, angiogenesis, apoptosis, inflammatory response, ferroptosis, and TGFB (Figure 6A–T). The results reflected a strong positive correlation between *FOXP3* expression and the tumor inflammation signature (p <0.001), implying that *FOXP3* may play a significant role in regulating inflammation within the tumor microenvironment, potentially impacting cancer progression and therapy response.

Discussion

FOXP3 has been identified as a contributing factor in the onset and advancement of a range of malignant neoplasms, such as those affecting the liver,[25] melanoma,[26] bladder, kidney cancers,[27] non-small cell lung cancer,[18] oral,[28] prostate,[29] colorectal[30] and thyroid cancers,[11] by regulating immune-related genes. However, a lack of comprehensive bioinformatics investigations on the expression and function of *FOXP3* proteins in BRCA has hindered the complete understanding of *FOXP3*'s role in the development, progression, and prognosis of certain malignancies. For the first time, we utilized bioinformatics techniques in this investigation to scrutinize the gene expression and variations of *FOXP3* in BRCA. Additionally, we assessed its biological role, molecular processes, and the influence it has on prognosis and immune infiltration among BRCA patients.

FOXP3 plays a vital role in the regulation of the maturation and immunological function of Treg cells in cancer resistance.[31] Evidence suggests that it can stifle the expansion of the MCF-7 breast cancer cell line and incite cell apoptosis.[32] Additionally, *FOXP3* is essential for suppressing oncogenes in BRCA with favorable prognosis.[32–34] In our investigation, database analysis and IHC revealed higher levels of *FOXP3* mRNA in BRCA tissues compared to normal tissues, irrespective of histological type, tumor stage, and race. Our Kaplan-Meier survival analysis revealed a complex role for *FOXP3* in BRCA prognosis. We discovered a significant correlation between the surge in *FOXP3* mRNA

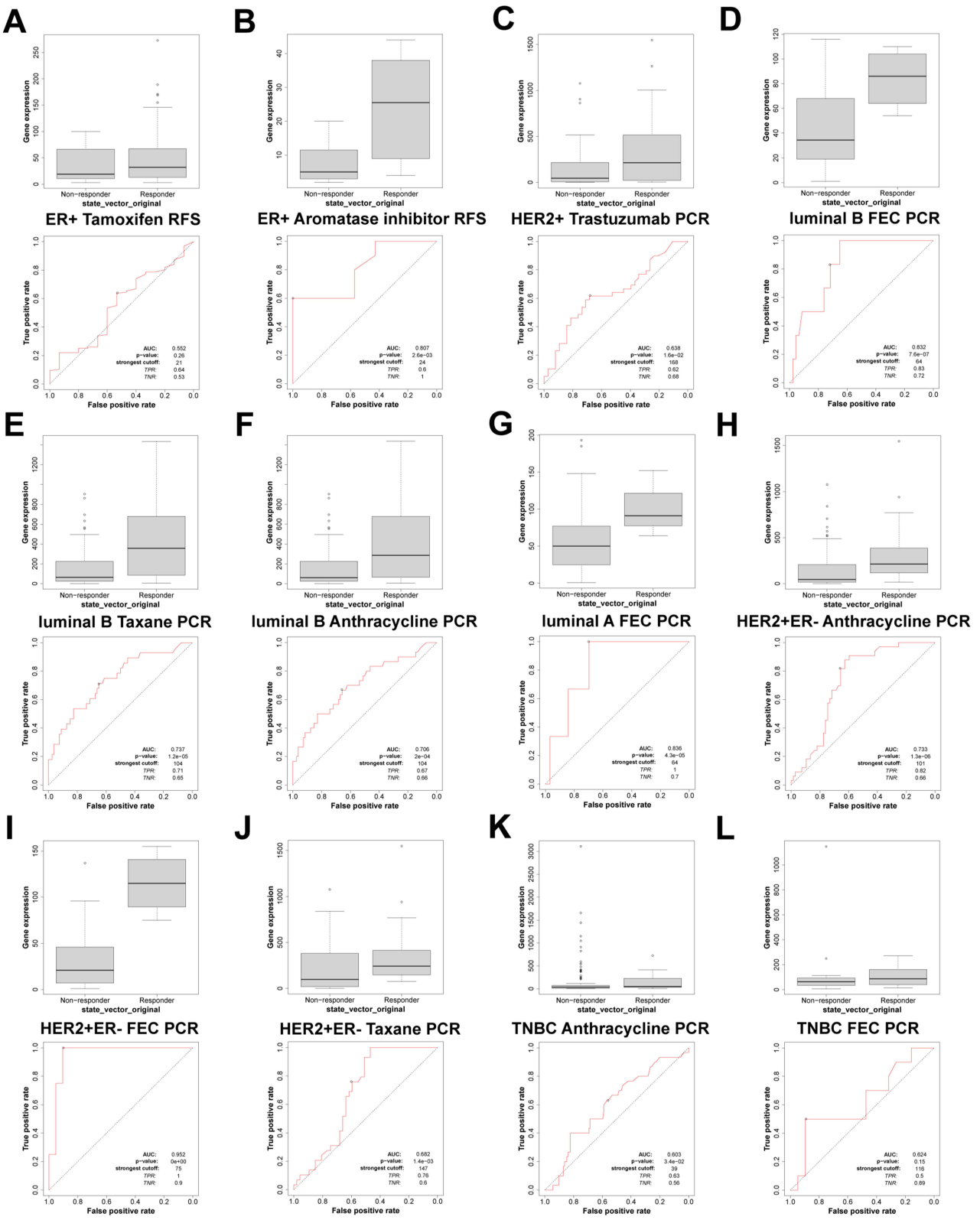

Figure 5 *FOXP3* expression in groups of responder and non-responder BRCA patients treated using the online platform ROC plot. (**A** and **B**) RFS of ER+ BRCA patients, (**C**) PCR of HER2+ BRCA patients, (**D**–**F**) PCR of Luminal B BRCA patients, (**G**) PCR of Luminal A BRCA patients, (**H**–**J**) PCR of HER2+ and ER- BRCA patients, (**K** and **L**) PCR of TNBC BRCA patients.

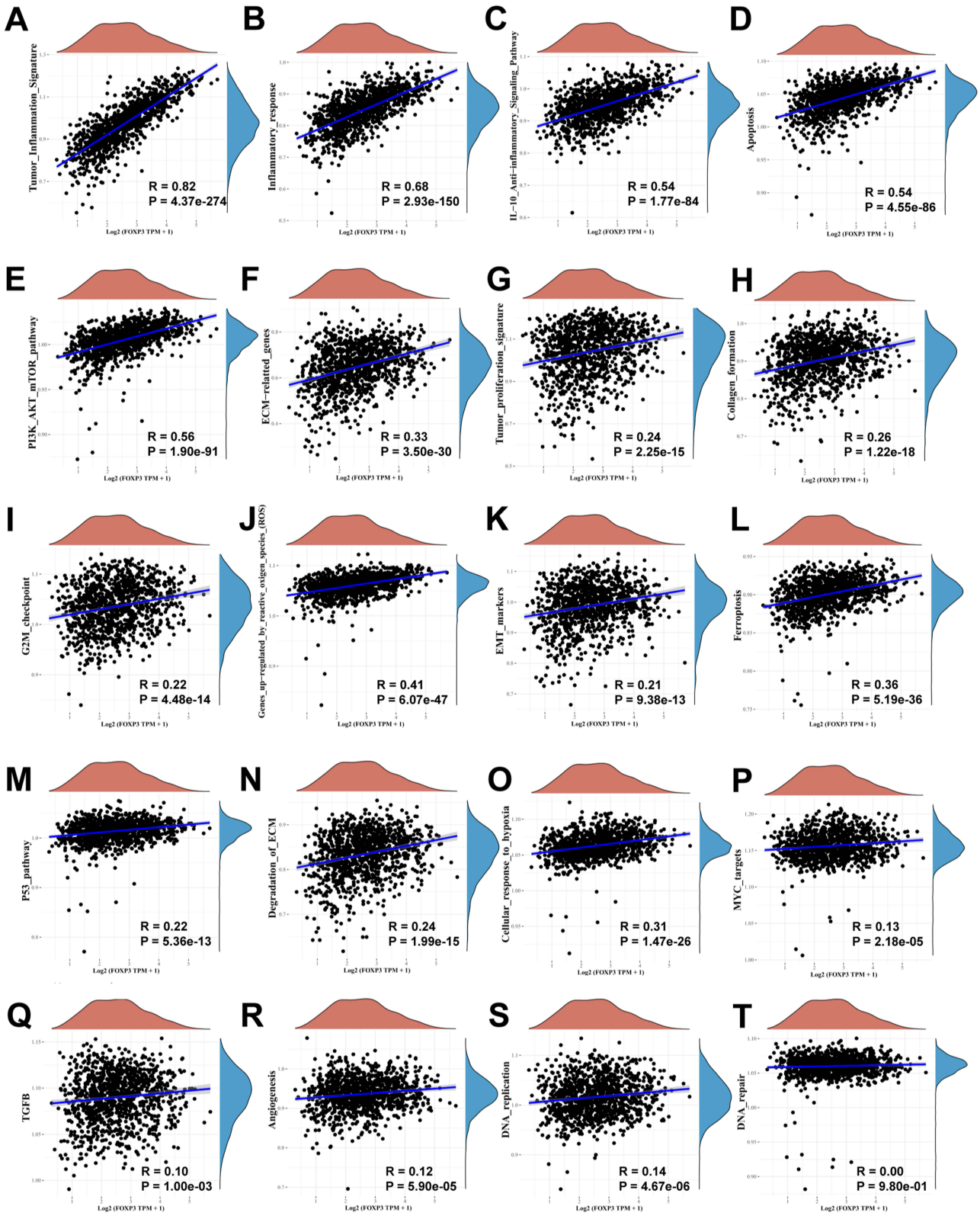

Figure 6 The correlations between *FOXP3* and pathway score was analysed with Spearman. The abscissa represents the distribution of the gene expression, and the ordinate represents the distribution of the pathway score. The density curve on the right represents the trend in distribution of pathway immune score, the upper density curve represents the trend in distribution of the gene expression. (**A**) Tumor inflammation signature, (**B**) Inflammatory response, (**C**) IL-10 anti-inflammatory signaling pathway, (**D**) Apoptosis, (**E**) *PI3K/AKT/mTOR* pathway, (**F**) ECM related genes, (**G**) Tumor proliferation signature, (**H**) Collagen formation, (**I**) G2M checkpoint, (**J**) Genes up-regulation by reactive oxygen species (ROS), (**K**) EMT markers, (**L**) Ferroptosis, (**M**) P53 pathway, (**N**) Degradation of ECM, (**O**) Cellular response to hypoxia, (**P**) MYC targets, (**Q**) TGFB, (**R**) Angiogenesis, (**S**) DNA replication, (**T**) DNA repair.

expression and improved OS among breast cancer patients. However, we found no substantial link between *FOXP3* mRNA expression and DMFS. Furthermore, our investigation revealed that an augment in *FOXP3* expression correlated with an extended RFS, while a decrease in gene expression was associated with a longer PPS. These observations imply that *FOXP3*'s influence on BRCA prognosis is intricate and deserves a more in-depth exploration, given that *FOXP3* may have varying effects on PFS and PPS among BRCA patients.

FOXP3 is an essential regulator of the development and immune function of Treg cells, which have been implicated in cancer progression. Previous studies have shown that *FOXP3* can suppress oncogenes in BRCA and is associated with a favorable prognosis. Our research additionally uncovered a high frequency of *FOXP3* gene mutations across diverse histological classifications of BRCA. Common occurrences included deletions, functionally impactful somatic mutations, and a decrease in the expression of the *FOXP3* gene. The observed association between *FOXP3* mutations and *HER-2/ERBB2* overexpression highlights the potential role of *FOXP3* as an X-linked BRCA suppressor gene and a critical regulatory factor of the *HER-2/ERBB2* oncogene.[32]

In our research, we showcased the intricate role of *FOXP3* in BRCA, indicating its potential utility as both a diagnostic biomarker and an immunomodulatory influence. Our analysis of the ROC curve revealed that *FOXP3* presented a notably elevated AUC value for identifying BRCA, boasting a detection accuracy rate of 95.7%. We also observed strong associations between *FOXP3* expression and immune checkpoint alterations in invasive BRCA and immune cell infiltration, further supporting its role as an immunomodulatory factor.

FOXP3 protein plays a crucial role in regulating immune function.[35,36] Our study's genomic analysis highlighted a strong association between *FOXP3* and alterations in numerous immune checkpoints in invasive BRCA. Notably, this association was concurrent but not mutually exclusive.

In our study, we discovered a potent positive linkage between *FOXP3* and *PDCD1, CD274*, and *CTLA4* within the context of BRCA. *FOXP3* was also involved in the infiltration of immune cells and the subsequent inflammatory reaction. More specifically, we found a significant association between *FOXP3* expression and the presence of infiltrating B cells, neutrophils, dendritic cells, macrophages, CD4+ T cells and CD8+ T cells. As stated in a study by West et al[37] patients with BRCA possessing *FOXP3*+ tumor-infiltrating lymphocytes (TILs) exhibited improved survival rates. These findings further endorse the potential function of *FOXP3* as an influential factor in the immunomodulation of invasive BRCA.

Former research has indicated a correlation between *FOXP3* and multiple genes or proteins. These include genes associated with the glucocorticoid-induced tumor necrosis factor receptor family,[38] *CTLA4* and CD25,[39] transforming growth factor-β,[40] nuclear factor-activated T cells[40] and the Runt-related transcription factor 1.[41] In the course of our study, we scrutinized the association between *FOXP3* and its adjacent genes or proteins, identifying 50 major genes related to *FOXP3*. Further investigation revealed that these genes primarily play roles in regulating lymphocyte function and differentiation.

Our investigation further substantiated that the quantification of *FOXP3* mRNA expression could act as a prognosticator for the reactivity of BRCA cells to anticancer therapeutics. Intriguingly, *FOXP3* exhibits sensitivity in forecasting the therapeutic impact of aromatase inhibitors on patients diagnosed with ER-positive BRCA, as evidenced by RFS rates. Drawing a parallel, Ladoire et al[42] disclosed that the presence of *FOXP3* in BRCA independently correlates with enhanced OS in patients undergoing anthracycline-based chemotherapy.

Regarding the pCR of BRCA, *FOXP3* is sensitive in predicting the treatment effects of trastuzumab, FEC, taxane, and anthracycline. Patients who achieved pCR after neoadjuvant treatment exhibited significantly higher *FOXP3* expression compared to those who did not achieve pCR after treatment with these drugs. Horlock et al[43] documented that the count of circulating Th17 cells is diminished in subjects with HER2-positive breast cancer. The alteration in the Treg: Th17 ratio seems to typify the oncogenic state, with trastuzumab therapy further destabilizing this equilibrium. Thereby, trastuzumab could potentially exert a therapeutic influence by modulating the cellular immune system's responsiveness in patients afflicted with breast cancer.

Increased infiltration of CD4+, CD8+, and *FOXP3*+ TILs was associated with pCR in triple-negative breast cancer (TNBC) patients receiving neoadjuvant anthracycline or anthracycline + taxane therapy.[44] Furthermore, patients with high *FOXP3*+ and CD8+ TIL infiltration after neoadjuvant paclitaxel followed by FEC experienced elevated pCR rates.[45]

However, to fully harness the potential of *FOXP3* in breast cancer and translate these findings into clinical practice, further experimental and clinical research is necessary. Future studies should investigate the molecular mechanisms,

immunoregulatory functions, and interactions with anti-cancer drugs of *FOXP3* in breast cancer, with the aim of providing more effective treatment strategies and personalized therapeutic options for breast cancer patients. In summary, our research emphasizes the significance of *FOXP3* in the realm of breast cancer studies and constructs a foundation for subsequent explorations into its prospective clinical utilization.

Conclusions

Our study elucidates the pivotal role of *FOXP3* in breast cancer, highlighting its potential as a diagnostic biomarker and immunoregulatory factor. The strong associations with immune checkpoint changes, immune cell infiltration, and anticancer drug responses emphasize the importance of *FOXP3* in breast cancer management. Further understanding of *FOXP3*'s molecular mechanisms may contribute to improved tumor detection, treatment strategies, and patient outcomes.

Abbreviation

ACC, Adrenocortical carcinoma; BC, Breast cancer; BLCA, Bladder Urothelial Carcinoma; BRCA, Breast invasive carcinoma; CHOL, Cholangiocarcinoma; COAD, Colon adenocarcinoma; CTD, Comparative Toxicogenomics Database; DLBC, diffuse large B-cell lymphoma; DMFS, Distant metastasis-free survival; ECM, Extracellular matrix; ER, Estrogen receptor; ESCA, Esophageal carcinoma; *FOXP3*, Forkhead box P3; GBM, Glioblastoma multiforme; GGI, Gene-gene interaction; GO, Gene Ontology; HNSC, Head and neck squamous cell carcinoma; LAML, Acute Myeloid Leukemia; IDC, Infiltrating Ductal Carcinoma; IHC, Immunohistochemistry; ILC, Infiltrating Lobular Carcinoma; KEGG, Kyoto Encyclopedia of Genes and Genomes; KICH, Kidney chromophobe; KIRC, Kidney renal clear cell carcinoma; KIRP, Kidney renal papillary cell carcinoma; LIHC, Liver hepatocellular carcinoma; LUAD, Lung adenocarcinoma; LUSC, Lung squamous cell carcinoma; OS, Overall survival; OV, Ovarian serous cystadenocarcinoma; PAAD, Pancreatic adenocarcinoma; pCR, Pathological complete response; PFS, Progression-free survival; PPI, Protein-protein interaction; PPS, Post-progression survival; PR, Progesterone receptor; READ, Rectum adenocarcinoma; RFS, Recurrence-free survival; ROC, Receiver Operating Characteristic; SKCM, Skin Cutaneous Melanoma; STAD, Stomach adenocarcinoma; THCA, Thyroid carcinoma; UCEC, Uterine Corpus Endometrial Carcinoma; TCGA, The Cancer Genome Atlas; THYM, Thymoma; TILs, Tumor-infiltrating lymphocytes; TGCT, Testicular germ cell tumors; TIMER, Tumour Immune Estimation Resource; TME, The tumor microenvironment; TNBC, Triple-negative breast cancer.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author (Jianing Yi) on reasonable request.

Ethics Approval and Consent to Participate

The study has been authorized by the Medical Ethics Committee of Hunan Provincial People's Hospital/The First Affiliated Hospital of Hunan Normal University (IRB Approval NO.2024-038) and practiced in accordance with the research principles described in the Helsinki Declaration.

Acknowledgments

We thank Bullet Edits Limited for the linguistic editing and proofreading of the manuscript.

Funding

This study was funded by The Scientific Research Programme of Hunan Provincial Health Commission (B202304016524); The Scientific Research Fund of Hunan Provincial Education Department (22B0081, 23B0091).

Disclosure

The authors declare that they have no competing interests in this work.

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