RESEARCH ARTICLE

Comprehensive Analysis of PLOD Family Prognostic Value and Related Regulatory ceRNA Network in Breast Cancer

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Abstract

Procollagen-lysine, 2-oxoglutarate 5-dioxygenases (PLODs) catalyze lysine hydroxylation, promoting collagen crosslinking and extracellular matrix stability, and are implicated in tumor aggressiveness. However, their expression and prognostic value in breast cancer (BC) remain unclear. We explored PLOD1-3 expression in BC using ONCOMINE, TIMER, CCLE, cBioPortal, UALCAN, GEPIA, and HPA. Prognostic associations were assessed via Kaplan-Meier Plotter, with enrichment analysis by clusterProfiler. A competing endogenous RNA (ceRNA) network was constructed using TCGA data, and immune infiltration analyzed by TIMER and CIBERSORT. PLOD1-3 were upregulated in BC versus normal tissues at both transcript and protein levels, with high expression predicting shorter survival. Enrichment analysis implicated PLODs in DNA replication, cytokinesis, and basement membrane formation. The ceRNA network for PLODs was successfully constructed. Immune infiltration analysis revealed significant correlations between PLOD expression and immune cell levels; high M2 macrophage or low plasma cell infiltration indicated poor prognosis. PLODs are highly expressed in BC and may serve as prognostic biomarkers and therapeutic targets.

Keywords: PLOD family, ceRNA network, breast cancer, prognostic.

1 Introduction

Worldwide, BC is the most frequently diagnosed type of cancer among women, with 23% of new malignancy cases [63]. The incidence and mortality of BC in China are still rising, causing a substantial socio-economic burden [38]. BC is a highly heterogeneous tumor that

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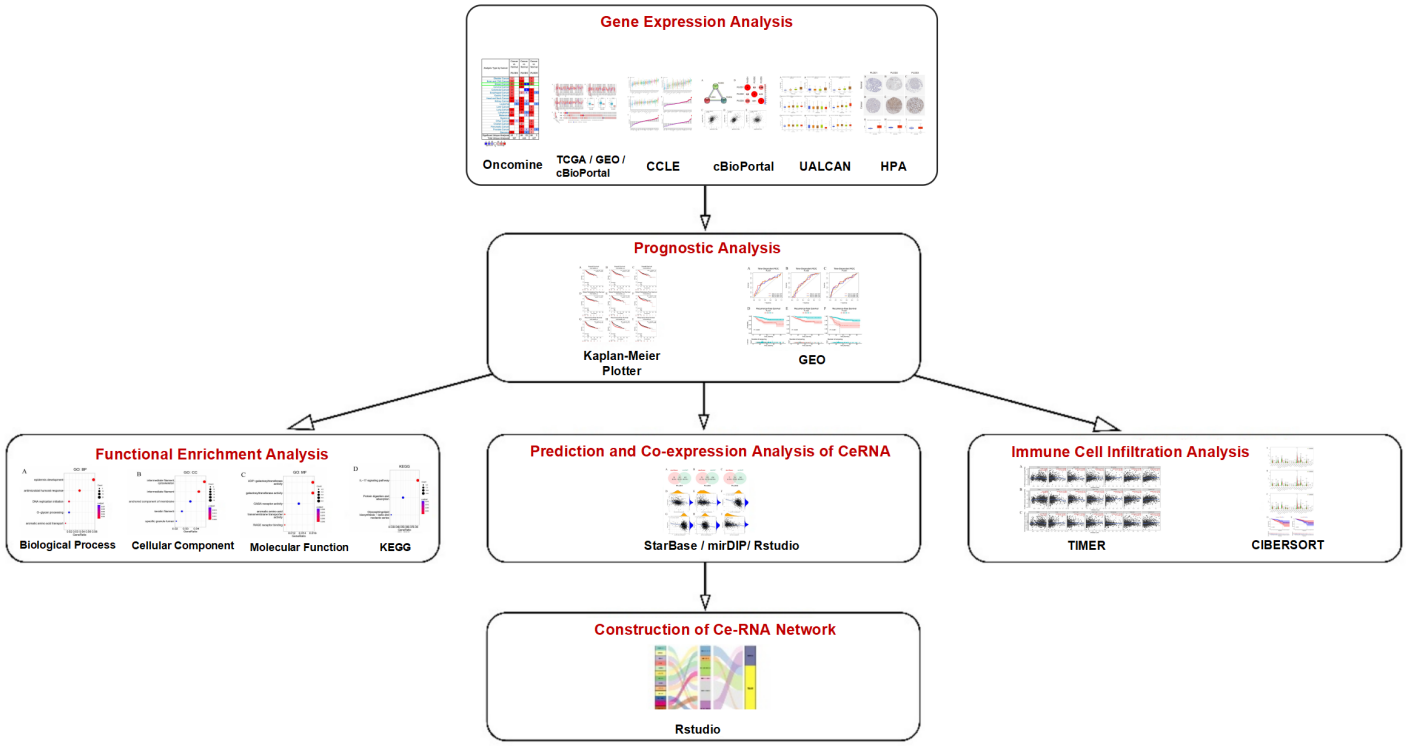

Figure 1. Schematic diagram of this study.

exhibits complex clinical, histological, and molecular diversity [79], with triple-negative BC being one of the more heterogeneous pathological subtypes of BC, with a higher incidence in younger women and a lower survival rate [80]. In BC patients, more than 90% of deaths are caused by the spread of tumor cells to distant sites and proliferation [30]. Therefore, early diagnosis and targeted treatment of BC are vital for the survival prognosis of patients. However, the commonly used clinical serum tumor markers for BC, CA153, CA125, and CEA, have low sensitivity and specificity, can only be used for adjuvant diagnosis, and cannot indicate prognosis [13, 39, 48, 73]. As a result, the discovery of additional biomarkers is essential to help individualize the treatment of BC and improve patient survival.

The tumor microenvironment (TME) is mainly made up of cellular and non-cellular components [3]. The extracellular matrix (ECM), a major part of the TME, has a crucial function in tumor formation, and collagen is the most fundamental constituent of the ECM [19, 28]. Collagen crosslinking and deposition is dependent on lysyl hydroxylation, catalyzed by PLODs [50]. PLODs are a set of enzymes that stabilize collagen by crosslinking and are engaged in lysine hydroxylation, and three members, namely PLOD1, PLOD2, and PLOD3, have been determined [25, 29, 51, 52]. All hydroxylated lysine residues of PLODs are located in the pre-collagen helix region and participate in the collagen synthesis process [69]. Notably, the active site located at the N-terminal of PLOD3 also has specific galactosyl hydroxylated lysine-glucosyltransferase activity, which is particularly important in promoting high glycation collagen to maintain the stability of basement membrane and vascular structural [50]. Therefore, by acting on the formation of the basement membrane, the PLOD family affects cell functions such as cell growth, adhesion, and migration, leading to the invasion and metastasis of malignant tumors. PLODs are expressed excessively in diverse cancers, and high expression of PLOD family genes has been linked to worse prognosis in gastric cancer (GC), non-small cell lung cancer (NSCLC), and glioma [23, 37, 49, 84]. Whereas, the expression, functional, and prognosis value of PLODs in BC are not yet totally understood.

In this work, the expression profile, biological functions, and immune relevance of PLOD1/2/3 in BC were researched on the basis of an online database and various tools and datasets. Using diverse online databases, the comprehensive analysis of the PLOD family highlighted the predictive value of PLODs for BC and explored its potential functions and regulatory mechanisms through enrichment analysis and ceRNA network construction. According to our results, PLODs were overexpressed in BC tissues as

opposed to normal tissues. Furthermore, high PLODs expression was significantly associated with reduced long-term survival. Therefore, our findings may offer new perspectives for understanding the biology of BC and its potential therapeutic targets. The schematic diagram of this study is shown in Figure 1.

2 Methods

2.1 Microarray data

GEO dataset GSE54002 was used to confirm the differential expression of PLOD family in BC compared to normal tissues, the prognostic value of PLOD1-3 in BC was verified using GEO dataset GSE25055, and the ceRNA network was constructed using mRNA, miRNA and lncRNA transcript data from the TCGA-BRCA database. Besides, the effect of PLOD family expression levels on immune cell infiltration in BC was analyzed using transcriptome data from the TCGA and the CIBERSORT algorithm.

2.2 ONCOMINE Data Analysis

The cancer microarray database ONCOMINE[1] contains a large dataset of gene expression arrays for online analysis of transcript levels of PLODs in different cancers. For this study, Student's t-test was applied to calculate the P-values for the variation in expression between cancer and the normal control. Fold change > 2, and P values less than 0.05 were considered statistically significant.

2.3 TIMER Analysis

TIMER[2] allows the multifaceted study of the connection between immune cells and different cancers. Primarily, the algorithm is applied to assess the abundance of tumor-infiltrating immune cells from gene expression profiles. For this research, we investigated the transcript levels of PLOD1/2/3 and their relationship with immune cells and tumor purity.

2.4 CCLE Datasets

The CCLE is an oncogenomics research project led by the Broad Institute Research Institute covering gene expression in thousands of cell lines from more than thirty tissue sources[3]. In this research, the CCLE dataset was used to confirm the high and low expression of PLOD1-3 in various tumor cell lines.

2.5 cBioPortal Data Analysis

The cBioPortal provides easy access to visualizing and analyzing cancer genomic data in multidimensional ways [16]. We performed a detailed assessment of copy number variants and gene types summarized in BC using cBioPortal. Additionally, a correlation analysis was performed between select commonly expressed genes of PLODs and PLOD family genes in BC using cBioPortal.

2.6 GEPIA Database

GEPIA is an online collection of gene-expression profiles compiled by comparing tumor tissues with matched normal tissues[4] [17]. Using the GEPIA database, our research has visualized co-expression profiles of PLOD1/2/3 in BC.

2.7 UALCAN Analysis

UALCAN[5] analyzed TCGA online RNA-seq data and clinical information for 31 cancer types [8]. In order to compare expression differences between different BC subgroups, we used it to make a comparison of the relative expression differences of PLODs in BC and comparison samples. Transcript levels were normalized to transcripts per million reads, and Student's t-tests considered P-values of less than 0.01 significant.

2.8 Human Protein Atlas Data Analysis

The Human Protein Atlas is divided into three sections, Cell, Tissue, and Pathology, mainly including protein expression in cells and normal and cancerous tissues. The protein expression levels of PLOD1-3 in different tissues were assessed by HPA.

2.9 Survival analysis with a Kaplan-Meier plotter

Kaplan-Meier Plotter(K-M Plotter) was utilized to estimate the impact of a number of genes on survival with the use of PLOD gene expression data. The samples were categorized into two high and low groups by the median mRNA expression of genes from the PLOD family. The prognostic value of PLODs was examined using log-rank tests and survival curves. Hazard ratios, 95% confidence intervals, and P values are shown on the curves. There was statistical significance for P-values <0.05.

2.10 Functional and Pathway Enrichment Analyses

An analysis and visualization of genetic function profiles was carried out using the R package

[1] https://www.oncomine.org/
[2] https://cistrome.shinyapps.io/timer/
[3] https://www.broadinstitute.org/ccle
[4] http://gepia.cancer-pku.cn/
[5] http://ualcan.path.uab.edu

clusterProfiler(v4.2.0), and the functional profiles of Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) were also investigated. P-value cut-offs were set to 0.05, and Q-value cut-offs were set to 0.05.

2.11 Construction of the ceRNA Network

StarBase and mirDIP websites integrate human-related target gene information from 30 source databases for predicting miRNAs targeting PLODs. The lncRNAs bound to the target miRNAs were also predicted on the starBase website [35, 66]. The ceRNA network was constructed using mRNA, lncRNA and miRNA transcript data from the TCGA database. The expression associations between target miRNAs and PLOD1/2/3 and lncRNAs and target miRNAs were visualized through co-expression analysis with the R package limma. The threshold of P value was set at <0.005.

2.12 Statistical Analysis

The data was analyzed and visualized using R (version 4.1.2) and RStudio (version 1.4.1103). We visualized the data with the R packages ggpubr (v0.4.0), ggplot2 (v3.3.6), limma (v3.50.3), survival (v3.4-0), and clusterProfiler (v4.2.2). K-M curves and log-rank tests were applied to compare the survival distributions of high and low gene expression groups. P<0.05 was used as the significance level for all paired statistical tests.

3 Results

3.1 PLOD Family Genes Show High Transcript Levels in BC

Firstly, PLOD1 to PLOD3 genes were compared to the levels of expression in 20 human cancers with their adjacent normal samples in Oncomine. Transcript levels of PLOD1-3 were observed to upregulation in 2, 6, and 6 datasets, respectively, in BC, compared to normal breast tissue, where PLOD2 was also found to down-regulation in 3 datasets (Figure 2). Next, we applied the TIMER to make a comparison of the expression of PLODs between BC samples and control samples, and in comparison with normal breast tissue, BC transcript levels of PLOD1/3 were significantly elevated (Figure 3(A-C)). Furthermore, the GSE54002 dataset was used to validate the differential expression of PLODs, which showed that PLOD1/2/3 were overexpressed in BC (Figure 3(D-F)). Then, we determined the frequency of genetic alterations in PLODs in BC patients. The gene alterations of PLODs were detected in BC patients, including amplification, deep deletion, mutation, mRNA high, and multiple mutations, with upregulation of mRNA being the first common type (Figure 3(G)). In addition, the percentage of PLOD1 alteration in BC patients was 5%, while the PLOD2 and PLOD3 alteration were both 9%, respectively (Figure 3(H)). Next, we also analyzed the transcript levels of PLOD1-3 in BC cell lines in the CCLE database. Consistent with the above results, as compared to a number of other cancer cell lines, BC cell lines have high transcription levels of PLODs (Figure 4(A-F)).

Analysis Type by Cancer	Cancer vs. Normal PLOD1		Cancer vs. Normal PLOD2		Cancer vs. Normal PLOD3	
Bladder Cancer	1		1		1	
Brain and CNS Cancer	3		11		3	
Breast Cancer	2		6	3	6	
Cervical Cancer	1		3			
Colorectal Cancer	3			6	9	
Esophageal Cancer	4		2	1	8	1
Gastric Cancer	3				8	
Head and Neck Cancer	4		8		9	
Kidney Cancer	2	1	8	2	11	
Leukemia		5	1	1		1
Liver Cancer			1		4	
Lung Cancer	3		14		2	
Lymphoma	5		8	3	12	
Melanoma	4			1	3	
Myeloma			4			
Other Cancer	11	1	8		4	
Ovarian Cancer	2		2		1	
Pancreatic Cancer			4		3	
Prostate Cancer			1	1	1	1
Sarcoma	5		5	4	1	
Significant Unique Analyses	53	7	86	22	86	3
Total Unique Analyses	387		444		427	

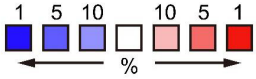

Figure 2. Comparative expression of PLOD1-3 in the ONCOMINE database. Graphs show the number of datasets with statistically different mRNA overexpression (red) or down-expression (blue). The number in each cell indicates how many analyses meet that threshold within that analysis and cancer type.

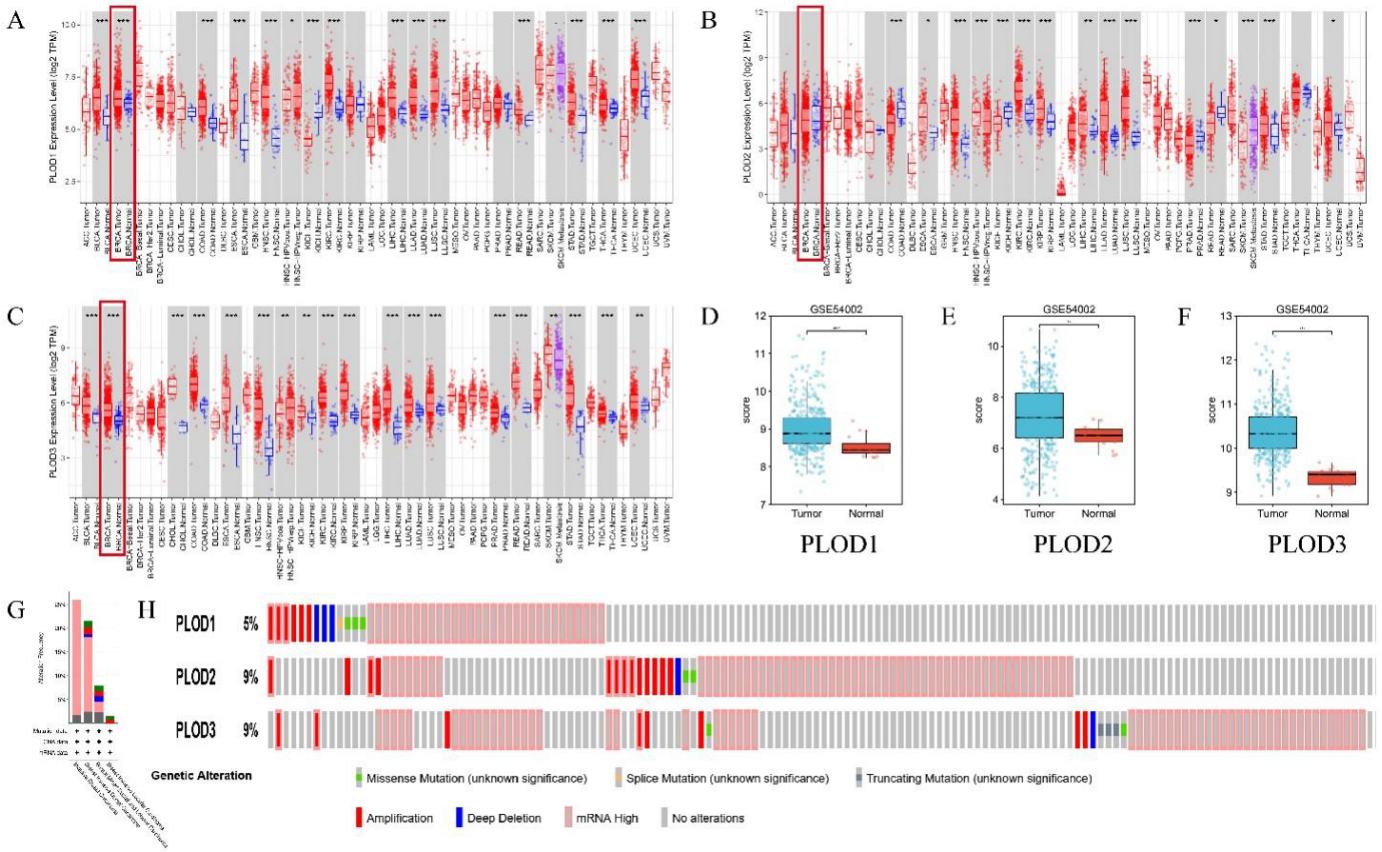

Figure 3. The expression of PLODs in BC. The expression of PLOD1-3 in pan-cancer (A-C). Expression levels of PLOD1/2/3 in BC in GSE54002 dataset (D-F). Genetic alteration of PLODs (G-H).

3.2 PLOD Family Genes Were Correlated with Clinicopathological Features of BC Patients

Next, we analyzed the relationship between the molecular subtypes of BC and the transcript levels of PLOD1-3. The expressions of PLODs were significantly higher in triple-negative than HER2-positive or luminal in BC of different molecular subtypes ($P < 0.05$)(Figure 5(A-C)). Second, considering that PLOD1/2/3 may be associated with the invasion of BC, we further evaluated the connection between PLOD1, PLOD2, and PLOD3 expression and tumor grade and lymph node stage in BC patients. The increase in PLOD1 and PLOD3 expression was observed with the significantly increasing pathological stage, while the expression level of PLOD1 and PLOD3 also increased with the significantly growing lymph node stage ($P < 0.05$)(Figure 5).

3.3 PLOD Family Genes Were Highly Expressed at the Protein Level in BC Patients

Following our study of PLOD expression at the mRNA level, we investigated PLOD expression at the protein level in the HPA database. Notably, the expression of PLODs proteins was higher than normal in BC tissues. As shown in Figure 6, PLOD1 protein was overexpressed in BC with weak intensity staining (Figure 6(A, D)). Similarly, the immunohistochemistry results also showed significant upregulation of PLOD2 and PLOD3 protein in BC tissues with medium staining , on validation at the protein expression level, PLODs in primary tumors and normal tissues had differences, with PLOD1 and PLOD2 being the most pronounced (Figure 6). Meanwhile, PLOD1 and PLOD3 were not detected in any normal glandular tissues. Thus, these results suggest that these three genes are also overexpressed at the protein level in BC patients.

3.4 High Expression of PLOD Family Genes was Associated with Poor Prognosis in BC Patients

The effect of PLOD1-3 expression on survival in BC was evaluated using the K-M Plotter database. The analysis indicates that high mRNA level expression of PLOD1 was linked to poorer OS (HR) = 1.28, $P < 0.05$), DMFS (HR = 1.32, $P < 0.05$), Recurrence-Free Survival (RFS) (HR = 1.26, $P < 0.05$) in BC, high mRNA level expression of PLOD2 was also associated with shorter OS (HR = 1.32, $P < 0.05$), DMFS (HR = 1.38, $P < 0.05$), RFS (HR = 1.5, $P < 0.05$), while overexpression of PLOD3 was associated with poorer

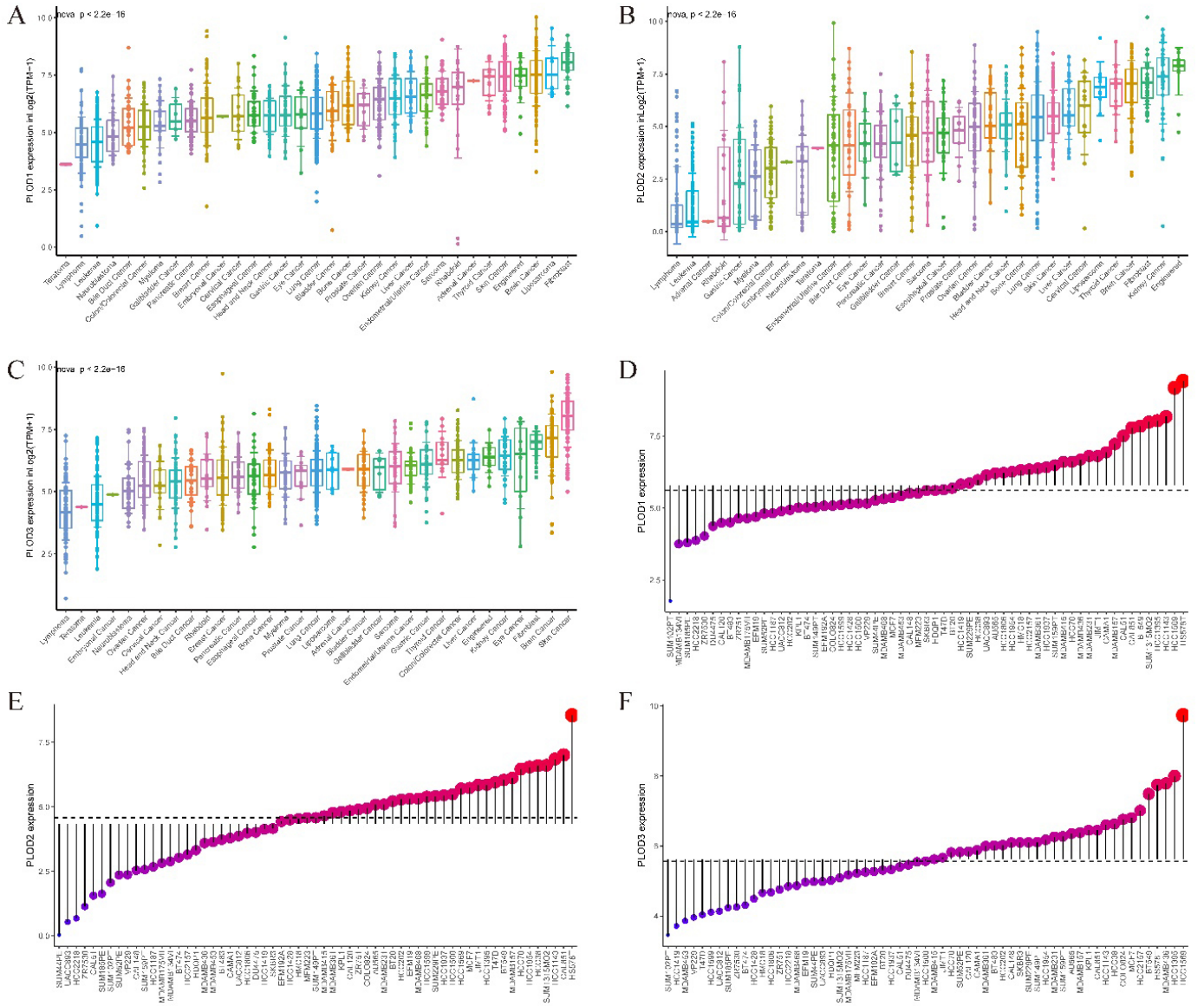

Figure 4. Expression of PLOD1, PLOD2 and PLOD3 in various cancer cell lines (A-C).

DMFS (HR = 1.38, P < 0.05), RFS (HR = 1.19, P < 0.05) in BC (Figure 7). The above results suggest that PLODs may be biomarkers of poor survival outcomes in BC.

Next, we validated the predictive value of each member of the PLODs in the GEO dataset GSE25055. ROC analysis of individual PLOD family members using the GEO dataset showed that PLOD1/2/3 were associated with BC recurrence. The AUC values for PLOD1 to predict 1-, 3-, and 5-year survival were 0.67, 0.64, and 0.62, respectively, and the AUC values for PLOD2 were 0.63, 0.65, and 0.66, while the respective AUC values for PLOD3 were 0.65, 0.64, and 0.65 (Figure 8(A–C)). In addition, Kaplan-Meier analysis of recurrence-free survival (RFS) for PLOD1/2/3 in the GEO dataset GSE25055 indicated that high expression of PLOD1-3 predicted poor RFS (P < 0.01; Figure 8(D-F)).

3.5 Co-expression and Interaction Analysis of PLOD Family Genes in BC

After obtaining the associations between PLODs and prognosis, we probed the correlation of the three genes and found that all genes of the PLODs were positively related to each other. Protein-protein interaction results revealed that the functions of PLOD1-3 were closely related (Figure 9(A)). From cBioPortal data analysis, PLOD1 and PLOD3 (R=0.58) were the most closely related, followed by PLOD1 and PLOD2 (R=0.2), and finally, PLOD2 and PLOD3 (R= 0.11) (Figure 9(B)). The approximate results were obtained by GEPIA analysis. PLOD1 and PLOD3 were the most correlated (R=0.53), then PLOD1 and PLOD2 (R=0.24), and lastly, PLOD2 and PLOD3

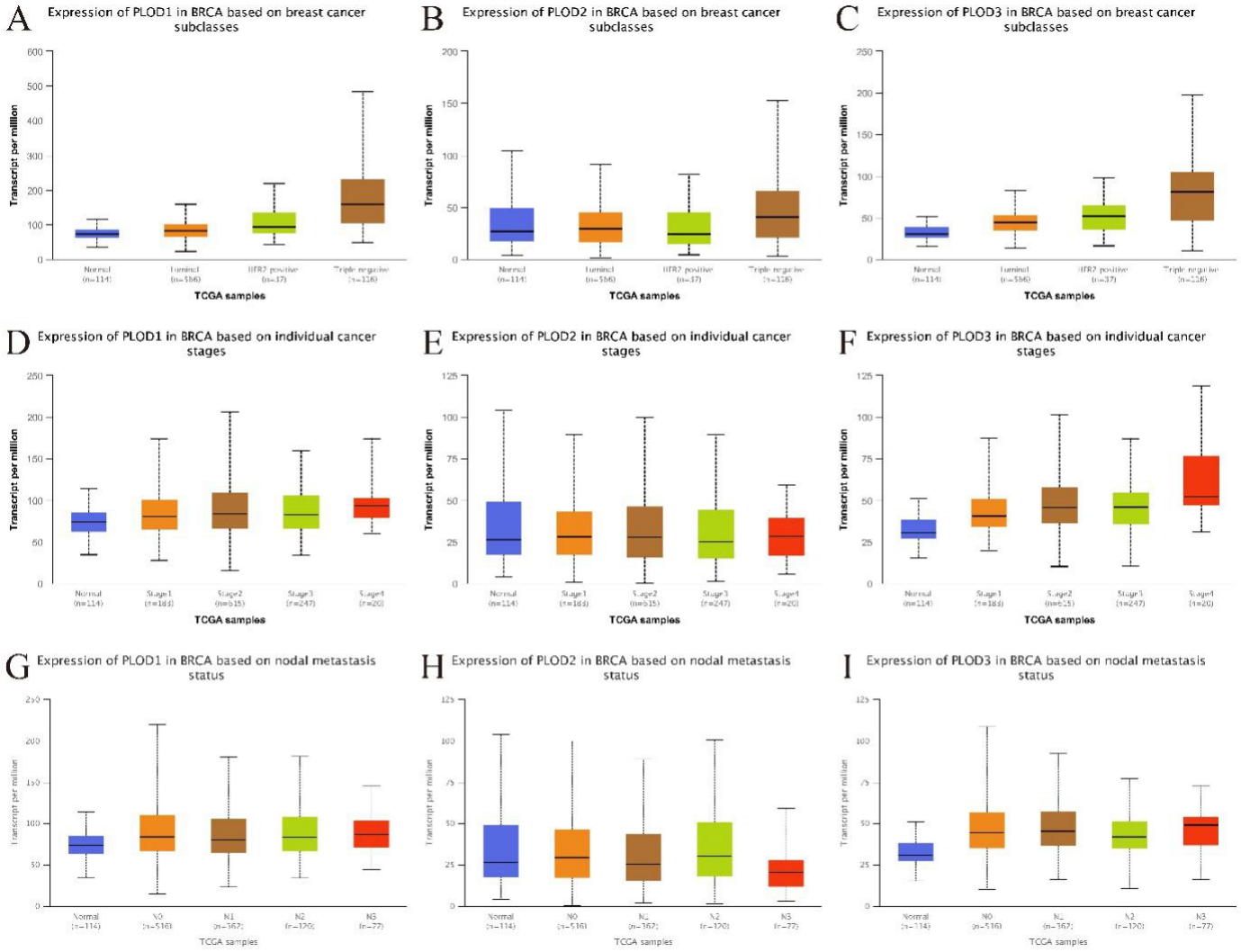

Figure 5. PLODs expression in BC according to BC subtypes (A-C), individual cancer stages (D-F) and nodal metastasis status (G-I).

(R=0.12)(Figure 9(C-E)).

3.6 PLOD1/2/3 GO Enrichment and KEGG Analysis

To further probe the biological functions of PLODs and their neighbor genes in BC, clusterProfiler was used to construct the GO and KEGG pathways. In biological processes (BP), it was discovered that these genes are majorly responsible for epidermis development, antimicrobial humoral response, DNA replication initiation, O-glycan processing, and aromatic amino acid transport (Figure 10(A)). The significantly enriched terms in the GO cellular component (CC) analysis were intermediate filament cytoskeleton, intermediate filament, anchored component of membrane, keratin filament, and specific granule lumen (Figure 10(B)). In addition, significantly enriched molecular function (MF) terms included UDP-galactosyltransferase activity, galactosyltransferase activity, GABA receptor activity, aromatic amino acid transmembrane transporter activity, and RAGE receptor binding (Figure 10(C)). KEGG pathway analysis showed enrichment in the IL-17 signaling pathway, Protein digestion and absorption, and Glycosphingolipid biosynthesis-lacto and neolacto series (Figure 10(D)). Enrichment analysis of PLOD 1/2/3 and their closely linked genes in BC suggests that the functions of these genes may be engaged in cellular DNA replication, cell division, and basement membrane formation.

3.7 The ceRNA Network of PLOD Family Genes in BC

We next predicted and constructed a ceRNA network associated with PLODs through the starBase and mirDIP websites and TCGA transcriptome data. 78 and 496 miRNAs were predicted by starBase and mirDIP against PLOD1, respectively, with 43

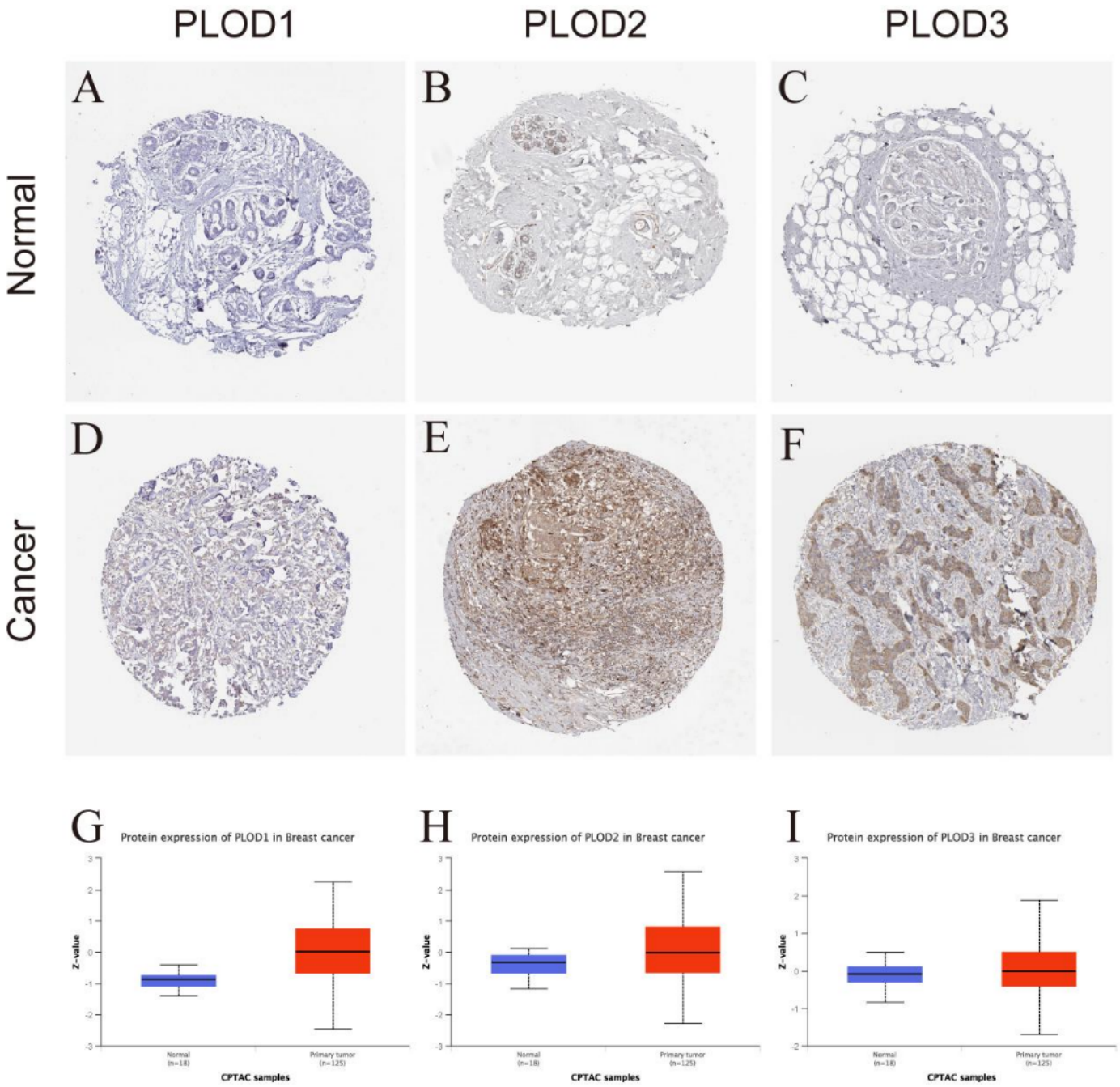

Figure 6. Image representation of PLOD1/2/3 immunohistochemistry in control breast tissue (A-C) and BC (E-F).

miRNAs found by both sites (Figure 11(A)). At the same time, co-expression analysis for these 43 miRNAs revealed that hsa-miR-335-5p ($R = -0.2$, $P < 0.01$) and hsa-miR-642a-5p ($R = -0.19$, $P < 0.01$) were negatively co-expressed with PLOD1 (Figure 11(D-E)). Besides, we picked up 126 and 709 miRNAs targeting PLOD2, respectively, 103 of which could be detected by these two tools (Figure 11(B)). Meanwhile, co-expression analysis of these 103 miRNAs suggested that hsa-miR-182-5p ($R = -0.25$, $P < 0.01$), hsa-miR-200b -3p ($R = -0.15$, $P < 0.01$) and hsa-miR-340-5p ($R = -0.15$, $P < 0.01$) were negatively correlated with PLOD2(Figure 11(F-H)). Moreover, the websites predicted 41 and 387 miRNAs that could bind to PLOD3, respectively, and 24 of these miRNAs were picked up by them (Figure 11(C)), while the correlation analysis of these 24 miRNAs with PLOD3 showed that only the expression of hsa-miR-184 ($R = -0.19$, $P < 0.01$) was negatively co-expressed with PLOD3 (Figure 11(I)).

Next, starBase predicted lncRNAs bound to hsa-miR-182-5p, hsa-miR-184, hsa-miR-200b-3p, hsa-miR-335-5p, hsa-miR-340-5p and hsa-miR-642a-5p. Co-expression revealed that the expression of

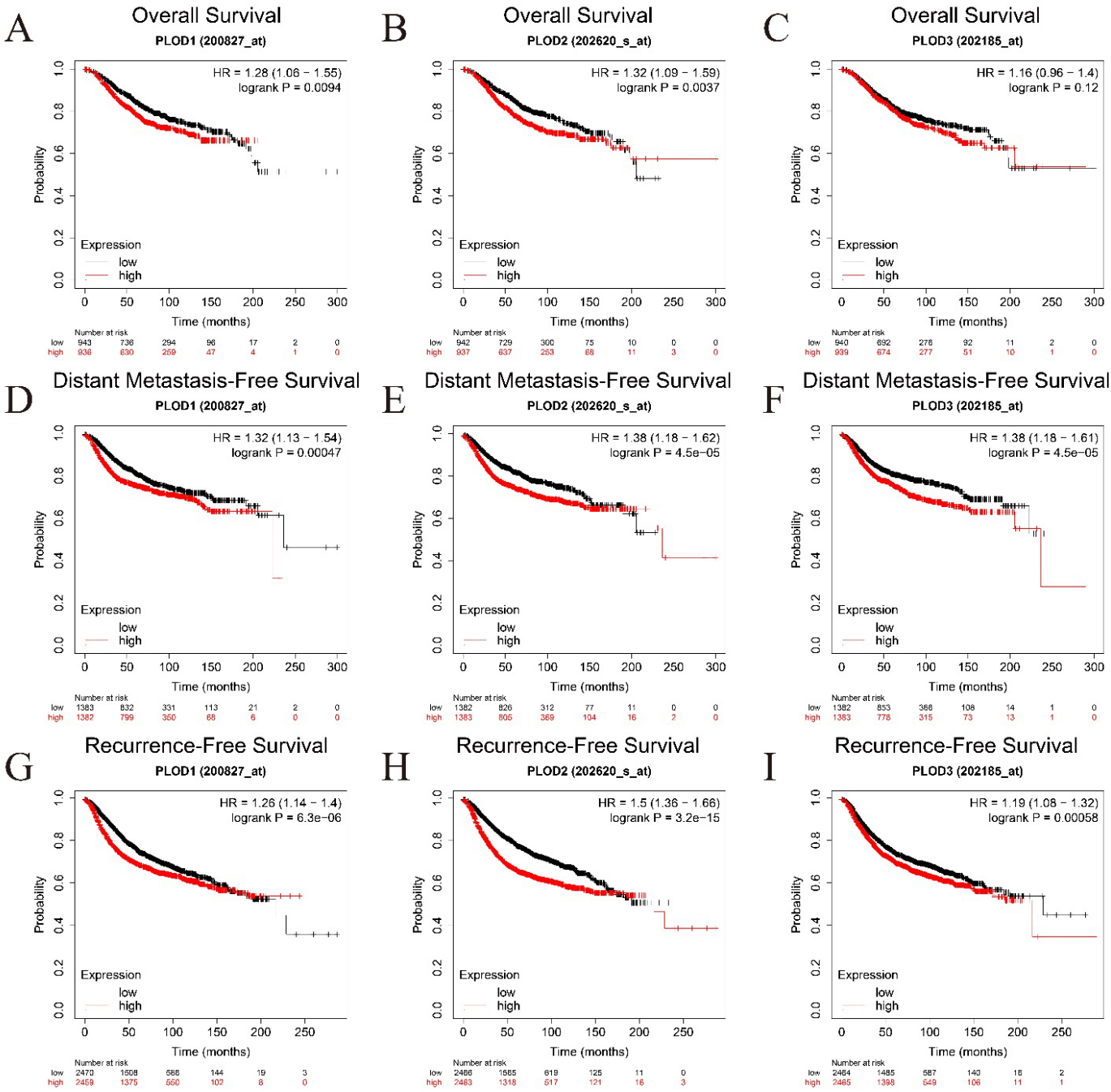

Figure 7. Analysis of the prognostic significance of the transcript levels of PLODs in BC. Relationships between the transcript levels of PLODs and OS (A-C), Distant Metastasis-Free Survival (DFS) (D-F) and RFS (G-I). OS, overall survival; DFS, distant metastasis-free survival; RFS, recurrence-free survival; HR, hazard ratio.

LINC01018 and PRR7-AS1 was in a negative relation with hsa-miR-182-5p (r=-0.15; r=-0.15) and in a positive relation with the transcript level of PLOD2 (r= 0.2; r=0.11). An adverse correlation was found between LINC00665 and hsa-miR-184 (r=-0.16), while a positive correlation was found between LINC00665 and PLOD3 (r=0.15). LINC01140, LINC01303 and MSC-AS1 have negative correlations with hsa-miR-200b-3p (r=-0.16; r=-0.15; r=-0.33) and positive correlations with PLOD2 expression level (r=0.17; r=0.21; r=0.37). LINC01503 was adversely co-expressed with hsa-miR-335-5p (r=-0.15) and had positive co-expression with PLOD1 (r=0.29). AP000766.1, EBLN3P, LINC01963 and OIP5-AS1 were adversely co-expressed with hsa-miR-340-5p (r=-0.23; r=-0.2; r=-0.17; r=-0.26) and positively co-expressed with PLOD2 (r=0.12; r=0.14; r=0.1; r=0.24). Additionally, the expression of AC131212.3, HCG11 and MELTF-AS1 was negatively correlated with hsa-miR-642a-5p (r=-0.13; r=-0.12; r=-0.19) and

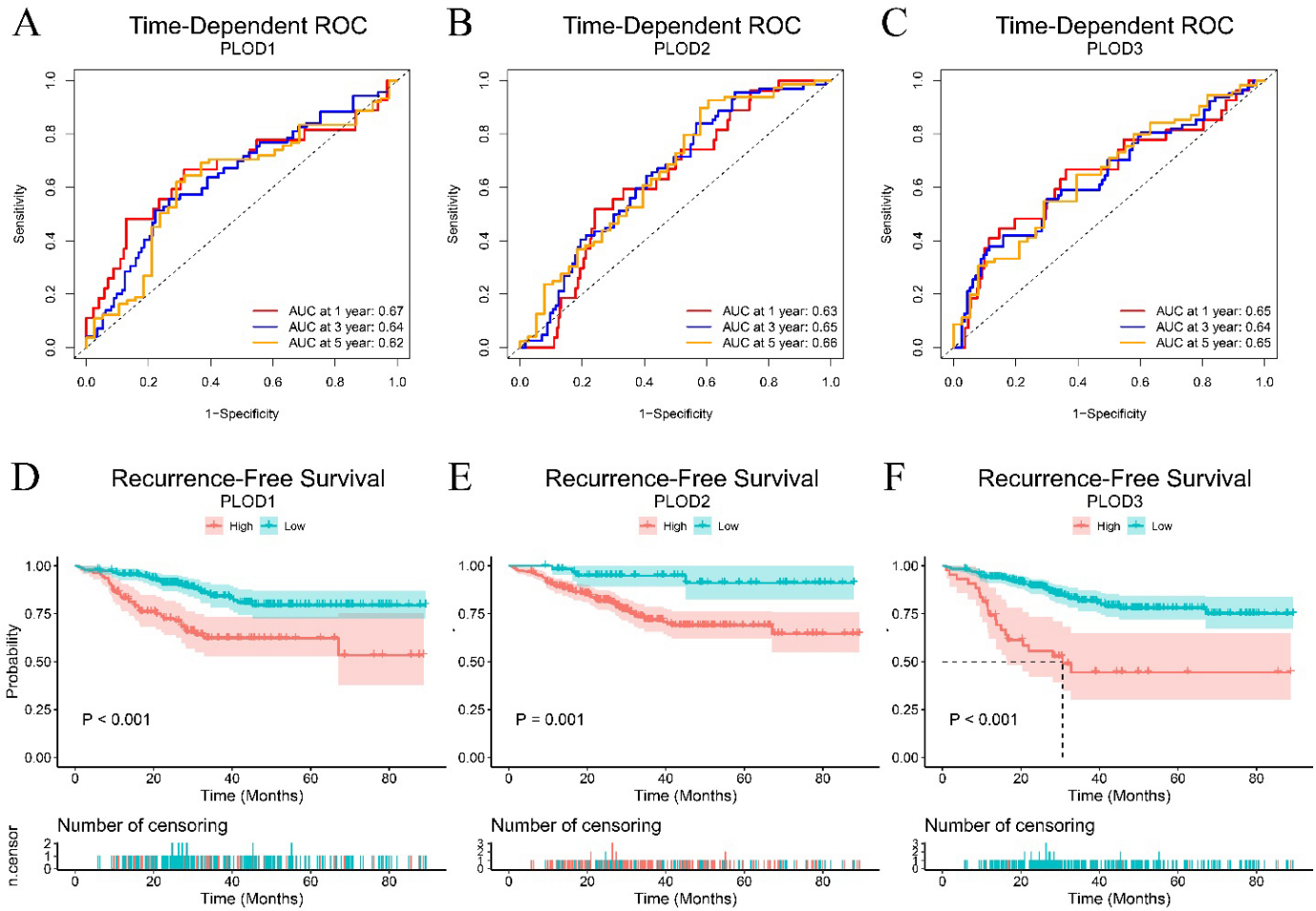

Figure 8. Prognostic value of PLODs in BC patients. Time-dependent ROC curves for PLOD1/2/3 in BC (A-C). RFS curves for PLOD1/2/3 in BC (D-F).

positively correlated with PLOD1 transcript levels (r=0.13; r=0.26; r=0.17). A ceRNA network was formed and shown in the Sankey diagram (Figure 12).

3.8 The Expression of PLOD Family Genes in BC Patients Correlates with the Level of Immune Infiltration

To explore the interplay between the tumor immune microenvironment and PLODs, we investigated the link between immune cell infiltration levels and differentially expressed PLODs in BC using the TIMER database. It was discovered that the expression of PLOD1 was actively connected with CD4+ T cells (r=0.1, P=2.00e-03), macrophages (r=0.126, P=7.73e-05), neutrophils (r=0.198, P=6.91e-10) and dendritic cells(DC) (r=0.211, P=5.19e-11), and positive correlations have also been found among PLOD2 and B cells (r=0.088, P=6.17e-03), CD8+ T cells (r=0.241, P=2.27e-14), CD4+ T cells (r=0.105, P=1.17e-03), macrophages (r=0.28, P=3.58e-19), neutrophils (r= 0.29, P=7.95e-20) and DC (r=0.243, P=2.77e-14) infiltration, while the expression of PLOD3 was positively related to the infiltration level of CD4+ T cells (r=0.091, P=4.53e-03) and DC (r=0.075, P=2.16e-02), but negatively correlated with CD8+ T cells (r=-0.154, P=1.37e-06) and macrophages (r=-0.125, P=8.55e-05) infiltration levels (Figure 13(A-C)). We also analyzed the expression of PLODs in relation to genetic markers of various immune cells in BC, including B cells, CD8+ T cells, DC, macrophages, monocytes, natural killer (NK) cells, neutrophils, T cells (general), T cell exhaustion, and regulatory T cells (Tregs)(Table 1). Our results suggested that PLOD1/2 expression remarkably correlated with the majority of immune markers of various immune cell subtypes in BC, while PLOD3 expression was weakly correlated with these immune markers. Furthermore, PLOD1 expression correlated more closely with immune markers of DC cells (NRP1), M1 macrophages (PTGS2, NOS2), M2 macrophages (VSIG4, CD163), and Th2 (GATA3). PLOD2 expression correlated more closely with immune markers of DC cells (NRP1),

Table 1. Correlation analysis between PLODs and markers of immune cells in TIMER.

Description	Gene markers	PLOD1				PLOD2				PLOD3			
		None		Purity		None		Purity		None		Purity	
		Cor	P	Cor	P	Cor	P	Cor	P	Cor	P	Cor	P
B cell	CD19	-0.007	0.812	-0.093	**	-0.069	*	-0.091	**	0.002	0.949	-0.011	0.718
	CD79A	0.018	0.554	-0.071	*	-0.055	0.067	-0.075	*	-0.008	0.794	-0.026	0.411
CD8+ T cell	CD8A	-0.038	0.213	-0.116	***	-0.005	0.867	0.004	0.898	-0.031	0.303	-0.037	0.241
	CD8B	0.057	0.060	-0.007	0.827	-0.012	0.689	-0.006	0.851	0.064	*	0.070	*
Dendritic cell	ITGAX	0.161	***	0.115	***	0.171	***	0.187	***	0.032	0.287	0.026	0.407
	NRP1	0.263	***	0.240	***	0.350	***	0.386	***	-0.030	0.313	-0.034	0.282
	CD1C	-0.043	0.154	-0.143	***	-0.109	***	-0.123	***	-0.073	*	-0.119	***
	HLA-DPA1	0.026	0.389	-0.052	0.101	0.068	*	0.082	*	-0.089	**	-0.113	***
	HLA-DRA	0.071	*	0.005	0.881	0.120	***	0.145	***	-0.072	*	-0.090	**
	HLA-DQB1	0.063	*	-0.010	0.754	0.004	0.899	-0.001	0.982	0.038	0.211	0.028	0.381
	HLA-DPB1	0.063	*	-0.013	0.689	-0.061	*	-0.070	*	0.024	0.420	0.017	0.596
M1 Macrophage	PTGS2	0.236	***	0.200	***	0.228	***	0.252	***	0.057	0.059	0.038	0.230
	IRF5	0.037	0.225	0.015	0.636	0.073	*	0.075	*	0.207	***	0.218	***
	NOS2	0.307	***	0.311	***	0.230	***	0.228	***	0.154	***	0.153	***
M2 Macrophage	MS4A4A	0.120	***	0.071	*	0.213	***	0.243	***	-0.010	0.737	-0.016	0.612
	VSIG4	0.233	***	0.203	***	0.209	***	0.232	***	0.057	0.059	0.057	0.074
	CD163	0.235	***	0.206	***	0.292	***	0.317	***	0.081	**	0.085	**
Monocyte	CSF1R	0.216	***	0.173	***	0.140	***	0.159	***	0.054	0.074	0.047	0.136
	CD86	0.177	***	0.132	***	0.256	***	0.286	***	-0.027	0.372	-0.034	0.282
Natural killer cell	KIR2DS4	0.074	*	0.041	0.195	0.061	*	0.069	*	0.037	0.223	0.043	0.180
	KIR3DL3	0.023	0.439	-0.003	0.916	0.064	*	0.060	0.057	0.032	0.290	0.044	0.167
	KIR3DL2	0.030	0.322	-0.026	0.405	0.040	0.185	0.051	0.107	0.022	0.465	0.001	0.971
	KIR3DL1	0.105	***	0.068	*	0.091	**	0.094	**	0.058	0.055	0.063	*
	KIR2DL4	0.095	**	0.056	0.077	0.097	**	0.099	**	0.056	0.063	0.060	0.060
	KIR2DL3	0.070	*	0.032	0.316	0.069	*	0.065	*	0.027	0.369	0.031	0.329
	KIR2DL1	0.086	**	0.049	0.119	0.038	0.212	0.037	0.238	0.036	0.235	0.044	0.170
Neutrophils	CCR7	-0.062	*	-0.164	***	-0.048	0.114	-0.053	0.097	-0.014	0.642	-0.036	0.254
	ITGAM	0.173	***	0.134	***	0.179	***	0.193	***	0.006	0.830	0.003	0.923
	CEACAM8	0.026	0.389	0.037	0.241	0.054	0.075	0.058	0.067	0.031	0.303	0.033	0.298
T cell (general)	CD3D	0.022	0.475	-0.072	*	-0.033	0.271	-0.038	0.226	0.012	0.682	0.000	0.991
	CD3E	0.016	0.602	-0.083	**	-0.017	0.562	-0.022	0.485	-0.001	0.986	-0.016	0.618
	CD2	0.021	0.488	-0.070	*	0.027	0.374	0.033	0.293	-0.020	0.513	-0.034	0.287
T cell exhaustion	CTLA4	0.126	***	0.066	*	0.133	***	0.148	***	0.058	0.053	0.059	0.065
	LAG3	0.119	***	0.088	**	0.061	*	0.070	*	0.154	***	0.166	***
	HAVCR2	0.167	***	0.123	***	0.264	***	0.290	***	-0.032	0.292	-0.031	0.333
	GZMB	0.147	***	0.097	**	0.102	**	0.121	***	0.107	***	0.114	***
	PDCD1	0.068	*	0.004	0.910	-0.022	0.470	-0.021	0.518	0.123	***	0.131	***
TAM	CCL2	0.183	***	0.128	***	0.239	***	0.260	***	0.074	*	0.058	0.068
	IL10	0.117	***	0.066	*	0.239	***	0.260	***	0.001	0.980	-0.008	0.802
	CD68	0.217	***	0.185	***	0.255	***	0.280	***	0.049	0.103	0.053	0.097
Tfh	BCL6	0.083	**	0.055	0.086	0.161	***	0.157	***	-0.128	***	-0.147	***
	IL21	0.010	0.747	-0.023	0.471	0.036	0.229	0.039	0.220	-0.012	0.701	-0.017	0.595
Th1	TBX21	0.037	0.214	-0.039	0.214	-0.015	0.609	-0.016	0.617	0.046	0.124	0.049	0.121
	STAT4	0.031	0.299	-0.050	0.116	0.104	**	0.122	***	-0.060	*	-0.078	*
	STAT1	0.071	*	0.051	0.106	0.280	***	0.300	***	-0.069	*	-0.062	*
	IFNG	0.070	*	0.017	0.594	0.087	**	0.099	**	0.023	0.450	0.028	0.373
	TNF	0.144	***	0.102	**	0.172	***	0.166	***	0.140	***	0.137	***
Th2	GATA3	-0.475	***	-0.457	***	-0.139	***	-0.137	***	-0.272	***	-0.277	***
	STAT6	-0.089	**	-0.094	**	-0.066	*	-0.058	0.069	0.010	0.737	0.000	0.993
	STAT5A	0.100	***	0.070	*	0.015	0.617	0.039	0.218	0.059	0.051	0.061	0.055
	IL13	0.084	**	0.072	*	0.075	*	0.081	*	0.042	0.168	0.046	0.150
Th17	STAT3	0.115	***	0.119	***	0.268	***	0.294	***	-0.120	***	-0.118	***
	IL17A	0.097	**	0.098	**	0.041	0.174	0.046	0.150	0.066	*	0.086	**
Treg	FOXP3	0.136	***	0.081	*	0.138	***	0.144	***	0.026	0.392	0.017	0.602
	CCR8	0.077	*	0.042	0.187	0.258	***	0.273	***	-0.055	0.067	-0.058	0.070
	STAT5B	-0.095	**	-0.107	**	0.062	*	0.086	**	-0.137	***	-0.140	***
	TGFB1	0.175	***	0.131	***	0.082	**	0.092	**	0.110	***	0.122	***

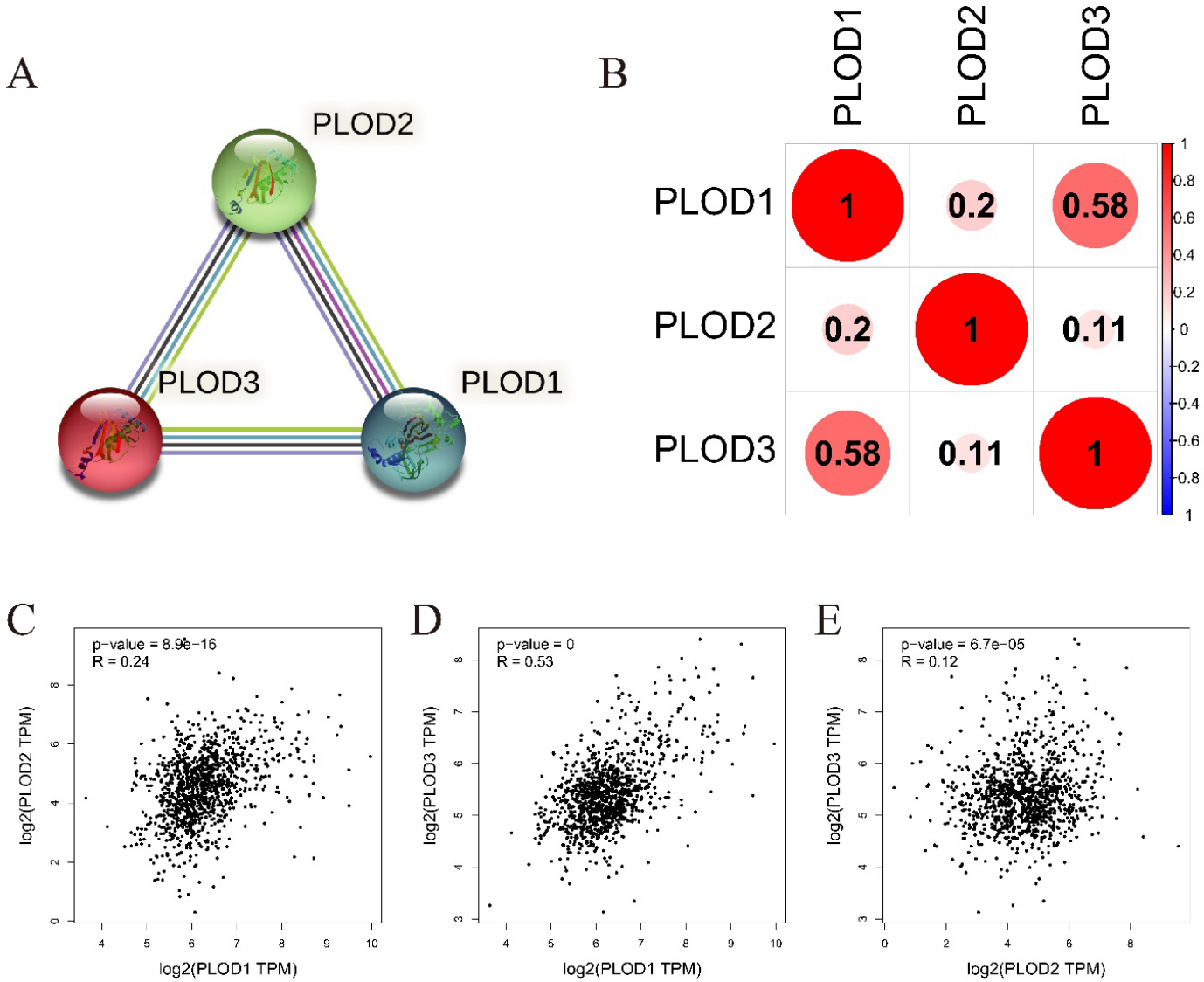

Figure 9. Correlation analysis of PLOD1, PLOD2, and PLOD3. Analysis of protein-protein interactions between PLOD1/2/3 (A). Co-expression analysis of PLOD1/2/3 in BC from the cBioPortal database (B). The co-expression analysis of PLOD1/2/3 in BC from the GEPIA database (C-E).

M1 macrophages (PTGS2, NOS2), M2 macrophages (MS4A4A, VSIG4, CD163), monocytes (CD86), T cell exhaustion (HAVCR2), TAM (CCL2, IL10, CD68), Th1 (STAT1), Th17 (STAT3), and Treg (CCR8). PLOD3 was closely connected with the immune markers of M1 macrophages (IRF5) and Th2 (GATA3).

Meanwhile, based on the CIBERSORT algorithm, we further explored the influence of PLODs expression on immune cell infiltration (Figure 14). We found that the abundance of NK cells resting, macrophages M0 and macrophages M2 were greater in the PLOD1 high expression subgroup, and the abundance of B cells naive, plasma cells (PCs), T cells CD8, T cells CD4 memory resting, T cells gamma delta, monocytes, macrophages M1, DC resting and mast cells (MCs) resting were higher in the PLOD1 low expression group ($P < 0.05$). In addition, the abundance of T cells CD4 memory resting, macrophages M0, macrophages M2, MCs activated and neutrophils were greater in the PLOD2 high expression group, while the abundance of B cells naive, PCs, T cells CD8, T cells follicular helper, Tregs, NK cells resting, NK cells activated, monocytes and MCs resting were more abundant in the PLOD2 low expression group ($P < 0.05$). Furthermore, the abundance of Tregs, NK cells resting, and macrophages M0 were higher in the PLOD3 high expression group, while the PLOD3 low expression group had higher T cells CD4 memory resting, T cells gamma delta, and MCs resting abundance ($P < 0.05$). Notably, we analyzed the impact of the level of infiltration of different immune cell subtypes on the prognosis of BC. The results indicated that the greater macrophage M2 abundance was correlated

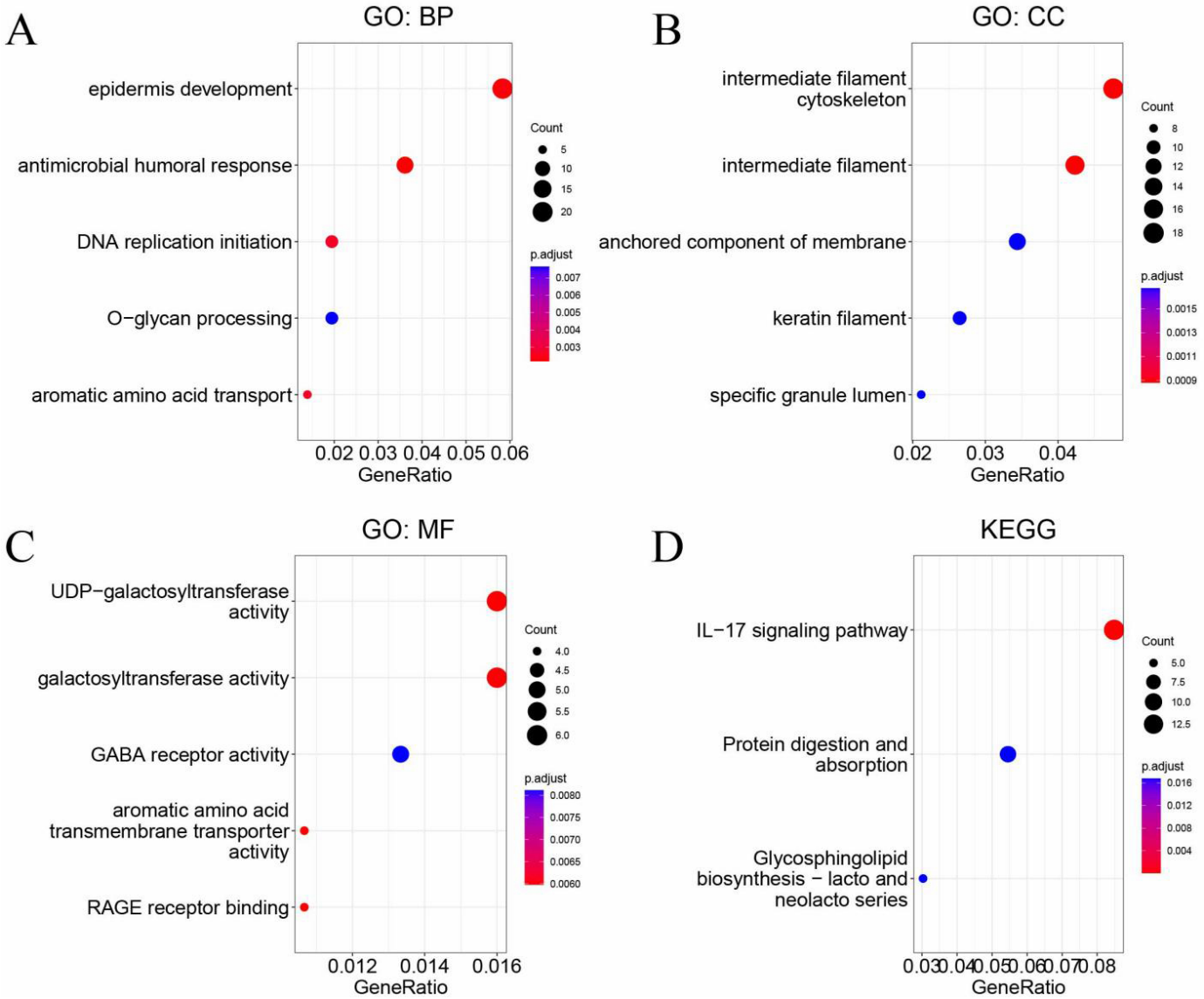

Figure 10. GO and KEGG pathway enrichment analysis of PLOD1/2/3 in BC.The top enriched BP (A), CC (B) and MF (C) terms as well as KEGG pathways (D). GO, gene ontology; KEGG, Kyoto encyclopedia of genes and genomes; BP, biological process; CC, cellular component; MF, molecular function.

with a worse outcome (P = 0.002), while higher PCs abundance corresponded to a better prognosis (P = 0.041) in BC.

4 Discussion

A multi-perspective exploration of the role of PLODs was conducted in this work, including the expression levels, disease survival, biological function, signaling pathway, and tumor immunity analysis.

PLOD gene family is a member of the 2-ketoglutarate-dependent dioxygenase family with lysine hydroxylase activity that forms homodimers in the availability of Fe^{2+} and 2-ketoglutarate (2-OG) to catalyze the hydroxylation of single-chain pre-collagen lysines and promote collagen crosslinking and maturation, thereby playing a vital role in maintaining ECM stability [9, 56]. PLOD genes consist of PLOD1/2/3, coding for 2-oxoglutarate 5- dioxygenase, which modulates collagen synthesis and crosslinking deposits [54]. Mutations and overexpression of PLODs produce hydroxylysine pyridine chains that lead to the over-deposition of collagen fibers, disrupting the ECM framework and promoting the development and metastasis of a variety of malignancies [50]. When solid tumors are hypoxic, hypoxia-inducible factor 1α(HIFNSCLC(-1α) can upregulate its expression by binding to the hypoxic response element(HRE) in the promoter region of candidate genes [43]. It has been demonstrated that PLOD1 and PLOD2 promoter regions in BC cells contain HRE [18]. Therefore, the hypoxic environment

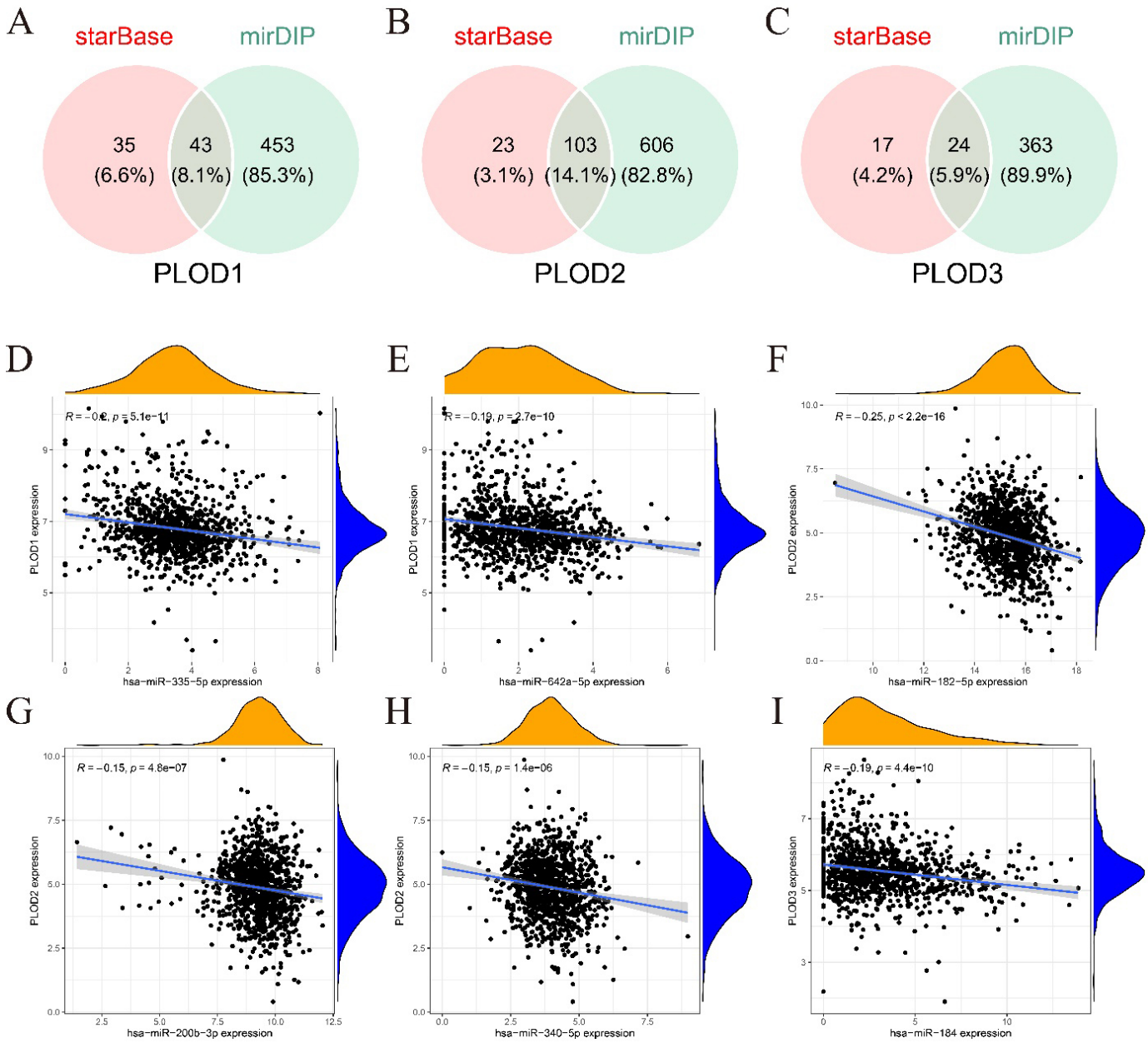

Figure 11. Comprehensive analysis of miRNAs targeting PLOD1/2/3 in BC. Venn graphs illustrating common miRNAs predicted to target PLOD1/2/3 by starBase and mirDIP (A–C). The miRNAs negatively co-expressed with PLOD1/2/3 (D-I).

can upregulate PLOD1 and PLOD2 expression by stabilizing HIF-1α, increasing the migration ability of BC cells, and promoting tumor metastasis and migration [18].

The PLOD1 gene, situated on chromosome 1p36.2-36.3, comprises 19 exons, promoting the hydroxylation process of lysine residues on V-type collagen [65]. It is involved in developing various tumors, and its high mRNA level may predict the malignancy of certain cancers [20]. The transcript level of PLOD1 was elevated in BC tissues and induced by hypoxic conditions in a HIF-1-dependent manner, and overexpression of PLOD1 promoted proliferation and lung and lymph node metastasis in BC by regulating collagen crosslinking [18]. In addition, aberrant PLOD1 expression has been detected in bladder cancer, and the malignant phenotype of tumor cells can be reduced if treated by siRNA-mediated PLOD1 knockdown or with PLOD1 inhibitors [78]. Meanwhile, PLOD1 gene mutation or overexpression has also been examined in esophageal squamous cell carcinoma, GC, and colorectal cancer(CRC) while linked to decreased patient survival, and PLOD1 is also tightly linked to NF-KB signaling [36, 71, 74]. Similar to the above results, our study revealed that PLOD1

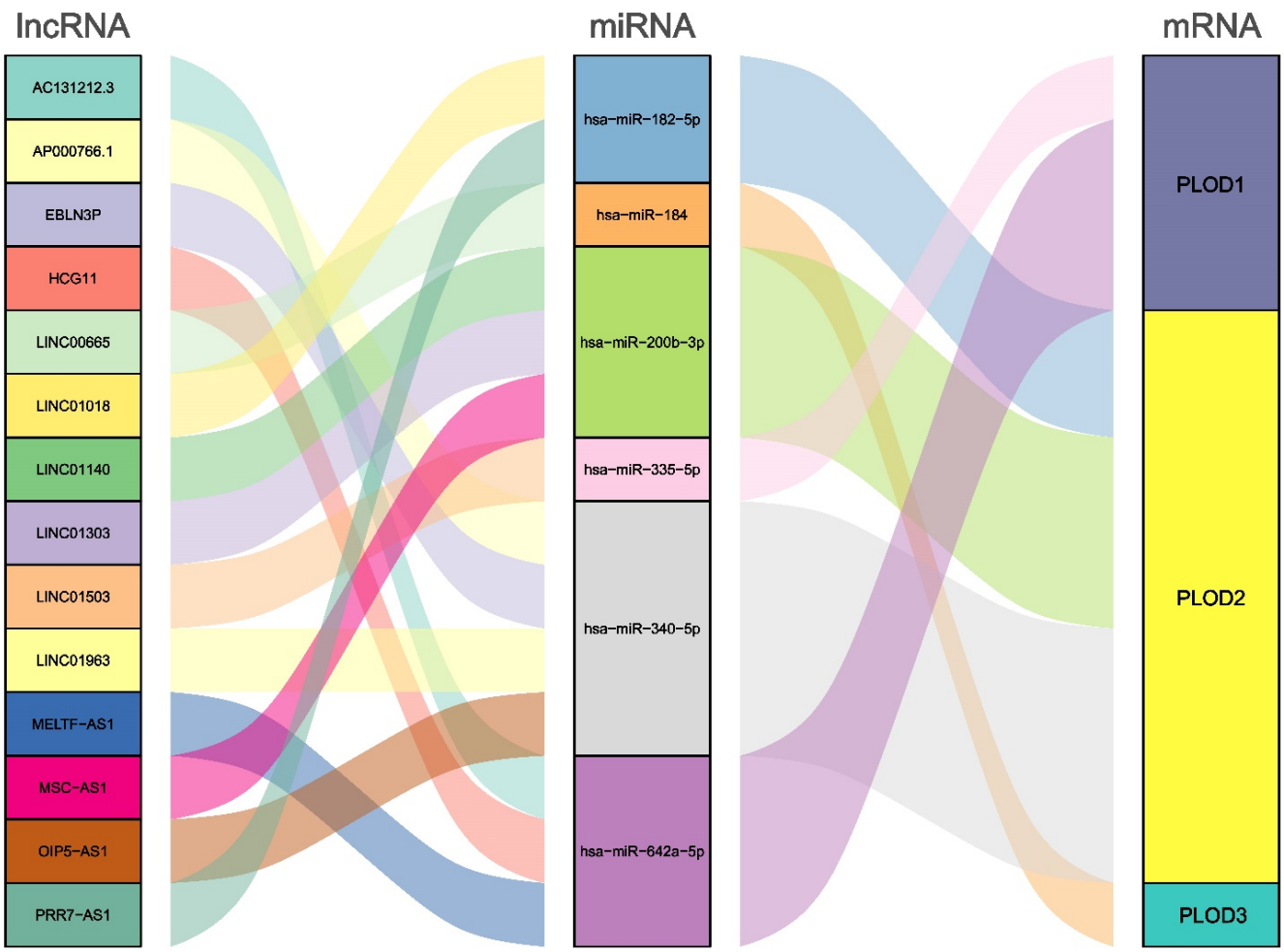

Figure 12. Sankey diagram of the ceRNA network targeting PLOD1/2/3 in BC.

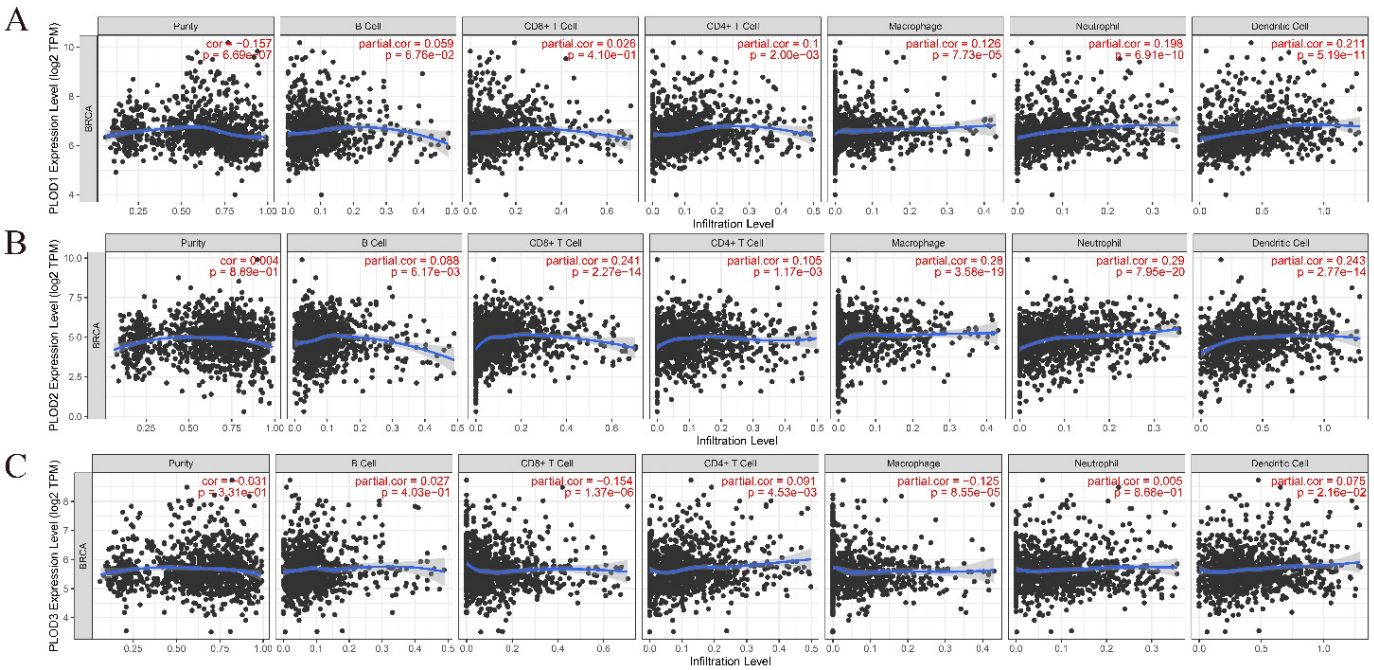

Figure 13. An evaluation of the relationship between PLOD1/2/3 expression and immune infiltration in BC. Correlation between various classes of TIICs (B cells, CD8+ T cells, CD4+ T cells, macrophages, neutrophils and DC) and PLOD1 (A), PLOD2 (B) and PLOD3 (C) expression.

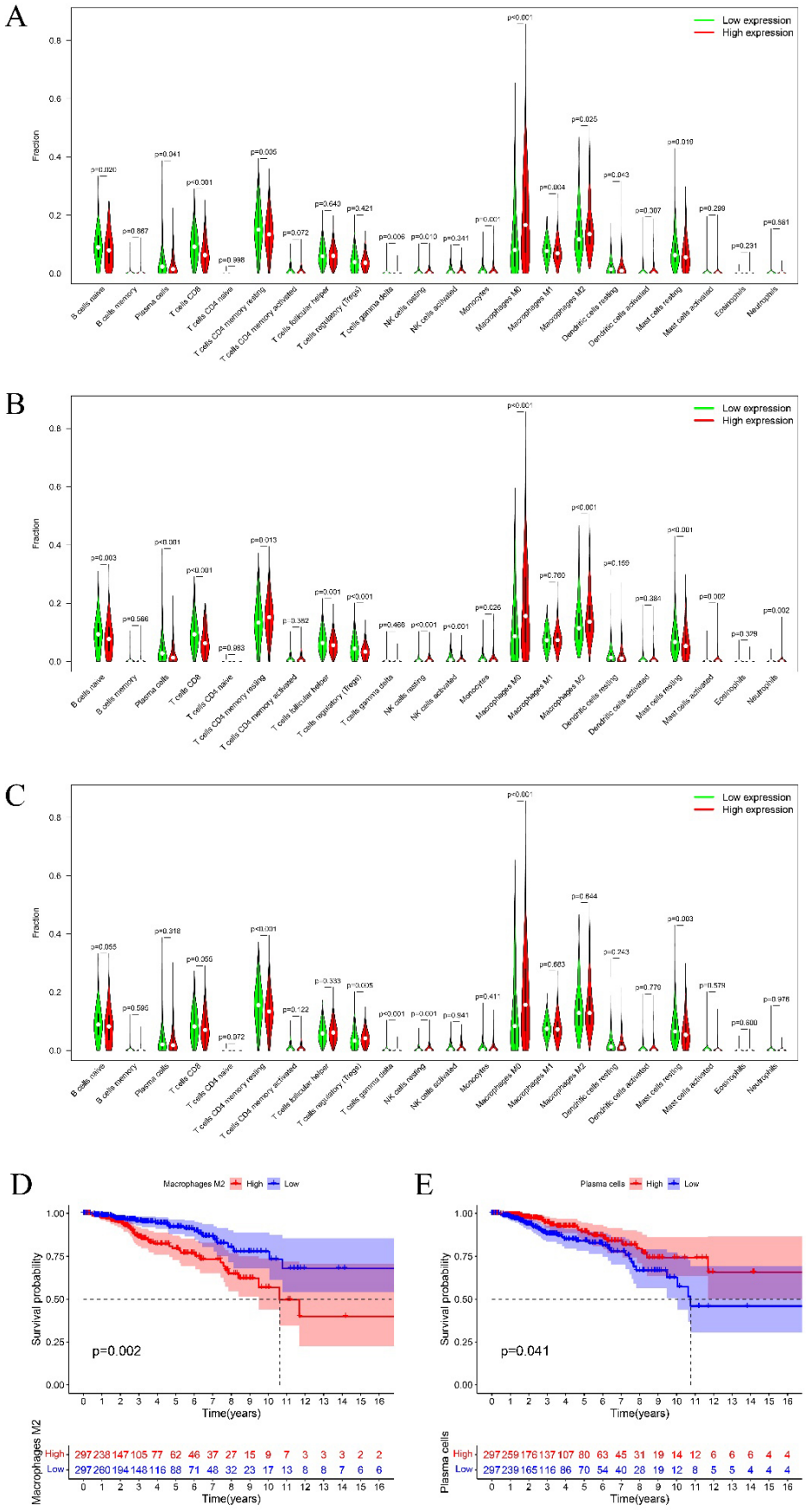

Figure 14. Comparison of the abundance of infiltrating immune cells between the low and high PLOD1/2/3 expressing groups (A-C). Relationship between expression of Macrophages M2 and Plasma cells and BC survival (D-E).

was upregulated in BC, and its high transcript levels were involved in the worsening of DMFS, OS and RFS in BC.

The PLOD2 gene, located on chromosome 3q23q24, contains 19 exons and is chiefly situated in the rough endoplasmic reticulum of many human tissues [64]. PLOD2 is thought to be a crosslinking enzyme between extracellular collagen molecules. It plays a crucial role in forming stable collagen crosslinks, specifically hydroxylating the lysine of pre-collagen-terminal peptides, affecting collagen covalent crosslinking and tissue stiffness. Overexpression of PLOD2 promotes collagen crosslinking, increases the stiffness of the ECM, and promotes tumor cell expansion and metastasis [9, 17, 69]. The role and mechanisms of PLOD2 in various tumors are being explored in depth. Former studies have proposed that PLOD2 was an independent prognostic indicator for bladder, renal cell, glioma, biliary tract, hepatocellular, gastric, and oral squamous cell cancers. Simultaneously, its high transcript levels were positively connected with peritoneal metastasis of GC, biliary tract cancer, and intrahepatic metastasis of hepatocellular carcinoma [31, 45, 53, 59]. The mechanism of HIF-1a promoting PLOD2 expression is employed to control sarcoma metastasis [11]. The aberrant expression of PLOD2 in BC is also regulated by HIF-1a, increasing tumor hardness of BC and promoting metastasis of the tumor to lymph nodes and lungs [10, 44, 47]. Additionally, our study similarly demonstrated that PLOD2 expression is upregulated in BC, and its high expression predicted poorer DMFS, OS and RFS in BC.

The PLOD3 gene, located on chromosome 7q36 [68], promotes the hydroxylation process of lysine residues in collagen, essential for collagen synthesis and modification [50]. It has been shown that PLOD3 was involved in the genesis and metastasis of a wide range of tumors, including colon, liver, lung, glioblastoma, and GC, and its mechanism of action may be related to epithelial-mesenchymal transition and angiogenesis involving collagen modifying enzymes [5, 24, 27, 42, 46, 57, 67, 71]. According to previous studies, down-regulation of PLOD3 expression can lead to G1 block in glioma cells mediated by P21 [67]. In addition, inhibition of PLOD3 expression can promote lung cancer cell apoptosis through endoplasmic reticulum stress-mediated IRE1α activation and overcome chemotherapy resistance [4]. Our work found that a high level of PLOD3 expression tends to correlate with shorter DFS, RFS.

Triple-negative BC is a particular type of BC with a low early detection rate, high malignancy, and recurrence rate [38]. According to our study, we detected higher levels of PLOD1/2/3 expression in triple-negative BC than in luminal or her^{2+} BC, and the high expression predicted a higher pathological stage, but the regulatory mechanism of the PLOD family is still unclear. KEGG enrichment analysis found that PLOD1/2/3 were connected with the IL-17 signal pathway, Protein digestion and absorption, and Glycosphingolipid biosynthesis-lacto and neolacto series were correlated. BC develops and progresses depending on many factors, and inflammation is one of the most critical factors affecting the progression of BC by activating inflammatory signaling pathways such as nuclear factor-kB and other potent pathways that lead to the expression of pro-inflammatory cytokines and chemokines such as IL17, thereby promoting tumor invasion [6, 33]. The Th17 cell has a critical position in the invasion of BC by producing pro-inflammatory cytokines (IL17), and researchers have now exploited novel targets involving the IL17B-IL17RB signaling pathway [2, 58]. Glycolipids (GSLs) are complex glycans linked to sphingomyelin and different fatty acid chains and are indispensable components of the exposed animal cell membrane surface. Various research has demonstrated that they play an irreplaceable function in cell proliferation, adhesion, motility, and differentiation [85], and their aberrant expression is a hallmark of cancer cells and is associated with their malignant properties [22]. In addition, most tumor cell surfaces show altered patterns of GSLs and abnormalities in GSLs signaling and biosynthesis, which play an essential role in tumor development [85]. BC glycolipid composition and metabolism are dramatically altered, and a close relationship may exist between these processes and the development of BC. As a result, PLOD genes occupy a unique role in the progression of BC.

In 2011, Salmena et al. [55] first proposed the ceRNA hypothesis that lncRNAs could modulate the expression of downstream genes by competitively binding miRNAs, lncRNAs and circRNAs serve as miRNA sponges to reduce the abundance of miRNAs, through microRNAs response elements (MREs) in miRNAs, thereby interfering with the repressive effects of miRNAs on downstream target genes. In this study, molecular tags of mRNA, miRNA and lncRNA related to BC prognosis were screened by mining the public cancer database, and a lncRNA-miRNA-mRNA-based ceRNA network was

constructed. In this ceRNA network, it was observed that hsa-miR-335-5p and hsa-miR-642a-5p may reduce the transcription level of PLOD1 by targeting bound its mRNA. The hsa-miR-182-5p, hsa-miR-200b-3p and hsa-miR-340-5p may target PLOD2 mRNA and thus affect its expression. The hsa-miR-184 may target PLOD3 mRNA and thereby reduce the expression level. Meanwhile, their potential lncRNA regulatory mechanisms were also explored in our research. Recent studies have shown that exosomal miR-335-5p promoted metastasis in CRC [62], miR-642a-5p acted as a tumor suppressor in prostate cancer [7], miR-182-5p could inhibit human bladder tumor growth by suppressing Cofilin 1 expression [72], miR-200b-3p played an oncogenic role in several cancers [40, 77], miR-340-5p inhibited colon cancer cell migration [1], and inhibition of miR-184 in malignant glioma would enter tumor aggressiveness [12]. The latest study also found that LINC00665 promotes BC progression by regulating the miR-379-5p/lin28b axis [26]. Therefore, the constructed ceRNA network provides new insights and directions for further research into the regulatory mechanisms of BC.

A number of recent studies have identified collagen metabolism, hypoxia, and the TME as the main mechanisms involved in PLODs in cancer development. The TME is composed chiefly of tumor cells, peripheral immune and inflammatory cells, tumor-associated fibroblasts, nearby mesenchymal tissue, microvasculature, diverse cytokines, as well as chemokines complex integrated system involved in the control of BC proliferation, metastasis, chemoresistance, and immunotherapy [60]. Collagen is the main ECM component, and PLODs can promote collagen synthesis and increase matrix hardness, thus accelerating tumor cell metastasis. In addition, glial cells and CAF are the central cell populations that express PLODs. On the one hand, Cancer-infiltrating immune cells, cancer genesis, cancer development, and metastasis are controlled by CAFs [14, 15, 76]. Among the essential elements of the TME, these cells contribute to tumor invasion, promoting tumorigenesis and allowing cancer cells to evade the immune system [21, 32, 41, 61]. On the other hand, the PLOD family accelerates tumor progression by promoting the differentiation of fibroblasts into myofibroblasts and remodeling the ECM. Notably, PLODs can also promote epithelial mesenchymal-suffusion transformation (EMT), leading to cell-to-cell adhesion and cell polarity changes, thus making tumor cells more susceptible to invading surrounding tissues and developing distant metastases [34, 86]. After further analysis of the interaction between the TME and PLODs, our study revealed that altered expression levels of PLOD1/2 in the PLOD gene family had a more significant effect on immune infiltration in BC than PLOD3. We also found that elevated levels of M2 macrophage infiltration in the tumor immune microenvironment were significantly associated with shorter BC survival time, while increased plasma cell infiltration levels suggested a better survival prognosis in BC. Former researchers have shown that cancer-associated fibroblast-induced M2 macrophages could significantly enhance the growth, migration and infiltration of pancreatic tumors [82], intrahepatic cholangiocarcinoma-induced M2-polarized macrophages promoted tumor proliferation and aggressiveness via IL-10/STAT3-induced EMT [81]. There was substantial evidence that plasma cell infiltration positively improved tumor prognosis [75]. Moreover, the PLOD family exerted pro-tumor effects in most cancers, and high expression of PLOD1-2 in pancreatic cancer promoted infiltration of tumor-infiltrating immune cells, which is probably related to the RalGEF-Ral signaling pathway [83]. Our results demonstrated that elevated levels of PLOD1/2 expression were linked to increased levels of M2 macrophages and decreased levels of PCs infiltration. Therefore, we hypothesized that the PLOD family might affect the immune microenvironment of BC by acting on M2 polarization of macrophages and plasma cell infiltration, thus impacting the survival prognosis of patients. Overall, PLODs and immune cell infiltration in BC are closely related, suggesting that PLODs are promising targets in BC immunotherapy.

PLOD enzymes may represent therapeutic targets because they control collagen hydroxylation and crosslinking, which influences extracellular matrix stiffness, invasion, and potentially immune infiltration. High-PLOD tumors, including hypoxia-enriched and triple-negative breast cancers, may benefit from approaches that limit PLOD-driven matrix remodeling, possibly in combination with systemic therapy or immunotherapy. Clinical translation remains challenging due to the lack of selective agents, potential toxicity from disrupting collagen homeostasis, and pathway redundancy, highlighting the need for patient stratification and preclinical validation.

Despite providing supportive bioinformatic evidence for the potential roles of the PLOD gene family as

biomarkers and therapeutic targets in breast cancer, this study has several limitations. First, the analyses were based on public datasets and computational inference, and therefore require validation in independent cohorts and clinical specimens. Second, the observed associations do not establish causality; functional experiments in vitro and in vivo are needed to clarify the biological roles and mechanisms of PLOD genes in breast cancer. Third, immune-infiltration results were inferred from transcriptomic data, and experimental validation using approaches such as flow cytometry or immunohistochemistry is warranted. Finally, although PLOD genes appear promising as targets, specific PLOD-directed therapeutics remain limited, and further drug development and preclinical evaluation are needed to assess their translational potential.

5 Conclusion

In conclusion, our integrative bioinformatic analyses suggest that the PLOD gene family is upregulated in breast cancer and is associated with poorer patient outcomes, supporting its potential value as prognostic biomarkers and candidate therapeutic targets. We also observed subtype-related differences, with higher PLOD expression in more aggressive disease. Further validation in independent cohorts, as well as mechanistic and preclinical studies, will be essential to clarify their clinical and therapeutic relevance.

Data Availability Statement

Data will be made available on request.

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Conflicts of Interest

The authors declare no conflicts of interest.

AI Use Statement

The authors declare that no generative AI was used in the preparation of this manuscript.

Ethical Approval and Consent to Participate

This work is a secondary analysis of publicly available, de-identified datasets and does not involve human subjects research requiring ethics committee approval or informed consent.

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