Diagnostic and prognostic value of mRNA expression of phospholipase C β family genes in hepatitis B virus-associated hepatocellular carcinoma

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Abstract. Four phospholipase C β (PLCB) isoforms, PLCB1, PLCB2, PLCB3 and PLCB4, have been previously investigated regarding their roles in the metabolism of inositol lipids and cancer. The present study aimed to explore the association between PLCB1-4 and hepatocellular carcinoma (HCC). Data from 212 patients with hepatitis B virus-associated HCC were used to analyze the diagnostic and prognostic significance of PLCB genes in. A nomogram predicted the survival probability. Gene set enrichment analysis explored gene ontology terms and the metabolic pathways associated with PLCB genes. Validation of the prognostic values of PLCB genes was performed using the Gene Expression Profiling Interactive Analysis website. *PLCB1* and *PLCB2* were revealed to have diagnostic value for HCC (0.869 and 0.836 area under the curve, respectively; both P≤0.05). The combination analysis of these genes had an advantage over each alone (0.905 PLCB1 and PLCB2, and 0.877 PLCB1 and PLCB3 area under the curve; P≤0.05). *PLCB1* was associated with overall survival (OS) and recurrence-free survival (RFS; adjusted P=0.002 and P=0.001, respectively). A nomogram predicted survival probability of patients with HCC at 1, 3- and 5-years. Gene set enrichment analysis indicated that *PLCB1* and *PLCB2* are involved in the cell cycle, cell division and the PPAR signaling pathway, among other functions. Validation using GEPIA revealed that *PLCB1* and *PLCB2* were associated with OS and *PLCB1* and *PLCB4* were associated with RFS. *PLCB1* and *PLCB2* exhibited diagnostic value for HCC and their combination had an advantage over each individually. *PLCB1* has OS and RFS prognostic value for patients with HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the main causes of tumor-associated mortality, being the fifth most common malignancy worldwide (1). In 2018, the number of new cases and liver cancer-associated mortalities was 841,080 and 781,631, respectively, worldwide (2). Many risk factors, including dietary aflatoxin exposure (3), hepatitis B and C virus (HBV) infection (4) and cirrhosis, contribute to the initiation and progression of HCC. To date, many diagnostic and treatment procedures, including ultrasound, computed tomography, liver resection, liver transplantation, radiofrequency, thermal and non-thermal ablation, trans-arterial chemoembolization (5), immunotherapies and therapeutic cancer vaccines (4), have been used for patients with HCC. However, the prognosis of HCC is remains poor and the 5-year relative survival rate is ~12% due to tumor metastasis and recurrence (6,7). Due to the characteristics of systemic disease, the evolution and progression of HCC involves deregulation of genes, cells and tissues (1). Therefore, it is crucial to identify novel biomarkers that may be involved in the course of tumor metastasis and recurrence, for early diagnosis and recurrence prediction for HCC.

Phospholipase C (PLC) is encoded by four genes, *PLCA*, *PLCB*, *PLCC* and *PLCD*, and is involved in the pathogenesis of several bacterial infections, including *Clostridium perfringens*, *Listeria monocytogene*, and *Pseudomonas aeruginosa* (8,9). The activity of PLCA and PLCB in *L. monocytogenes* appears to overlap in the course of intracellular infection (10). In *Listeria*, three genes, *PLCA*, *PLCB* and *PLCC*, are clustered together on the same chromosome, whereas the *PCLD* gene

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is located in another region (11,12). Under the transcriptional control of PrfA regulator, *PLCA*, *PLCB* and *HLY* (encoding listeriolysin O precursor) have a role encoding the Listeria Pathogenicity Island 1, leading to the escape from endocytic and secondary vacuoles (13-15). *PLCB* isoforms in mice include *PLCB1*, *PLCB2*, *PLCB3* and *PLCB4,* which are stimulated by G protein activation (G$\alpha_{q/11}$ and/or G$\beta\gamma$) (16,17). The roles of PLCB isoforms in immune defense and escape, and their functions in tumors are currently being investigated. *PLCB1* has been reported to be associated with HCC prognosis in tumor proliferation (1) and an aberrant expression pattern has been reported in patients with schizophrenia (18). The *PLCB2* and *PLCB4* genes were found to be differentially expressed in human breast cancer MCF-7 cells, and to be associated with multidrug resistance using RNA-seq technology (19). PLCB3 has been reported to be regulated by multiple protein kinases and to control hormonal signaling (20).

HBV infection is regarded as a main risk factor for the development of HCC (4). HBV is classified into ten genotypes, from A to J, and >40 associated sub-genotypes (21). The 10 genotypes are based on an intergroup divergence of ≥8% in the complete nucleotide sequence; whereas the sub-genotypes are based on a divergence of 4-7.5% (22,23). Notably, genotypes A and B are associated with earlier hepatitis B e antigen seroconversion, less active liver disease, and a slower rate of progression to cirrhosis and HCC compared with genotypes C and D (24-27).

Some PLCB isoforms have been explored with regard their associations with tumor development; therefore, the present study aimed to explore the association between four *PLCB* genes and HCC.

Materials and methods

Patient data collection. The GSE14520 dataset was used for analysis (ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14520; accessed June 10th, 2018) (28,29). This dataset contains two platforms: GPL571 (GeneChip® Human Genome U133A 2.0 Array; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and GPL3921 (GeneChip® HT Human Genome U133 Array Plate Set; Thermo Fisher Scientific, Inc.). To avoid a batch effect, patients from GPL3921 were used. Patients with HBV infection were used, including a total of 212 patients. In addition, patient survival, including overall survival (OS) and recurrence-free survival (RFS), validated findings in the GSE14520 dataset using the Gene Expression Profiling Interactive Analysis (GEPIA; gepia.cancer-pku.cn/index.html; accessed June 10th, 2018) website with data from The Cancer Genome Atlas (TCGA) database (30).

Gene, protein and tissue expression, and the body map. Gene expression, the body map and transcripts per million of the *PLCB* genes were collected from the GEPIA website (gepia. cancer-pku.cn/index.html; accessed June 12th, 2018). Tissue and protein expression of the *PLCB* genes were collected from the GTEx portal (gtexportal.org/home/; accessed June 12th, 2018) (31) and The Human Protein Atlas (proteinatlas.org/; accessed June 12th, 2018) (32) websites, respectively.

Gene set enrichment analysis (GSEA). GSEA (software. broadinstitute.org/gsea/index.jsp) was performed to explore

potential mechanisms that PLCB genes are involved in, including biological processes and metabolic pathways. Datasets of c2.cp.kegg.v6.1.symbols.gmt, c5.bp.b6.1.symbols. gmt, c5.cc.v6.1.symbols.gmt, c5.mf.v6.1.symbols.gmt and c5.all.v6.1.symbols.gmt were used to analyze statistically significant Gene Ontology (GO) terms, including biological process (BP), cellular component (CC), and molecular function (MF), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (33,34).

Association and interaction analysis. The Pearson correlation matrix among PLCB genes was constructed using R version 3.5.0 (r-project.org/). Pearson correlation and associations between PLCB gene expression and tumor stage were validated using the GEPIA website. The co-expression interactive network of gene-gene interactions was constructed using the geneMANIA plugin of Cytoscape software version 3.6.0 (35,36). The protein-protein interaction (PPI) network was constructed using the STRING (string-db.org/cgi/input. pl, accessed June 20th, 2018) website (37). GO enrichment analysis was visualized using the BiNGO plugin of Cytoscape software version 3.6.0 (38).

Diagnostic and prognostic analysis and stratified, joint-effect analysis. Diagnostic receiver operating characteristic (ROC) curves were constructed using the expression of PLCB genes in tumor and non-tumor tissues. Gene expressions were categorized into two groups of low and high expression at a cut-off value of median expression levels. OS and RFS were calculated using the Kaplan-Meier and Cox proportional hazards regression models. Statistically significant clinical factors were adjusted for multivariate Cox models. Then, prognosis-associated genes were further stratified for analysis by clinical factors. In addition, prognosis-associated genes were combined for a joint-effect analysis with α-fetoprotein (AFP) based low and high expression.

Expression model and nomogram construction. To further explore prognosis-associated genes for HCC survival, expression models for OS and RFS prediction were constructed. Gene expression, patient survival status, expression heatmaps and prognostic ROC curves were constructed in the model (39-42). Nomograms for OS and RFS were also constructed using clinical factors and genes to predict patient survival probability at 1, 3 and 5 years.

Genome-wide analysis of prognosis-associated genes. Prognosis-associated genes were further explored in genome-wide analysis. A cut-off value of 0.4 was determined for further analysis. The cut-off 0.4 can filter a lot of genes with weak relationships with PLCB1 and leads to a better presentation of GO and pathway results compared with other cut-off values. Gene-gene interactions, and BP, CC and MF were constructed using Cytoscape software.

Statistical analysis. Unpaired t test was used to analyze expressions of PLCBs in tumor and non-tumor tissues. Box plots and survival plots were generated using GraphPad software version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Survival analyses were performed using SPSS software
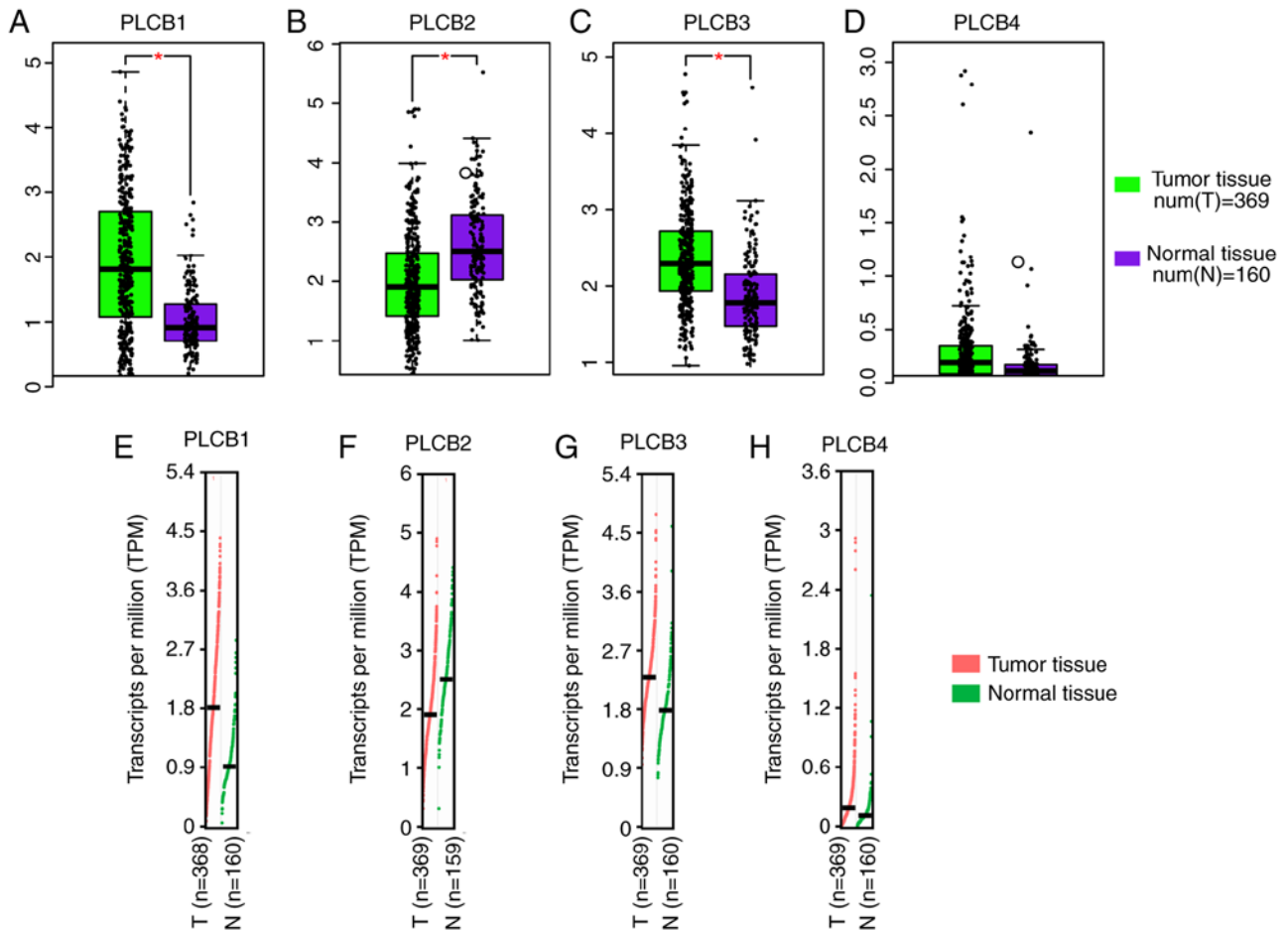
Figure 1. Relative mRNA expressions and transcriptional levels of PLCB1-4 in tumor and non-tumor tissues. Relative mRNA expressions of (A) PLCB1, (B) PLCB2, (C) PLCB3 and (D) PLCB4 in tumor and non-tumor tissues Transcriptional levels of (E) PLCB1, (F) PLCB2, (G) PLCB3 and (H) PLCB4 in tumor and non-tumor tissues. PLCB, phospholipase C β.

version 16.0 (SPSS, Inc., Chicago, IL, USA). Median survival time and log-rank P-value were calculated by the Kaplan-Meier method, and the 95% confidence interval (CI) and hazard ratio (HR) were calculated by univariate and multivariate Cox proportional hazards regression models, respectively. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Demographic and clinical characteristics. Data from 212 patients (GSE14520) with HBV-associated HCC were used in the study. AFP, BCLC stage, tumor size and cirrhosis were associated with OS ($P=0.049$, $P<0.0001$, $P=0.002$ and $P=0.041$, respectively). Gender, cirrhosis and BCLC stage were associated with RFS ($P=0.002$, $P=0.036$ and $P<0.0001$, respectively). Other factors were not associated with prognosis ($P>0.05$; Table SI).

Gene, protein, tissue expressions and transcription analysis. *PLCB1* and *PLCB3* were highly expressed in tumor tissues compared with normal tissue, whereas *PLCB2* had the opposite result (all $P≤0.05$; Fig. 1A-C). However, there was no difference in *PLCB4* expression between the tumor and normal tissue (Fig. 1D). Transcriptional analysis indicated that *PLCB1*, *PLCB3* and *PLCB4* consistently exhibited higher
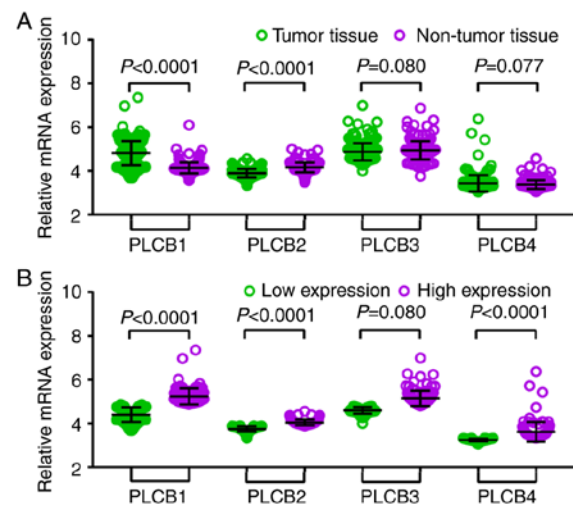

Figure 2. Relative mRNA expressions of PLCB1-4 in tumor and normal tissues and low, high expression groups. (A) Relative mRNA expressions of PLCB1-4 in tumor and normal tissues; (B) Relative mRNA expressions of PLCB1-4 in low and high expression groups. PLCB, phospholipase C β.

transcripts per millions in tumor tissues compared with normal tissues (Fig. 1E-H). Tissue and protein expression of the *PLCB* genes were collected from the GTEx portal.
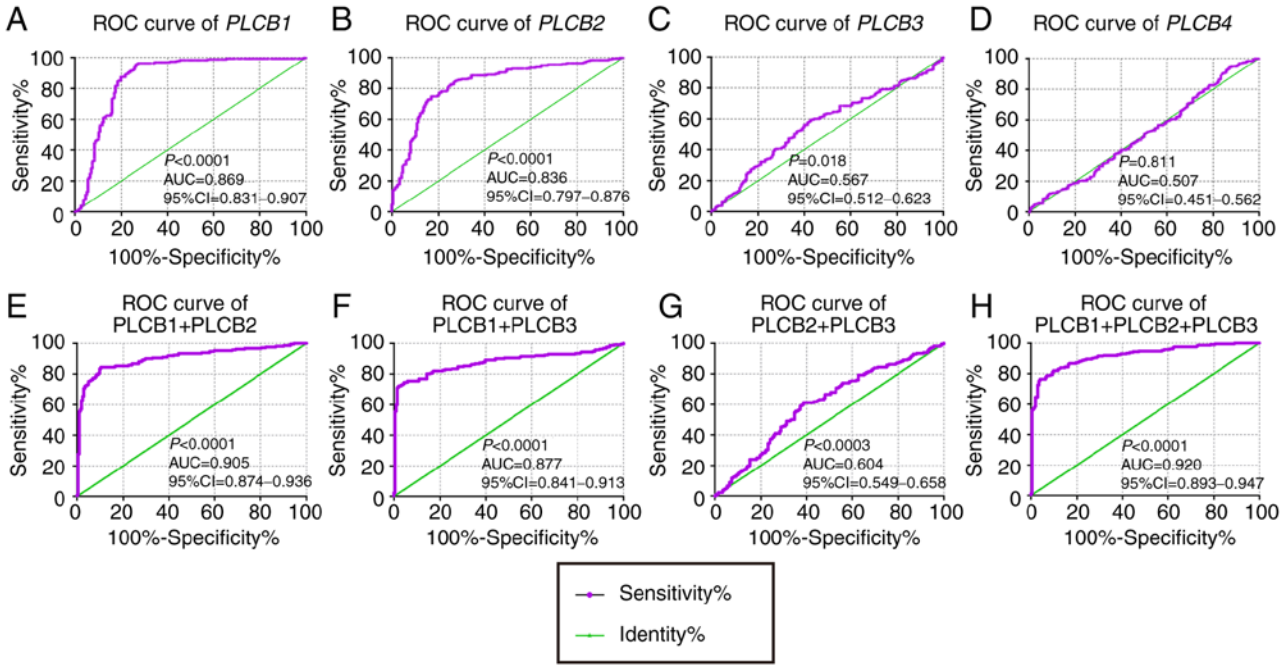
Figure 3. Diagnostic ROC curves of PLCB1-4. A-D: Diagnostic ROC curves of (A) PLCB1, (B) PLCB2, (C) PLCB3 and (D) PLCB4; Diagnostic ROC curves of combination of (E) PLCB1 and PLCB2, (F) PLCB1 and PLCB3, (G) PLCB2 and PLCB3, and (H) PLCB1, PLCB2 and PLCB3. ROC, receiver operating characteristics; PLCB, phospholipase C β; AUC, area under the curve; CI, confidence interval.
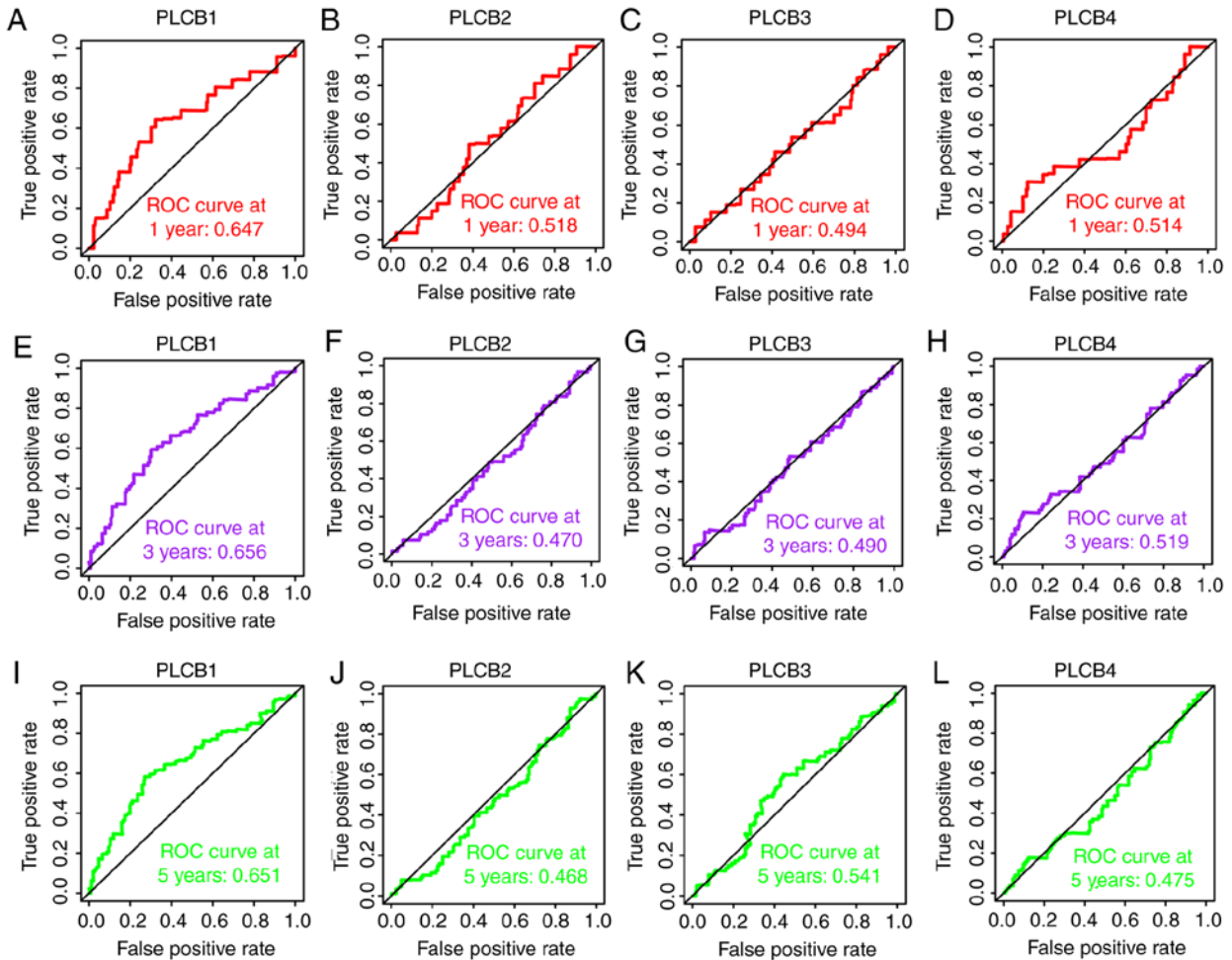

Figure 4. Overall survival ROC curves of PLCB1-4 at 1, 3 and 5 years. ROC curves of (A) PLCB1, (B) PLCB2, (C) PLCB3 and (D) PLCB4 at 1 year; ROC curves of (E) PLCB1, (F) PLCB2, (G) PLCB3 and (H) PLCB4 at 3 years; ROC curves of (I) PLCB1, (J) PLCB2, (K) PLCB3 and (L) PLCB4 at 5 years. PLCB, phospholipase C β; ROC, receiver operating characteristics.

Table I. Prognostic analysis of PLCB genes for overall survival.

Variable	Patients (n=212)	No. of events	MST (months)	HR (95% CI)	Crude P-value	HR (95% CI)	Adjusted P-value[a]
PLCB1							
Low expression	106	29	NA	Ref.		Ref.	
High expression	106	53	53	2.246 (1.426-3.536)	**<0.001**	**2.100 (1.310-3.367)**	**0.002**
PLCB2							
Low expression	106	41	NA	Ref.		Ref.	
High expression	106	41	NA	0.902 (0.585-1.391)	0.641	1.041 (0.660-1.641)	0.863
PLCB3							
Low expression	106	36	NA	Ref.		Ref.	
High expression	106	46	NA	1.394 (0.900-2.159)	0.137	1.035 (0.659-1.625)	0.882
PLCB4							
Low expression	106	44	NA	Ref.		Ref.	
High expression	106	38	NA	0.877 (0.568-1.354)	0.555	0.870 (0.555-1.363)	0.534

[a]P-values were adjusted for tumor size, cirrhosis, Barcelona Clinic Liver Cancer stage and α-fetoprotein; bold indicates significant P-values. Ref., reference value (1); NA, not available; MST, median survival time; HR, hazard ratio; 95% CI, 95% confidence interval; PLCB, phospholipase B.
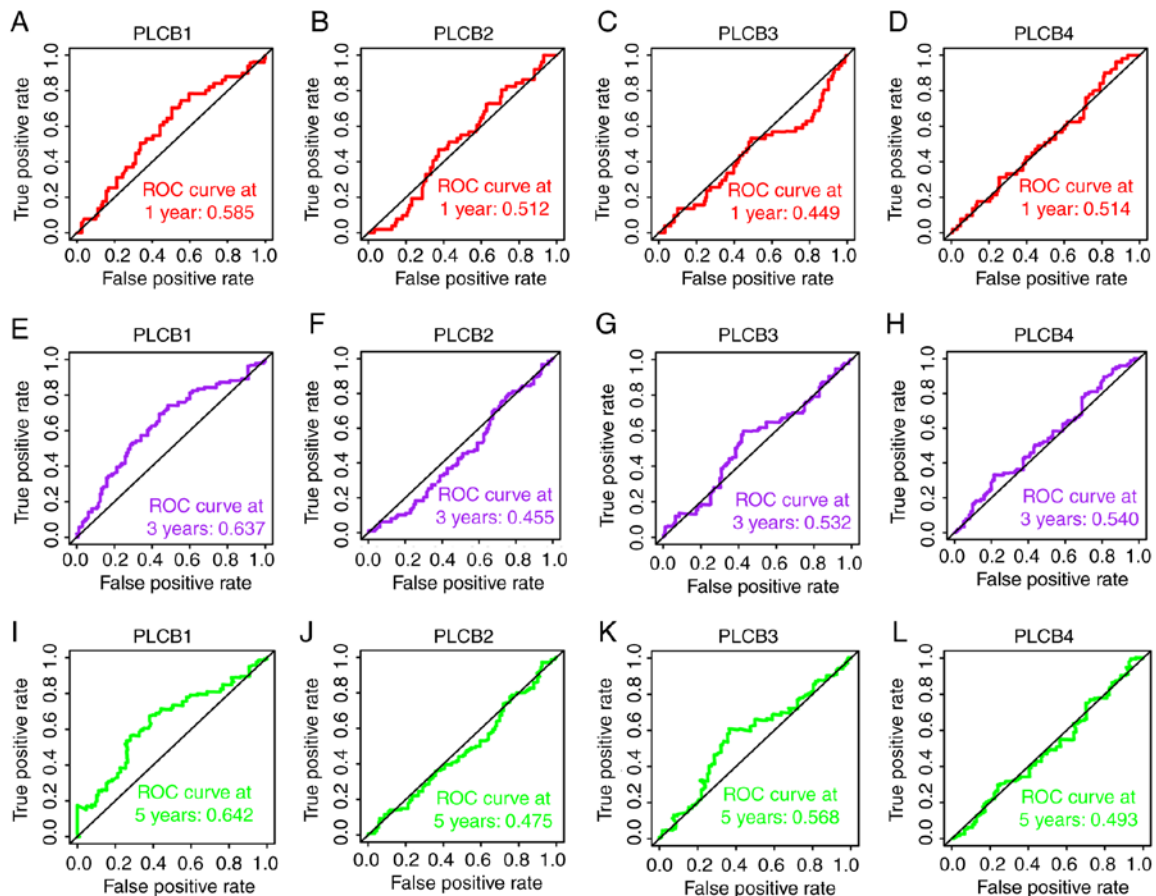

Figure 5. Recurrence-free survival ROC curves of PLCB1-4 at 1, 3 and 5 years. ROC curves of (A) PLCB1, (B) PLCB2, (C) PLCB3 and (D) PLCB4 at 1 year; ROC curves of (E) PLCB1, (F) PLCB2, (G) PLCB3 and (H) PLCB4 at 3 years; ROC curves of (I) PLCB1, (J) PLCB2, (K) PLCB3 and (L) PLCB4 at 5 years. PLCB, phospholipase C β; ROC, receiver operating characteristics.

Gene expression levels in 212 patients with HBV-HCC (GSE14520) indicated that there were significant differences in *PLCB1* and *PLCB2* expression between tumor and non-tumor tissues, whereas there was not difference in *PLCB3* and

Table II. Prognostic analysis of PLCB genes for recurrence-free survival.

Variable	Patients (n=212)	No. of events	MST (months)	HR (95% CI)	Crude P-value	HR (95% CI)	Adjusted P-value[a]
PLCB1							
Low expression	106	46	NA	Ref.		Ref.	
High expression	106	70	26.9	**1.914 (1.318-2.781)**	**0.001**	**1.861 (1.273-2.271)**	**0.001**
PLCB2							
Low expression	106	59	36.0	Ref.		Ref.	
High expression	106	57	51.1	0.863 (0.599-1.243)	0.429	0.956 (0.654-1.398)	0.817
PLCB3							
Low expression	106	52	54.8	Ref.		Ref.	
High expression	106	64	29.9	**1.466 (1.015-2.118)**	**0.042**	1.244 (0.853-1.814)	0.257
PLCB4							
Low expression	106	59	46.3	Ref.		Ref.	
High expression	106	57	43.2	1.015 (0.705-1.461)	0.936	0.962 (0.664-1.395)	0.840

[a]P-values were adjusted for gender, cirrhosis and Barcelona Clinic Liver Cancer stage. Ref., reference value (1); MST, median survival time; HR, hazard ratio; 95% CI, 95% confidence interval; PLCB, phospholipase B. Bold indicates significant P-values.
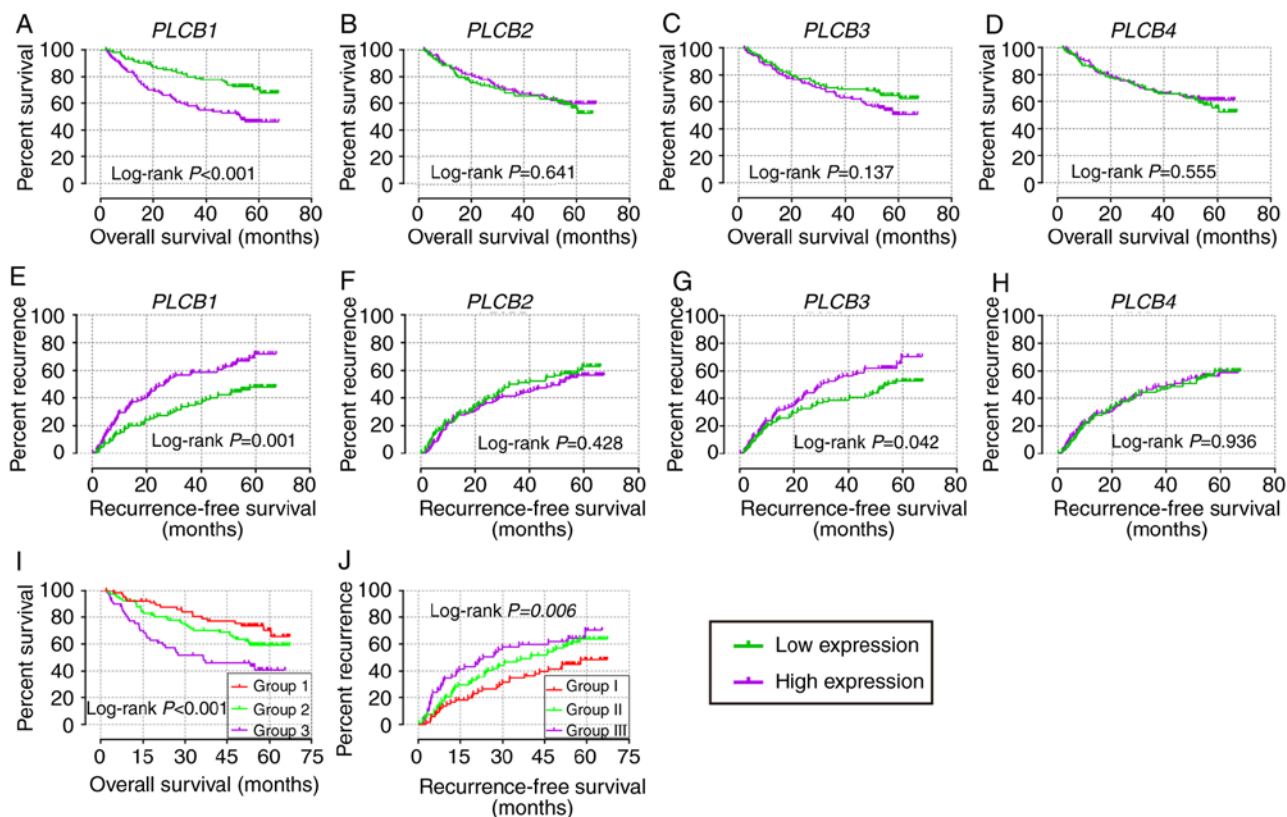

Figure 6. Overall survival and recurrence-free survival analysis plots of PLCB1-4. Overall survival analysis plot of (A) PLCB1, (B) PLCB2, (C) PLCB3 and (D) PLCB4; recurrence-free survival analysis plot (E) PLCB1, (F) PLCB2, (G) PLCB3 and (H) PLCB4; joint-effects analysis of (I) α-fetoprotein and (J) PLCB1 for overall survival and recurrence-free survival. Group 1, AFP low expression and PLCB1 low expression; Group 2, AFP low expression and PLCB1 high expression, and AFP high expression and PLCB1 low expression; Group 3, AFP high expression and PLCB1 high expression; Group I, AFP low expression and PLCB1 low expression; Group II, AFP low expression and PLCB1 high expression, and AFP high expression and PLCB1 low expression; Group III, AFP high expression and PLCB1 high expression. PLCB, phospholipase C β.

PLCB4 between the samples (Fig. 2A). In addition, when tumor samples were divided into high and low expression groups using the median as the cutoff there were significant differences in *PLCB1*, *PLCB2* and *PLCB4*; whereas *PLCB3* did not exhibit significance (Fig. 2B). The bodymap distribution of PLCB genes in different organs is shown in Fig. S1. Protein

Table III. Stratified analysis of *PLCB1* for overall survival and recurrence-free survival.

Variable	Overall survival				Recurrence-free survival			
	Low	High	Adjusted HR (95% CI)	Adjusted P-value	Low	High	Adjusted HR (95% CI)	Adjusted P-value
Sex								
Male	86	89	**1.967 (1.174-3.24)**	**0.010**	86	89	**1.877 (1.249-2.820)**	**0.002**
Female	20	17	2.619 (0.711-9.652)	0.148	20	17	0.754 (0.168-3.382)	0.713
Age (years)								
≤60	91	92	**2.252 (1.370-3.702)**	**0.001**	91	92	**1.736 (1.129-2.670)**	**0.012**
>60	15	14	0.850 (0.140-5.148)	0.860	15	14	2.043 (0.701-5.953)	0.191
HBV								
AVR-CC	20	36	1.987 (0.695-5.687)	0.200	20	36	1.486 (0.663-3.332)	0.336
CC	86	70	**1.957 (1.114-3.438)**	**0.020**	86	70	**1.864 (1.166-2.979)**	**0.009**
Tumor size (cm)								
≤5	75	62	**2.100 (1.149-3.838)**	**0.016**	75	62	**1.627 (1.012-2.616)**	**0.045**
>5	30	44	1.790 (0.821-3.901)	0.143	30	44	**2.204 (1.049-4.629)**	**0.037**
Cirrhosis								
Yes	93	102	**1.922 (1.196-3.091)**	**0.007**	93	102	**1.678 (1.124-2.503)**	**0.011**
No	13	4	476.586 (5.21E-12-4.36E16)	0.707	13	4	3.758 (0.379-37.311)	0.258
Multinodular								
Yes	21	24	1.399 (0.544-3.598)	0.487	21	24	1.186 (0.474-2.965)	0.716
No	85	82	**2.662 (1.522-4.656)**	**0.001**	85	82	**2.163 (1.395-3.355)**	**0.001**
AFP (ng/ml)								
≤300	68	47	**2.098 (1.097-4.015)**	**0.025**	68	47	**2.180 (1.307-3.635)**	**0.003**
>300	35	59	1.886 (0.934-3.806)	0.077	35	59	1.294 (0.710-2.359)	0.399
BCLC stage								
0	8	12	0.535 (0.033-8.559)	0.658	8	12	0.597 (0.097-3.665)	0.577
A	79	64	**2.214 (1.210-4.051)**	**0.010**	79	64	**1.928 (1.200-3.097)**	**0.007**
B	10	12	0.746 (0.225-2.478)	0.633	10	12	0.903 (0.310-2.627)	0.851
C	9	18	2.746 (0.836-9.021)	0.096	9	18	2.370 (0.774-7.252)	0.131

Ref., reference value (1); PLCB, phospholipase B; HR, hazard ratio; 95% CI, 95% confidence interval; HBV, hepatitis B virus; AVR-CC, acute viral replication-chronic carrier; CC, chronic carrier; AFP, AFP, α-fetoprotein; BCLC, Barcelona Clinic Liver Cancer. Bold indicates significant P-values.

expression levels demonstrated that PLCB2 is the most highly expressed of the PLCB family (Fig. S2). The different tissue expression levels of PLCB family members demonstrated that all were expressed at low levels in the liver (Fig. S3).

Diagnostic and prognostic analysis. In the diagnostic analysis of PLCB genes, PLCB1 and PLCB2 exhibited diagnostic value for HCC, while PLCB3 showed potential diagnostic value [P<0.0001, P<0.0001 and P=0.018, respectively; area under the curve (AUC), 0.869, 0.836 and 0.567, respectively; Fig. 3A-C]. However, PLCB4 did not have any diagnostic value (P=0.811; Fig. 3D). In the combined diagnostic analysis for PLCB1, PLCB2 and PLCB3, the combinations of PLCB1 + PLCB2, PLCB1 + PLCB3, and PLCB1 + PLCB2 + PLCB3 exhibited diagnostic value for HCC with an advantage over PLCB1, PLCB2 or PLCB3 alone (AUC, 0.905, 0.877 and 0.920, respectively; all P<0.05; Fig. 3E, F and H). The combination of PLCB2 and PLCB3 exhibited potential diagnostic value for HCC

(AUC, 0.604; P=0.0003; Fig. 3G). In the prognostic analysis (Figs. 4 and 5), only PLCB1 expression was associated with patient OS at 1-, 3- and 5-years (all AUC >0.6; Fig. 4A, E and I). In addition, PLCB1 expression was associated with patient RFS at 3- and 5-years (both AUC >0.6; Fig. 5E and I).

In the univariate analysis (Tables I and II; Fig. 6), *PLCB1* expression was associated with OS (crude P=0.002; Fig. 6A); *PLCB1* and *PLCB3* expression was associated with RFS (crude P=0.001 and P=0.042, respectively; Fig. 6E and G). In the multivariate analysis, *PLCB1* expression was associated with OS and RFS (adjusted P=0.002 and 0.001, respectively; Tables I and II). Other genes were not associated with prognosis (adjusted P>0.05; Tables I and II).

Stratified and joint-effect survival analysis. Stratification analysis was performed for *PLCB1* on OS and RFS. Male gender, age <60 years, chronic carrying of HBV, cirrhosis, single nodular, AFP levels <300 ng/ml, and A stage in the

Table IV. Joint-effect analysis of PLCB1 and AFP for overall survival and recurrence-free survival.

A, Overall survival

Group	AFP expression	PLCB1 expression	Events/total	MST (months)	Adjusted HR (95% CI)	Adjusted P-value
1	Low	Low	18/68	NA	Ref.	**0.008**
2	Low	High	32/82	NA	**2.162 (1.143-4.089)**	**0.018**
	High	Low				
3	High	High	32/59	36.4	**4.382 (1.703-11.276)**	**0.002**

B, Recurrence-free survival

Group	AFP expression	PLCB1 expression	Events/total	MST (months)	Adjusted HR (95% CI)	Adjusted P-value
I	Low	Low	29/68	NA	Ref.	0.075
II	Low	High	50/82	40.1	**1.613 (1.019-2.555)**	**0.041**
	High	Low				
III	High	High	37/59	23.0	**1.670 (1.012-2.755)**	**0.045**

Group 1, AFP low expression and PLCB1 low expression; Group 2, AFP low expression and PLCB1 high expression, and AFP high expression and PLCB1 low expression; Group 3, AFP high expression and PLCB1 high expression; Group I, AFP low expression and PLCB1 low expression; Group II, AFP low expression and PLCB1 high expression, and AFP high expression and PLCB1 low expression; Group III, AFP high expression and PLCB1 high expression. Ref., reference value (1); PLCB, phospholipase B; AFP, α-fetoprotein; MST, median survival time; HR, hazard ratio; 95% CI, 95% confidence interval. Bold indicates significant P-values.

BCLC staging system were associated with OS and RFS (all adjusted P≤0.05; Table III). Tumor size <5 cm was associated with OS and any group of tumor size was associated with RFS (all adjusted P≤0.05; Table III).

In the joint-effect analysis (OS/RFS: group 1/I, AFP low + PLCB1 low; group 2/II, AFP low + PLCB1 high, and AFP high + PLCB1 low; groups 3/III, AFP high + PLCB1 high), when combining *PLCB1* and AFP, prognostic significance was observed among the three groups for OS (adjusted P=0.008; Table IV); group 3 exhibited the worst prognosis [adjusted P=0.002, adjusted HR (95% CI)=4.382 (1.703-11.276); Table IV]. Prognostic significance was not observed among the three groups in RFS (adjusted P=0.075; Table IV). However, group III exhibited the worst prognosis [adjusted P=0.045, adjusted HR (95% CI)=1.670 (1.012-2.755); Table IV].

GSEA. Both diagnostic- and prognostic-associated genes were explored to investigate the mechanisms that PLCBs are involved in. Enriched GO terms and KEGG pathways annotated with *PLCB1* included 'G protein coupled receptor activity', 'sodium channel activity', 'extracellular ligand gated ion channel activity' and 'taste transduction pathway', among others (Fig. 7). Enriched GO terms and KEGG pathways annotated with *PLCB2* included 'mRNA processing', 'cell division', 'cell cycle checkpoints', 'DNA repair', 'PPAR signaling pathway', 'metabolism of xenobiotics by cytochrome P450' and 'adipocytokine signaling pathway' among others (Fig. 8).

Expression model and nomogram construction. An expression model was constructed for OS and RFS prognosis prediction (Fig. 9). PLCB1 expression, OS and RFS survival status, and

PLCB1 expression heatmaps are shown in Fig. 9A, and prognostic ROC curves demonstrated that PLCB1 expression has prognostic value for OS and RFS (Fig. 9B and C).

Furthermore, nomograms were constructed for clinical factors and *PLCB1*. High expression always led to low points. The same points indicated a higher probability of survival at 1 year, yet a lower probability of survival at 5 years for both OS and RFS. Survival probability at 3 years was seated in the middle (Fig. 10).

Interaction and co-expression networks and enrichment analysis. Associations between gene expression and TNM stage (I, II, III) were visualized; *PLCB1* gene expression of 212 HBV-HCC was significantly different in early (I, II) stages compared with advanced (III) stage in (P≤0.01; Fig. 11A). Associations between gene expressions and TNM stage (I, II and III) in GEPIA indicated that *PLCB1* expression was different in different tumor stages (Fig. 11B). Gene-gene co-expression interactions and PPI networks demonstrated interactions between PLCB members (Fig. 11C and D). The Pearson correlation matrix showed an association between PLCB members (Fig. 11E).

Furthermore, enriched GO terms are presented in Fig. S4A-C. KEGG pathways that PLCB members are involved in are presented in Fig. S5. All members were involved in diacylglycerol and IP3 metabolism and finally induced sustained angiogenesis, thus evading apoptosis and proliferation effects.

Analysis of PLCB1 and associated genes genome-wide. Pearson correlation analysis was performed for PLCB1 genome-wide. A total of 53 genes were identified at r≥0.4. Gene-gene interaction analysis was constructed and presented
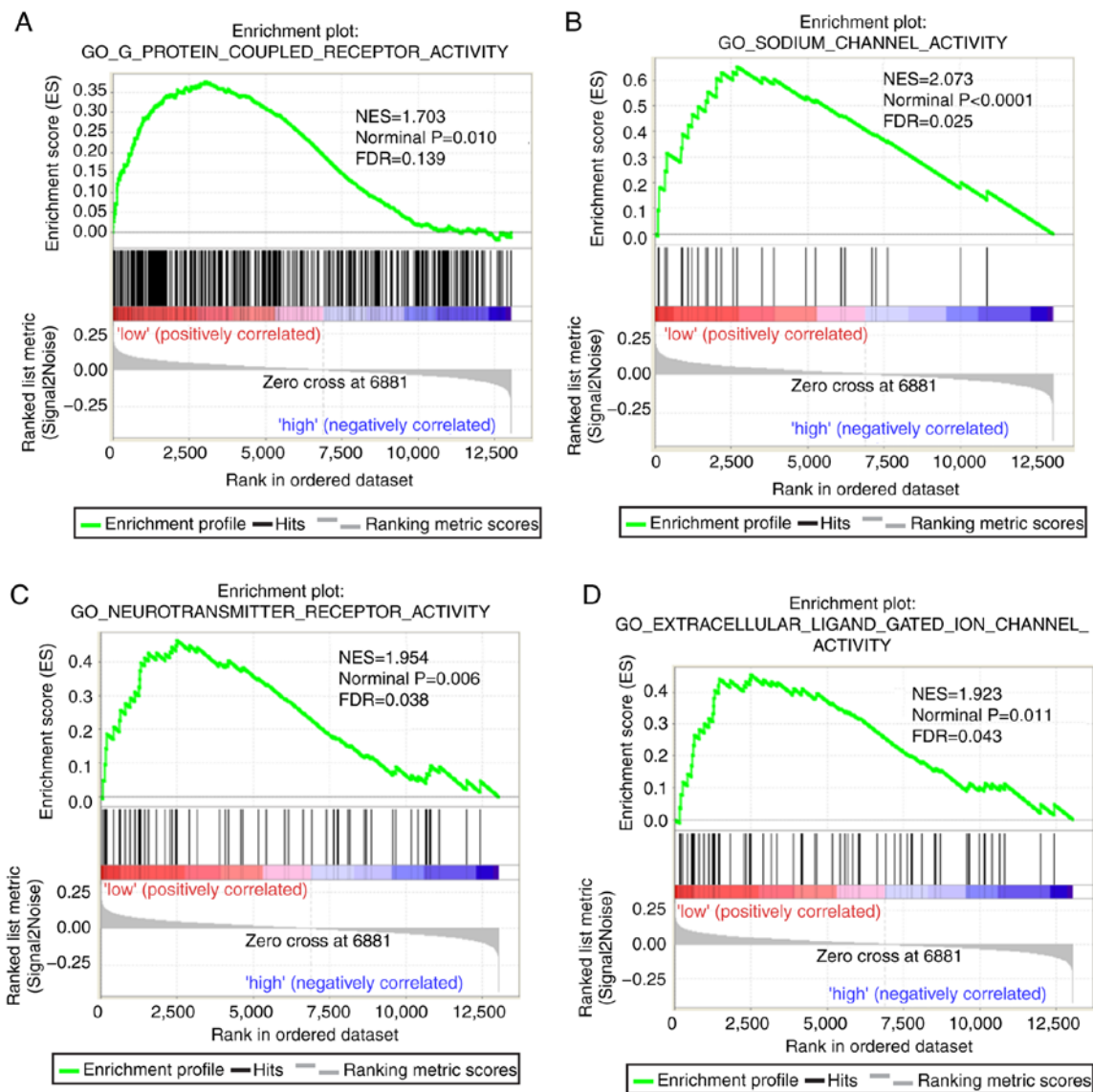
Figure 7. Gene set enrichment analysis results of *phospholipase C β1* gene. Results of gene ontologies: (A) G-protein coupled receptor activity; (B) sodium channel activity; (C) neutotransmitter receptor activity; (D) extracellular ligand gated ion channel activity. GO, gene ontology; NES, normalized enrichment score; FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes.

in Fig. 12. Networks of BP, CC and MF terms were constructed (Fig. 13). Enriched GO terms and KEGG pathways annotated by PLCB1 and correlated genes are presented in Table V.

Validation of prognostic values of PLCB genes. PLCB genes were further validated in GEPIA for OS and RFS (Fig. 14). *PLCB1* and *PLCB2* were associated with OS (P=0.0075 and P=0.041, respectively; Fig. 14A and B). In addition, *PLCB1* and *PLCB4* were associated with RFS (P<0.0001 and P<0.018, respectively; Fig. 14E and H). Other genes were not associated with OS or RFS (all P>0.05; Fig. 14). Pearson correlation in GEPIA (Fig. 15) indicated that *PLCB1* was positively correlated with *PLC3* and *PLCB4*, while *PLCB3* was positively correlated with *PLCB4*, which is consistent with Fig. 11E.

Discussion

In the current study, it was identified that *PLCB1* and *PLCB2* genes are differently expressed in tumor and normal tissues.

PLCB1 and *PLCB2* have diagnostic value for HCC, while *PLCB3* has potential diagnostic value for HCC. Combinations of these genes have an advantage over *PLCB1*, *PLCB2* or *PLCB3* alone with regard to HCC diagnosis. In addition, *PLCB1* has prognostic value of OS and RFS for HCC. Combining *PLCB1* and AFP had an advantage over PLCB1 alone for OS and RFS. GSEA indicated that PLCB1 and PLCB2 were involved in 'G protein coupled receptor activity', 'sodium channel activity', 'cell division', 'cell cycle checkpoint', 'DNA repair', 'PPAR signaling pathway', 'metabolism of xenobiotics by cytochrome P450' and 'adipocytokine signaling pathway', among others. Nomograms and gene expression models were constructed for HCC prognosis prediction. The validation of the prognostic values of PLCB genes revealed that *PLCB1* and *PLCB2* were associated with OS, and *PLCB1* and *PLCB4* were associated with RFS.

PLC proteins are key enzymes that metabolize inositol lipids and have a pivotal role in multiple transmembrane signaling transduction pathways that modulate a series of cellular
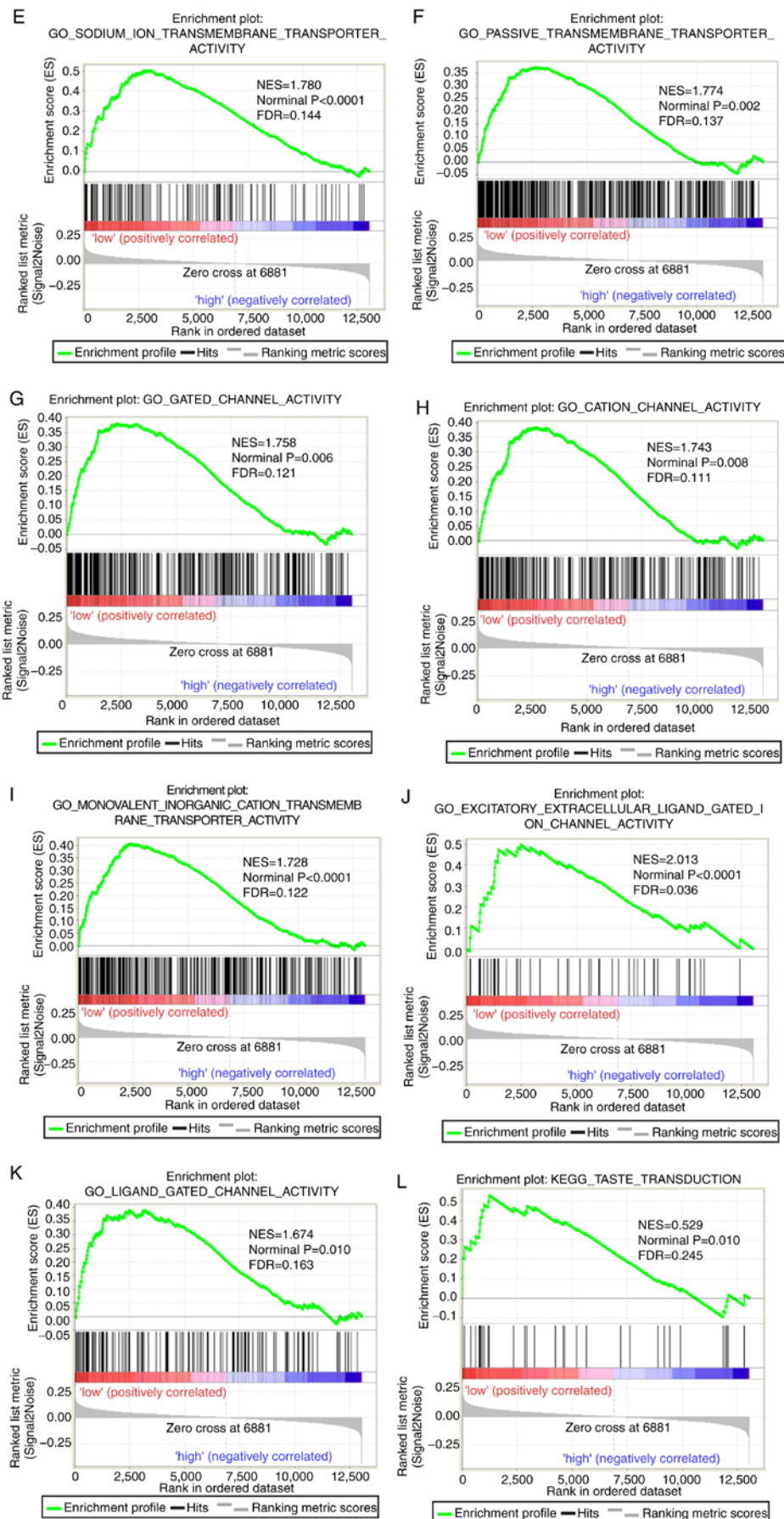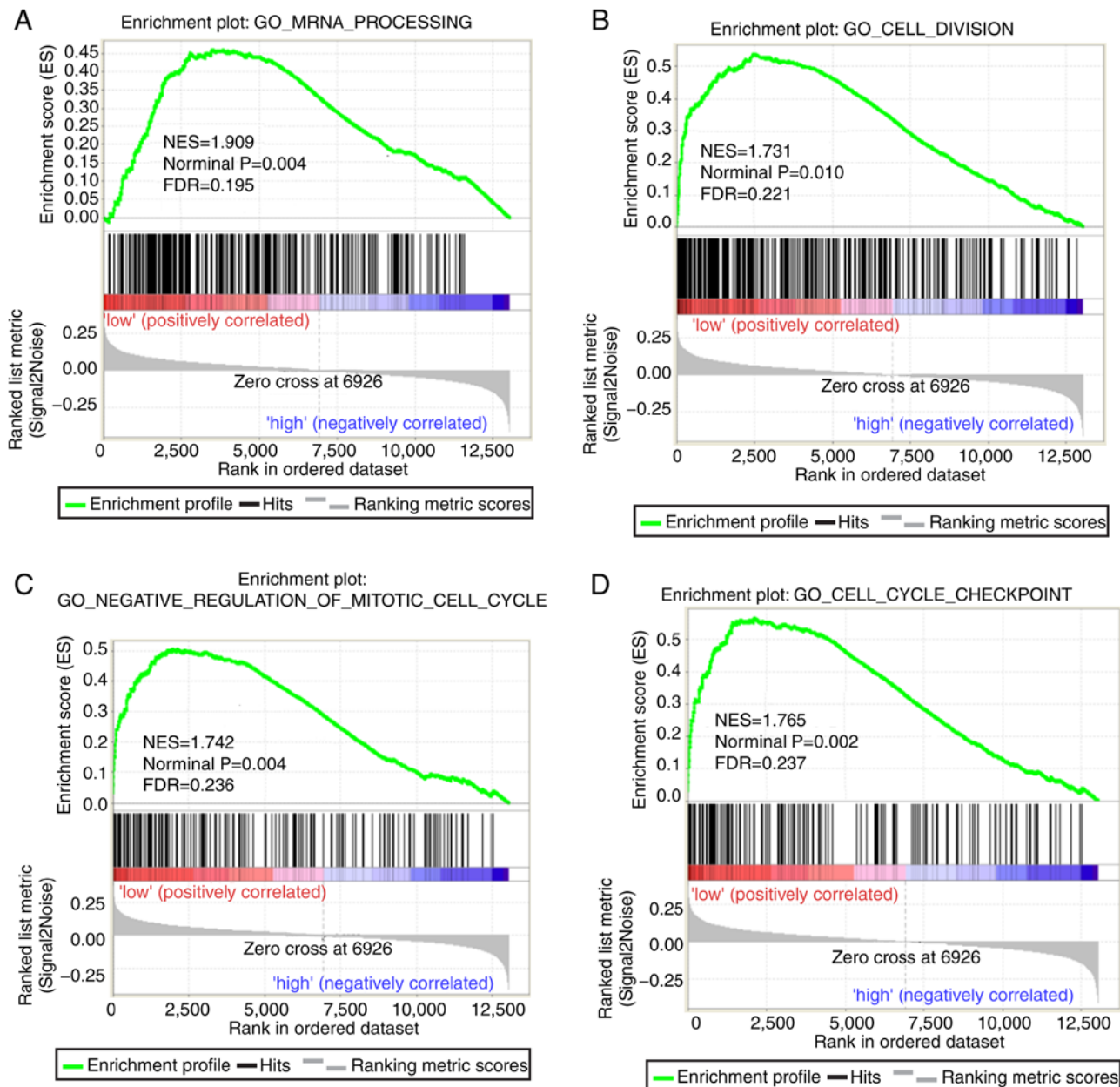
Figure 7. Continued. Gene set enrichment analysis results of *phospholipase C β1* gene. Results of gene ontologies: (E) sodium ion transmembrane transporter; (F) passive transmembrane transporter; (G) gated channel activity; (H) cation channel activity; (I) monovalent inorganic cation transmembrane transporter activity; (J) excitatory extracellular ligand gated ion channel activity; (K) ligand gated channel activity. (L) Taste transduction KEGG pathway. GO, gene ontology; NES, normalized enrichment score; FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Figure 8. Gene set enrichment analysis results of *phospholipase C β2* gene. Results of gene ontologies: (A) mRNA processing; (B) cell division; (C) negative regulation of mitotic cell cycle; (D) cell cycle checkpoint. GO, gene ontology; NES, normalized enrichment score; FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPAR peroxisome proliferator-activated receptor.

processes, including cell proliferation and mobility (16). In mammalian cells, there are four PLCB isoforms: PLCB1, PLCB2, PLCB3 and PLCB4. PLCB2 and PLCB3 are activated by Gβγ dimers, which are released upon the activation of Gα protein coupled receptor families (43). PLCB2 can also be activated by Rho family members of monomeric G proteins, with the strongest activation by Rac1; these participate in the cytoskeletal rearrangements that accompany cell mobility (44).

The PLCB1 enzyme is encoded by the *PLCB1* gene, which is located at chromosome of 20p12 (1). It was originally identified as a G protein coupled receptor-associated PLCB isoform that is able produce inositol 1,4,5-trisphosphate and diacylglycerol from phosphatidylinositol 4,5-bisphophate (45). The deregulation of signaling transduction pathways always

leads to advantages for tumor patients (1). *PLCB1* is activated by Gα and induces a variety of events, which may increase the total intracellular calcium levels (46); one possible result of this process is aberrant proliferation in the cell (1). PLCB1 has been reported to have a role in promoting cell cycle progression by targeting cyclin-cyclin kinase complexes (47).

In addition, *PLCB1* has been documented to have a pivotal role in myoblast differentiation, regulating the delayed differentiation of skeletal muscle in myotonic dystrophy myoblasts (48). *PLCB1* may also reduce cell damage under oxidative conditions and prevent α-synuclein aggregation (49). The amplification of *PLCB1* increased K562 cell viability and enables cells to evade apoptosis (50,51); the overexpression of *PLCB1* keeps Swiss 3T3 cells in the S phase of the cell cycle (52). Li *et al* (1) reported that
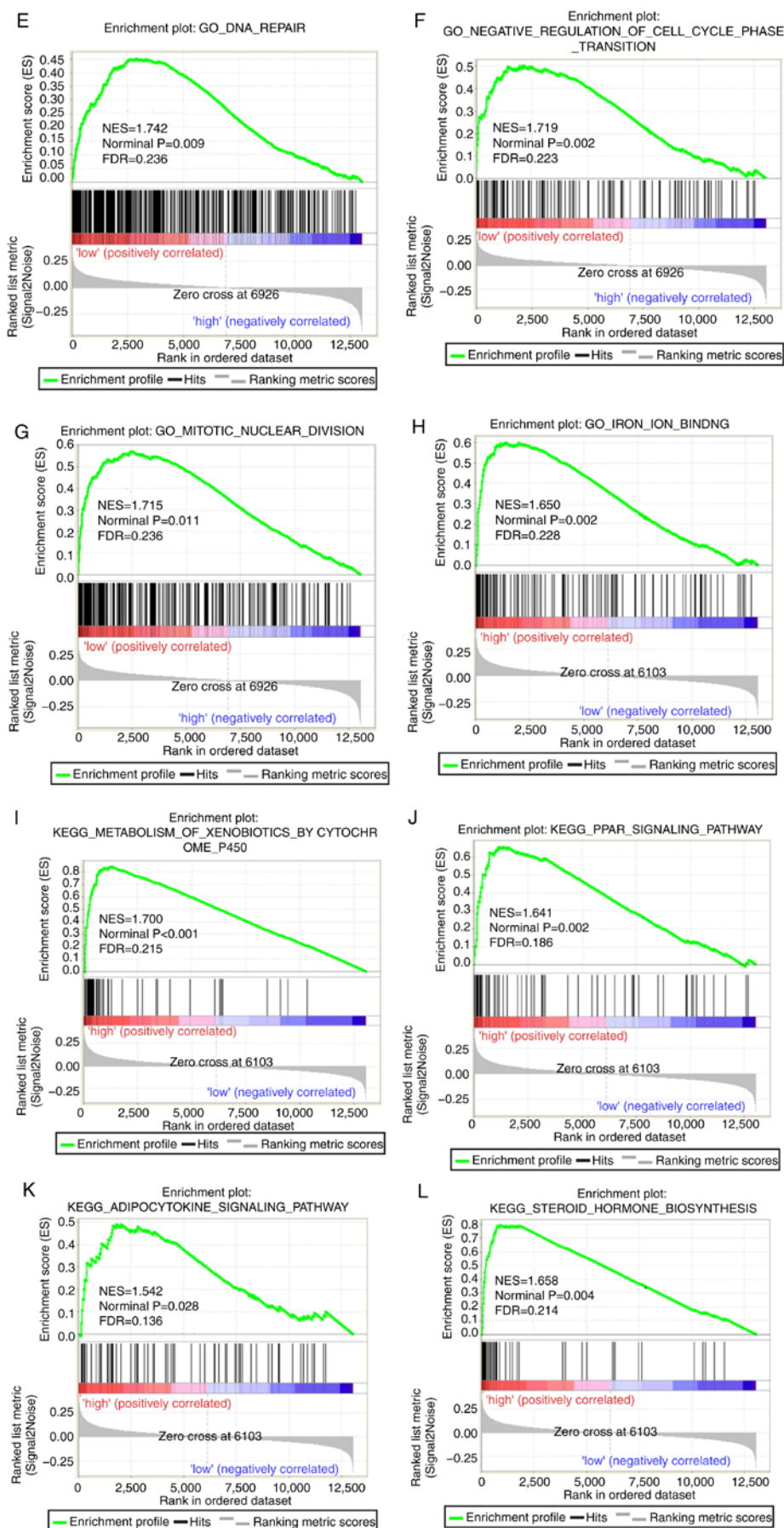
Figure 8. Continued. Gene set enrichment analysis results of *phospholipase C β2* gene. Results of gene ontologies: (E) DNA repair; (F) negative regulation of cell cycle phase transition (G) mitotic nuclear division; (H) iron ion binding. Results of KEGG pathways: (I) metabolism of xenobiotics by cytochrome P450; (J) PPAR signaling pathway; (K) adipocytokine signaling pathway; (L) steroid hormone biosynthesis. GO, gene ontology; NES, normalized enrichment score; FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes; PPAR peroxisome proliferator-activated receptor.
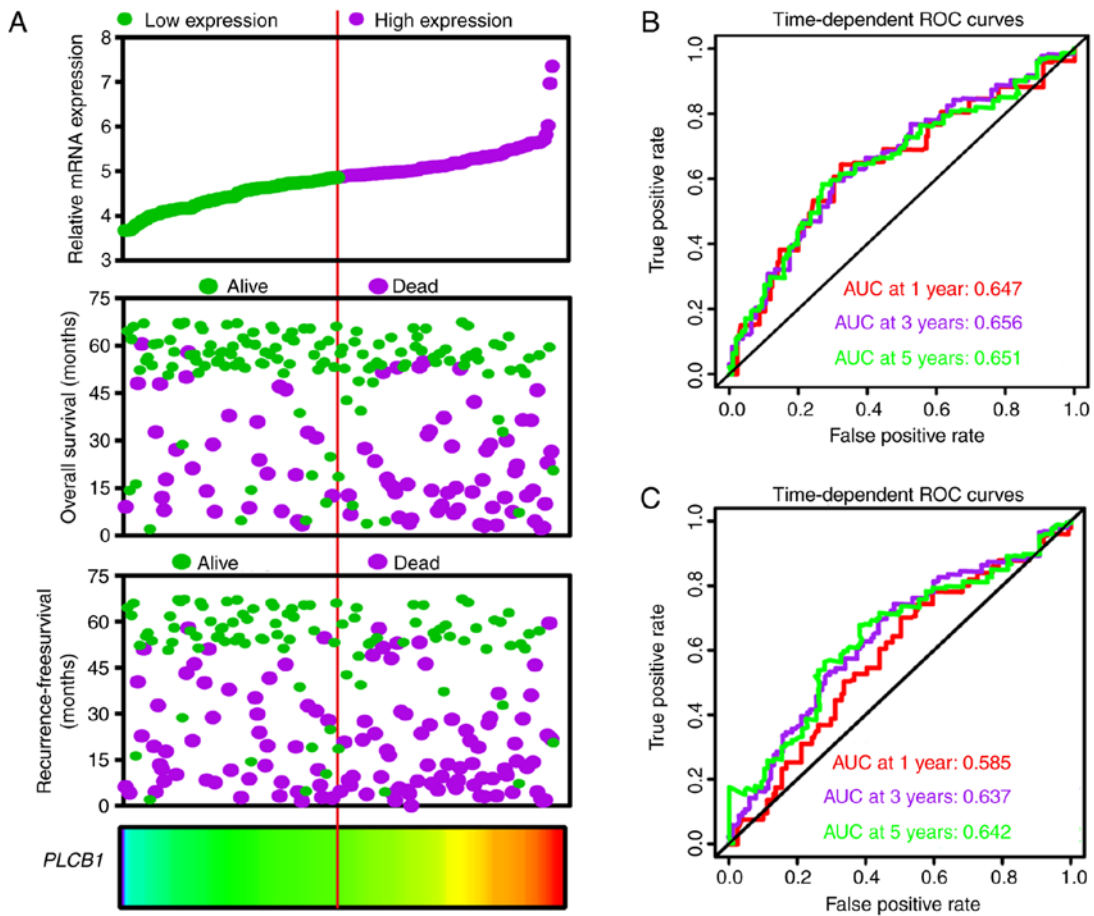
Figure 9. Expression model constructed using *PLCB1* gene. (A) Expression model including expression, overall survival status, recurrence-free survival status and heatmap. (B) Time dependent ROC curves of overall survival at 1, 3- and 5- years. (C) Time dependent ROC curves of recurrence-free survival at 1, 3- and 5-years. PLCB1, phospholipase C β1; ROC, receiver operating characteristics; AUC, area under the curve.
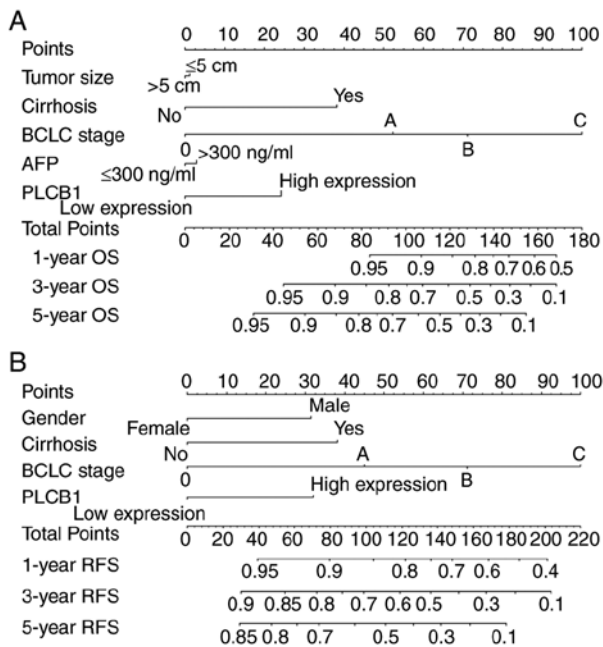

Figure 10. Nomograms constructed using overall survival and recurrence-free survival-related clinical factors and genes. (A) Nomogram of OS-associated genes and clinical factors. (B) Nomogram of RFS-associated genes and clinical factors. BCLC, Barcelona Clinic Liver Cancer; AFP, α-fetoprotein; PLCB1, phospholipase C β1; OS, overall survival; RFS, recurrence-free survival.

upregulated *PLCB1* expression is associated with tumor cell proliferation and infers a poor prognosis for HCC. The present study revealed that high expression has is undesirable for HCC prognosis (OS and RFS), which is consistent with the results of Li *et al* (1). Furthermore, *PLCB1* had diagnostic value for HCC.

PLCB2 mediates mitogenic, proliferative and migratory events by interacting with heterotrimeric and monomeric G proteins, and can interact with γ-synuclein to regulate G protein activation (43). Bertagnolo *et al* (53) reported that *PLCB2* induces cell cycle transition from G0/G1 to the S/G2/M phases, which is critical for tumor progression, without affecting cell cycle-associated enzymes. They also indicate that *PLCB2*, by modifying the phospholipase pool, may be responsible for the inositol lipid-associated modifications of the cytoskeleton architecture that occur in the course of division, motility and invasion of tumor cells (53). The current findings with regard to the role of *PLCB2* in the cell cycle and cell division are consistent with the results of Bertagnolo *et al* (53).

PLCB2 has been reported to promote mitosis and the migration of human breast cancer-derived cells (54), is highly expressed in breast cancer and associated with poor prognosis (55); however, little is known about HCC *PLCB2* expression, and the role in HCC diagnosis and prognosis. In the current study, *PLCB2* expression as not associated with HCC prognosis, but may be a diagnostic signature for HCC.
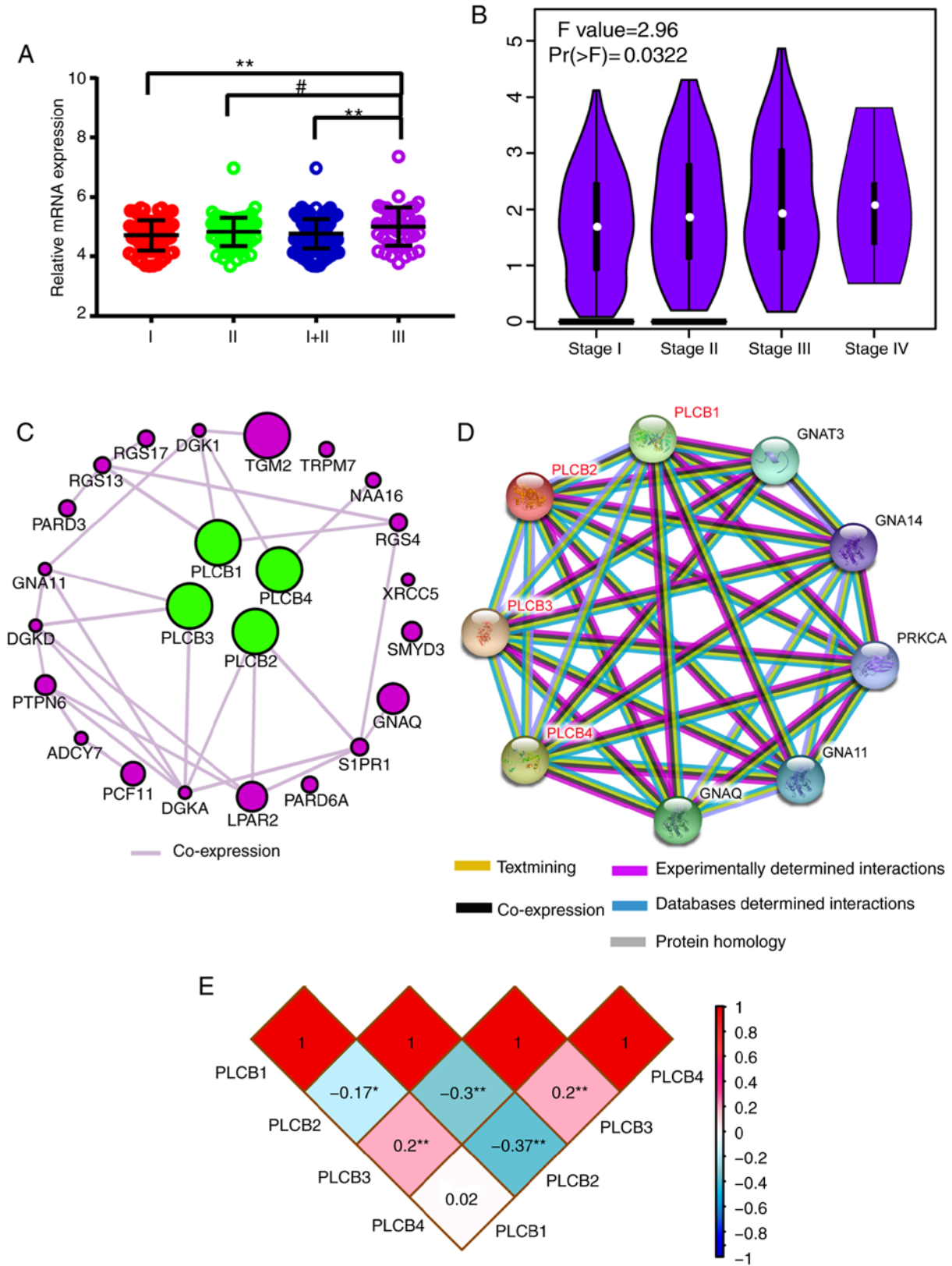
Figure 11. Scatter plots, matrix and interaction networks analysis. (A) Scatter plot and (B) violin plot of *PLCB1* expressions. (C) Co-expression network of PLCB1-4 genes. (D) Protein-protein interaction network of PLCB1-4. (E) Pearson correlation matrix of PLCB1-4. PLCB, phospholipase C β.

PLCB3 is located on chromosome 11q13 in the vicinity of the multiple endocrine neoplasia type 1 gene; its loss leads to the development of neuroendocrine tumors (56). The transfection of *PLCB3* to a human endocrine pancreatic tumor cell line can induce the activation of the human mismatch repair protein 3 gene (56). *PLCB3* interacts with Na(+)/H(+) exchange regulatory cofactor NHERF-1, providing a structural basis for CXCR2 signaling in pancreatic cancer (57).
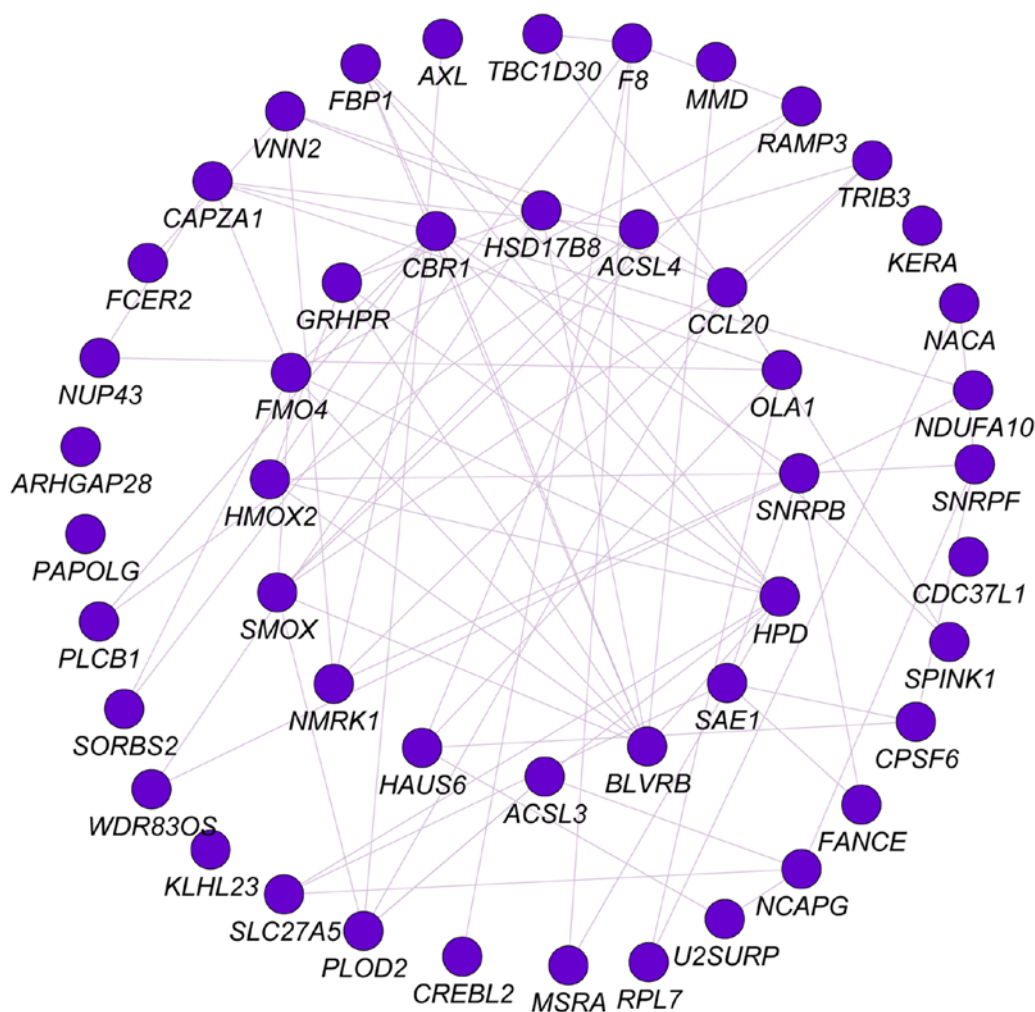
Figure 12. Co-expression network of *PLCB1* gene with correlation-associated genes in genome-wide analysis. PLCB1, phospholipase C β1.

Hoeppner *et al* (58) identified a novel role for *PLCB3*, functioning as a negative regulator of vascular endothelial growth factor-mediated vascular permeability by regulating intracellular Ca^{2+} release. *PLCB3* may have a tumor suppressor role via SHP-1-mediated dephosphorylation of Stat5 (59). Ju *et al* (60) reported that PLCB and $G_{q\alpha}$ may have important roles in scar remodeling, cardiac hypertrophy and fibrosis following myocardial infarction rat hearts. In the present study, *PLCB3* expression exhibited potential diagnostic value for HCC and without association with HCC prognosis. *PLCB3* may have a weak role in HCC if at all, which requires further investigation.

Compared with other PLCB genes, *PLCB4* is less well characterized, and associations between *PLCB4* and cancer are unclear. The expression of *PLCB4* and *PLCB3* was previously explored in Purkinje cell subsets of the mouse cerebellum (61). *PLCB4* and *PLCB3* are differentially expressed in microarray databases of non-small cell lung cancer, but neither are associated with the prognosis and development of lung cancer (62). Orchel *et al* (63) reported that *PLCB4* is differentially expressed in 50 endometrium samples from women with endometrial cancer, but is not associated with the treatment of endometrial cancer. Furthermore, the present study did not find any association

between *PLCB4* and HCC. Therefore, further studies are required to explore the relationship between *PLCB4* expression and malignancy.

The findings of the present study indicate that *PLCB1* expression was associated with OS, whereas *PLCB1* and *PLCB3* expression was associated with RFS in univariate analysis. In multivariate analysis, *PLCB1* expression was associated with OS and RFS. Multivariate cox analysis contains several significant clinicopathological characteristics, which produces new adjusted results and conclusions. In addition, *PLCB1* expression was associated with OS, whereas *PLCB1* and *PLCB3* expression was associated with RFS in univariate analysis. However, *PLCB1* expression was associated with OS and RFS in multivariate analysis. Different results may be due to varied clinicopathological characteristics in the GSE14520 and TCGA dataset. Of course, HBV is a pivotal factor associated with HCC.

There are some limitations to the present study that should be recognized. Firstly, larger sample cohorts are required to validate these findings. Additionally, the results are based on a HBV-associated HCC population; therefore, further explorations are needed in a study including HBV-infected and non-infected patients. Finally, functional trials are required to further explore the roles of PLCB genes in HCC initiation,

Table V. Enrichment results of gene ontologies and KEGG pathways of phospholipase B1 with genome-wide associated genes.

Category	Term	Count	P-value	False discovery rate	Genes
Biological process	Oxidation-reduction process	11	1.15E-06	0.001521	FMO4, CBR1, MSRA, PLOD2, BLVRB, F8, SMOX, GRHPR, NDUFA10, HPD, HSD17B8
Molecular function	Long-chain fatty acid-CoA ligase activity	3	0.000399	0.455909	ACSL4, ACSL3, SLC27A5
Cellular component	Extracellular exosome	16	0.000748	0.826682	NACA, FCER2, CAPZA1, FBP1, AXL, SPINK1, GRHPR, CBR1, MSRA, RPL7, PLOD2, BLVRB, SNRPB, ACSL4, PLCB1, HPD
Biological process	Long-chain fatty acid metabolic process	3	0.001186	1.559498	ACSL4, ACSL3, SLC27A5
Cellular component	Cytosol	17	0.001409	1.552995	CAPZA1, FBP1, ARHGAP28, TRIB3, SAE1, GRHPR, CBR1, MSRA, RPL7, NCAPG, BLVRB, SNRPB, SMOX, PLCB1, SNRPF, NUP43, HPD
Cellular component	Endoplasmic reticulum membrane	7	0.012018	12.55815	FMO4, HMOX2, PLOD2, ACSL4, ACSL3, SLC27A5, HPD
Cellular component	Actin cytoskeleton	4	0.012886	13.40731	MSRA, SORBS2, NCAPG, CAPZA1
Cellular component	U7 snRNP	2	0.015645	16.05626	SNRPB, SNRPF
Biological process	Heme catabolic process	2	0.01697	20.28229	HMOX2, BLVRB
Molecular function	Decanoate-CoA ligase activity	2	0.018337	19.08795	ACSL4, ACSL3
Molecular function	Very long-chain fatty acid-CoA ligase activity	2	0.02287	23.26214	ACSL4, SLC27A5
Cellular component	U4 snRNP	2	0.024478	24.04788	SNRPB, SNRPF
Cellular component	Methylosome	2	0.026674	25.92421	SNRPB, SNRPF
Biological process	Cellular protein modification process	3	0.027107	30.50786	MSRA, PLOD2, SAE1
Cellular component	Small nucleolar ribonucleoprotein complex	2	0.028865	27.75428	SNRPB, SNRPF
Biological process	Histone mRNA metabolic process	2	0.028919	32.20282	SNRPB, SNRPF
Biological process	Positive regulation of nitric-oxide synthase biosynthetic process	2	0.031291	34.36424	CCL20, FCER2
Molecular function	RNA binding	5	0.036659	34.78158	RPL7, SNRPB, CPSF6, PAPOLG, SNRPF
Cellular component	Intracellular ribonucleoprotein complex	3	0.037507	34.57769	RPL7, SNRPB, CPSF6
Cellular component	Small nuclear ribonucleoprotein complex	2	0.037582	34.63438	SNRPB, SNRPF
Cellular component	SMN-Sm protein complex	2	0.037582	34.63438	SNRPB, SNRPF
Cellular component	U1 snRNP	2	0.041912	37.82519	SNRPB, SNRPF
Biological process	Nuclear import	2	0.043071	44.18227	SNRPB, SNRPF
Cellular component	U12-type spliceosomal complex	2	0.056916	47.81769	SNRPB, SNRPF
Biological process	Metabolic process	3	0.063294	57.93576	GRHPR, ACSL4, ACSL3
Biological process	Drug metabolic process	2	0.063922	58.30767	FMO4, CBR1
Biological process	Spliceosomal snRNP assembly	2	0.066211	59.63802	SNRPB, SNRPF
Biological process	mRNA polyadenylation	2	0.066211	59.63802	CPSF6, PAPOLG
Biological process	Regulation of glucose transport	2	0.077575	65.68049	TRIB3, NUP43

Table V. Continued.

Category	Term	Count	False discovery rate	P-value	Genes
Biological process	Regulation of G-protein coupled receptor protein signaling pathway	2	71.75149	0.091035	RAMP3, PLCB1
Biological process	Long-chain fatty-acyl-CoA biosynthetic process	2	74.37219	0.097693	ACSL4, ACSL3
KEGG pathway	PPAR signaling pathway	3	29.78018	0.031778	ACSL4, ACSL3, SLC27A5
KEGG pathway	Fatty acid biosynthesis	2	45.0671	0.053251	ACSL4, ACSL3
KEGG pathway	Metabolic pathways	10	48.31368	0.058505	CBR1, FBP1, GRHPR, ACSL4, PLCB1, NDUFA10, ACSL3, SLC27A5, HPD, HSD17B8

KEGG, Kyoto Encyclopedia of Genes and Genomes.
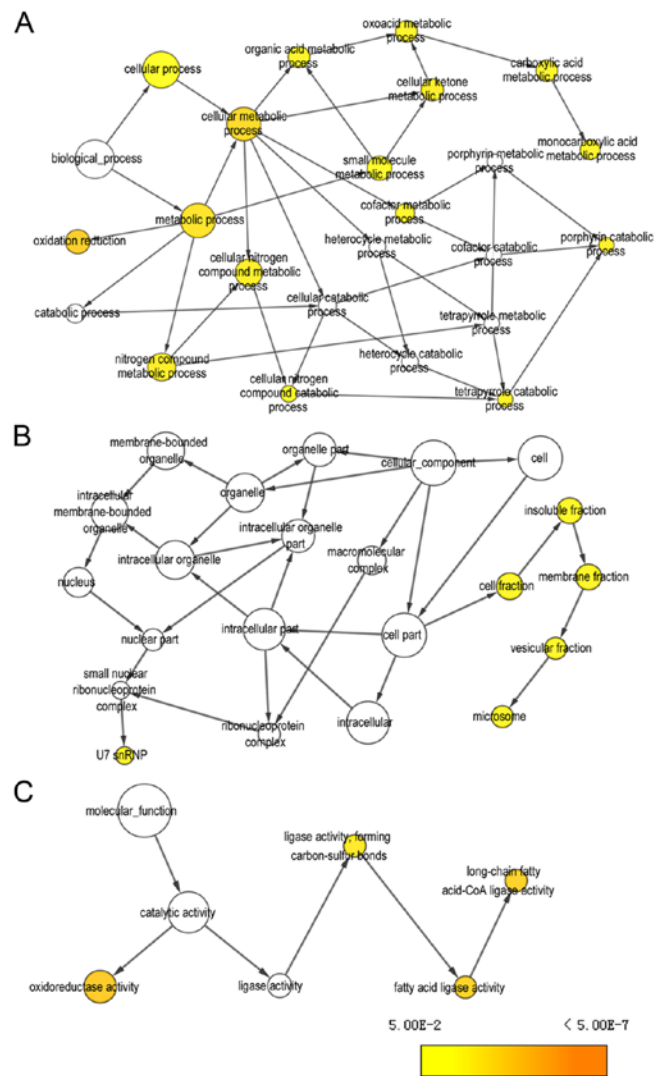
Figure 13. Visualized gene ontologies of *phospholipase C β1* and correlation-associated genes in genome-wide analysis. (A) Biological process, (B) cellular component and (C) molecular function.

development, metastasis, proliferation and angiogenesis. BCLC stage is an important factor associated with HCC and treatments concerning BCLC stage should mentioned in the material section.

The present study demonstrated that the *PLCB1* and *PLCB2* genes are differentially expressed between tumor and normal tissues and have diagnostic values for HCC. *PLCB3* has a potential diagnostic value for HCC. The combinations of these genes have an advantage over *PLCB1*, *PLCB2* or *PLCB3* used alone for HCC diagnosis. In addition, *PLCB1* has OS and RFS prognostic value for HCC. Combining *PLCB1* and AFP was advantageous over *PLCB1* alone for predicting OS and RFS. Nomogram and gene expression models were used to construct and predict HCC prognosis. GO terms and metabolic pathways associated with *PLCB1* and *PLCB2* are include 'G protein coupled receptor activity', 'cell division', 'cell cycle checkpoint', 'DNA repair', 'PPAR signaling pathway' and 'metabolism of xenobiotics by cytochrome P450'. Validation of the prognostic value of the PLCB genes revealed that *PLCB1*, *PLCB2* and *PLCB4* are associated with HCC prognosis.
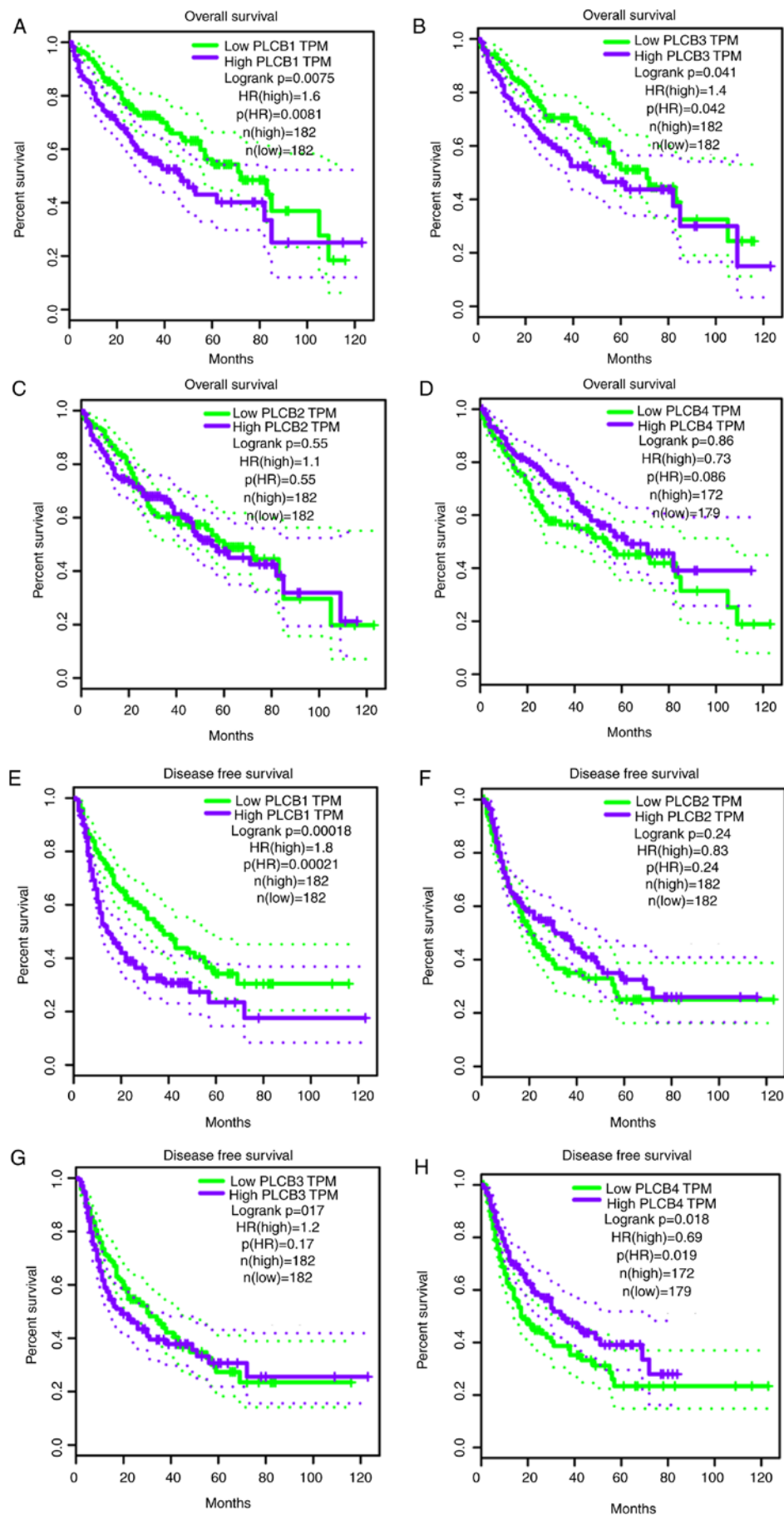
Figure 14. Overall survival and disease recurrence-free survival analysis plots of PLCB1-4. Overall survival analysis plots of (A) PLCB1, (B) PLCB2, (C) PLCB3 and (D) PLCB4. Disease recurrence-free survival analysis plot (E) PLCB1, (F) PLCB2, (G) PLCB3 and (H) PLCB4. PLCB, phospholipase C β; TPM, transcripts per million; HR, hazard ratio.
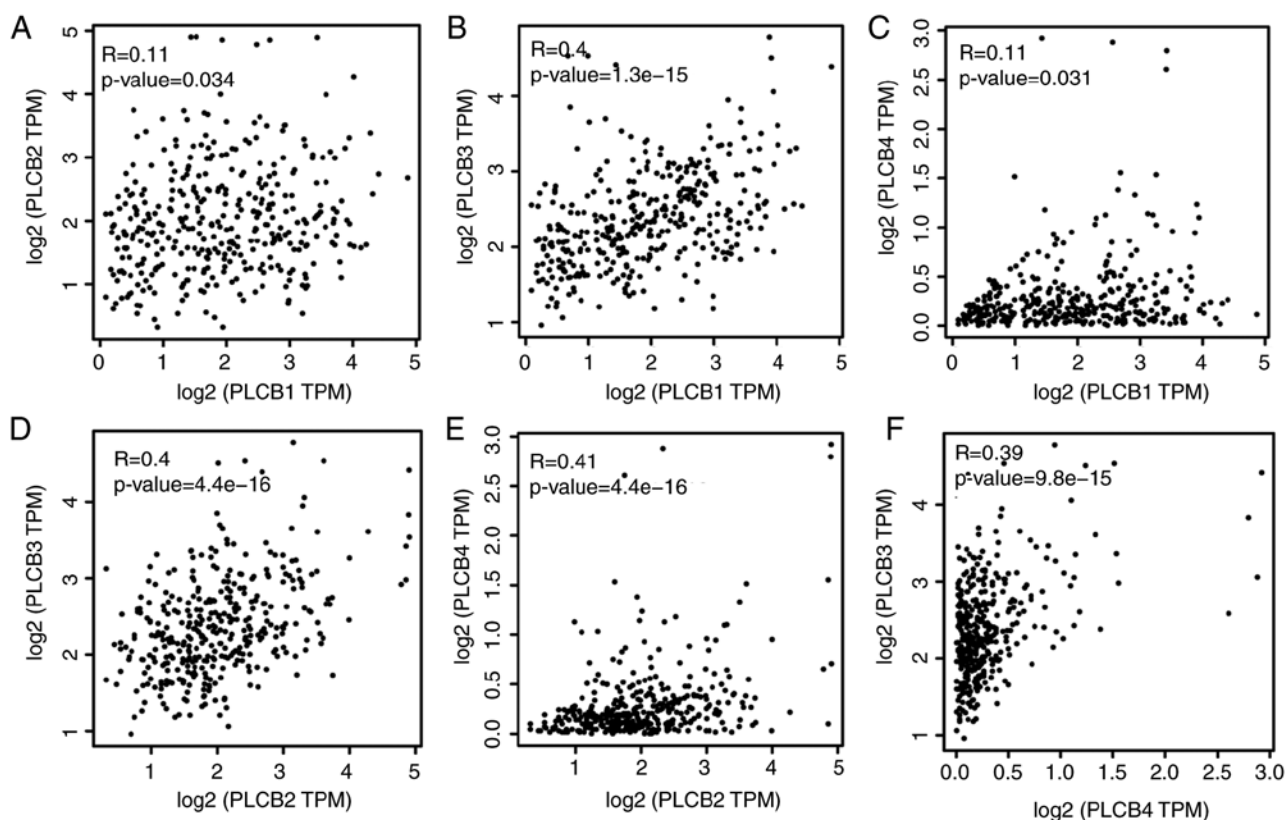
Figure 15. Pearson correlation plots of PLCB1-4 genes. (A) PLCB1 vs. PLCB2; (B) PLCB3 vs. PLCB1; (C) PLCB4 vs. PLCB1; (D) PLCB3 vs. PLCB2; (E) PLCB4 vs. PLCB2; (E) PLCB3 vs. PLCB4. PLCB, phospholipase C β; TPM, transcripts per million.

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Availability of data and materials

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XW and TP designed this study and the manuscript. KH, XZ, ZL, XL, CY, TY, CH, GZ, WQ and TP conducted the study and analyzed the data. XW wrote the manuscript and TP guided the writing. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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