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Research Paper

Genetic variants and Expression of Cytochrome p450 Oxidoreductase Predict Postoperative Survival in Patients with Hepatitis B Virus-Related Hepatocellular Carcinoma

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Abstract

Our current study investigates the prognostic values of genetic variants and mRNA expression of cytochrome p450 oxidoreductase (*POR*) in hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). A total of 19 candidate single nucleotide polymorphisms (SNPs) located in the exons of *POR* were genotyped using Sanger sequencing from 476 HBV-related HCC patients who underwent hepatectomy between 2003 and 2013. The mRNA expression of *POR* in 212 patients with HBV-related HCC was obtained from GSE14520 dataset. Survival analysis was performed to investigate the association of *POR* variants and mRNA expression with overall survival (OS) and recurrence-free survival (RFS). Nomograms were used to predict the prognosis of HBV-related HCC patients. Gene set enrichment analysis (GSEA) was used to investigate the mechanism of *POR* in HBV-related HCC prognosis. The polymorphism *POR*-rs1057868 was significantly associated with HBV-related HCC OS (CT/TT vs. CC, hazard ratio [HR] = 0.69, 95% confidence interval [CI] = [0.54, 0.88], P = 0.003), but not significantly associated with RFS (CT/TT vs. CC, P = 0.378). *POR* mRNA expression was also significantly associated with HBV-related HCC OS (high vs. low, HR = 0.61, 95% CI = [0.38, 0.97], P = 0.036), but not significantly associated with the RFS (high vs. low, P = 0.201). Two nomograms were developed to predict the HBV-related HCC OS. Furthermore, GSEA suggests that multiple gene sets were significantly enriched in liver cancer survival and recurrence, as well as *POR*-related target therapy in the liver. In conclusion, our study suggests that *POR*-rs1057868 and mRNA expression may serve as a potential postoperative prognosis biomarker in HBV-related HCC.

Key words: hepatocellular carcinoma, cytochrome p450 oxidoreductase, prognosis, hepatitis B virus, hepatectomy

Introduction

Liver cancer is the seventh most common cancer and the third leading cause of cancer-related death worldwide, and approximately 70%–90% of primary liver cancer is hepatocellular carcinoma (HCC) [1, 2]. Hepatitis B virus (HBV) promotes cirrhosis, which is found in approximately half of the patients with HCC worldwide and this rate is higher in China[3]. Although the rapid development of a comprehensive surgery-based treatment has improved the clinical outcome for HCC patients greatly, the long-term clinical outcome after hepatectomy of HCC remains unsatisfactory[4, 5]. Multiple clinical indicators of HCC such as tumor size, vascular invasion, portal vein tumor thrombus (PVTT), serum alpha-feto-protein (AFP) levels, Barcelona Clinic Liver Cancer (BCLC) stage, and antiviral therapy have been indicated as useful predictors for HCC patient prognosis after hepatectomy[6-10]. However, these factors still cannot accurately predict HCC prognosis. Considering that prognosis might be affected by genetic and clinicopathological factors, it would be rational to identify potential biomarkers for a more effective prognosis prediction, thus improving clinical outcomes in HCC patients.

During the past few years, an increasing number of genes that encode metabolic enzymes have been confirmed as tumor susceptibility genes[11, 12]. Cytochrome p450 oxidoreductase (*POR*) encodes a metabolic enzyme, which is expressed extensively in multiple normal and tumor tissues. POR is known as the unique electron donor for all microsomal cytochrome p450 (CYP) enzymes, which play a critical role in the metabolism of steroid hormones, fatty acids, bile acids, and drugs[13, 14]. Variants in *POR* have also been reported as risk factors in multiple cancers. Haiman et al. demonstrated that synonymous variants of *POR* G5G might increase the risk of breast cancer in African American populations[15]. Xiao et al. reported that the *POR* variant A503V is correlated with a risk of bladder cancer in a Chinese population[16]. However, the association of *POR* variants and expression with HCC prognosis remains unclear. Therefore, our current study is to investigate the prognostic values of genetic variants and expression of *POR* in HBV-related HCC prognosis after hepatectomy.

Materials and Methods

Study population

This study was approved by the Ethics Committee of The First Affiliated Hospital of Guangxi Medical University (Approval number: 2015[KY-E-032]). The inclusion criteria of patients in the present study were listed as follows: 1) Chinese population; 2) Patients with HBV-related HCC; 3) Patients underwent hepatectomy. The exclusion criteria of patients in the present study were listed as follows: 1) Patients with missing clinical features and prognostic data; 2) Patients survived less than one month. To explore the application value of *POR* genetic variation in prognosis of HCC, a total of 476 HBV-related HCC patients who underwent hepatectomy between 2003 and 2013 in The First Affiliated Hospital of Guangxi Medical University, Guangxi, China, and identified as cohort 1. To explore the application value of *POR* mRNA expression in prognosis of HCC, by retrieving the public database, only the whole genome expression profile data set of GSE14520 compliance with the above inclusion conditions, and identified as cohort 2. Cohort 2 consisted of 212 HBV-related HCC patients who underwent hepatectomy from the GSE14520 dataset (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14520)[17, 18].

Clinical data and definitions

The clinical features were retrospectively collected from medical records. HBV-related HCC patients were defined as patients with a seropositive of the HBV surface antigen (HBsAg) and with a postoperative sample pathological diagnosis of HCC. Tumor stage was classified according to the BCLC staging system[19]. PVTT was classified according to the classification system of the Liver Cancer Study Group of Japan[20]. Liver reserve function was measured according to the Child–Pugh score[21]. Preoperative AFP was classified into two groups with a cut-off value of 400 ng/ml[22]. The degree of cirrhosis was determined according to the pathological report. Antiviral therapy is defined here as receiving anti-HBV treatment systematically after surgery. Recurrence-free survival (RFS) was defined as the interval between surgery and the date of diagnosis of the first recurrence or the date of the last follow-up. Overall survival (OS) was defined as the interval between surgery and the date of death, or the date of the last follow-up.

Follow-up

The patients were followed up via telephone or hospital visit after surgery until patients died or up to the date of the last follow-up. The last follow-up was in January 2018.

Identification of *POR* variants

The 19 previously reported candidate single nucleotide polymorphisms (SNPs) located in the exons of *POR* were selected to investigate the prognostic values in HBV-related HCC. All SNPs were genotyped in HCC tumor tissues using the

Sanger sequencing method[23]. The primers used for amplification of the *POR* SNPs were according to a previous study (Supplementary **Table S1**)[16]. As shown in supplementary **Table S2**, two *POR* gene variants (rs1135612, rs1057868) were observed to have a polymorphic distribution in the cohort 1 population. The SNPs with a minor allele frequency (MAF) < 10% were excluded from the present study, and only the data of rs1135612 and rs1057868 were included in further study. The MAF of rs1135612 and rs1057868 was 43.8% and 35.9%, respectively. Both of these SNPs met the Hardy–Weinberg equilibrium (rs1135612: 0.619; rs1057868: 0.378). Rs1135612 is a synonymous mutation (P130P) and rs1057868 is a missense mutation (A503V).

Gene expression profile

The gene expression dataset was obtained from the Gene Expression Omnibus public database with a serial number of GSE14520[17, 18]. In order to avoid the batch effect between different platforms, only the dataset of the Affymetrix HT Human Genome U133A Array (GPL3921) of GSE14520 was included in the present study. Processing of raw data was according to the manufacturer's guidelines and shown in https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM362960. For multiple probe sets, the average value corresponding to the same gene was regarded as the expression value of the gene and normalized by the *limma* package in the R platform. Only 212 HBV-related HCC patients who underwent hepatectomy were analyzed further in the present study.

Gene set enrichment analysis

To investigate the potential mechanism of *POR* in HCC prognosis, a gene set enrichment analysis (GSEA, https://software.broadinstitute.org/gsea/)[24] was conducted using the gene expression dataset of GSE14520 based on the molecular signature database (MSigDB) of c2 (c2.all.v6.1.symbols.gmt) and c5 (c5.all.v6.1.symbols.gmt) gene sets[25]. A *P*-value < 0.05 and false discovery rate (FDR) < 0.25 were considered statistically significant.

Statistical analysis

Survival was calculated using the Kaplan–Meier method and compared with a log-rank test. Cox proportional hazards model was used to identify independent predictors of survival. All clinical features with a *P*-value < 0.1 in the univariate analysis were filtered into the multivariate analysis. A nomogram was developed to predict the survival based on Cox regression. The significant factors associated with prognosis in the multivariate analysis were fitted into nomograms. The nomogram was validated using the concordance index (c-index)[26]. All analyses were performed using R version 3.3.2 (http://www.R-project.org). A *P*-value < 0.05 was considered statistically significant.

Results

Characteristics of the study population

Cohort 1 consisted of 476 HBV-related HCC patients from The First Affiliated Hospital of Guangxi Medical University. The median age was 46 years. The relationship between *POR* variants and clinicopathological factors are summarized in **Table 1**. No significant correlation with rs1057868 and rs1135612 was found with gender, age, smoking, drinking, Child–Pugh, AFP, PVTT, BCLC, cirrhosis or antiviral therapy (all *P*> 0.05). The OS follow-up rate was 100%, while the RFS follow-up rate was 72%.

Cohort 2 consisted of 212 HBV-related HCC patients from GSE14520. The median age was 50 years. The patients were divided into low- and high-expression groups according to the median value of *POR* mRNA expression. The relationship between *POR* expression and clinicopathological factors are summarized in **Table 2**. No significant correlation was found with gender, age, alanine aminotransferase (ALT), and cirrhosis (all *P* > 0.05). However, *POR* expression was significantly associated with gender (*P* = 0.046), AFP (*P*< 0.001), and BCLC (*P* = 0.023). Both OS and RFS follow-up rate were 100%.

Table 1. The relationship between *POR* variants and clinicopathological factors in the cohort 1.

Variable		rs1057868			*P*-value*	rs1135612			*P*-value*
		CC	CT	TT		AA	AG	GG	
Gender	Male	172	199	50	0.665	137	199	85	0.584
	Female	19	29	7		16	30	9	
Age	≤46years	107	126	26	0.360	78	125	56	0.419
	>46years	84	102	31		75	104	38	
Smoking	No	122	157	34	0.330	94	160	59	0.186
	Yes	69	71	23		59	69	35	
Drinking	No	111	143	34	0.624	87	144	57	0.499
	Yes	80	85	23		66	85	37	
Child-Pugh	A	164	197	52	0.563	132	205	76	0.110
	B	27	31	5		21	24	18	
AFP	<400ng/ml	98	131	35	0.284	87	133	44	0.165
	≥400ng/ml	93	97	22		66	96	50	
PVTT	No	155	195	50	0.343	132	191	77	0.621
	vp1-4	36	33	7		21	38	17	
BCLC	0/A	110	136	35	0.945	93	135	53	0.900
	B	29	37	9		25	36	14	
	C	52	55	13		35	58	27	
Cirrhosis	No	20	30	7	0.699	18	29	10	0.874
	Yes	171	198	50		135	200	84	
Antiviral	No	116	153	39	0.326	101	148	59	0.874
	Yes	75	75	18		52	81	35	

Abbreviations: AFP, alpha-fetoprotein; PVTT, portal vein tumor thrombus; BCLC, Barcelona Clinic Liver Cancer stage. Note: **P*-value was calculated using two-sided χ^2 test.

Table 2. The relationship between *POR* expression and clinicopathological factors in the cohort 2.

Variable		POR expression		*P*-value*
		Low	High	
Gender	Male	86	97	0.046
	Female	20	9	
Age	≤50years	51	58	0.410
	>50years	55	48	
ALT	≤50U/L	59	65	0.486
	>50U/L	47	41	
AFP	≤300ng/ml	40	75	<0.001
	>300ng/ml	65	29	
BCLC	0/A	74	89	0.023
	B/C	32	17	
Cirrhosis	No	6	11	0.312
	Yes	100	95	

Abbreviations: ALT, alanine aminotransferase; AFP, alpha-fetoprotein; BCLC, Barcelona Clinic Liver Cancer stage. Note: *P-value was calculated using two-sided $\chi 2$ test.

Association of *POR* variants with OS

The result of univariate and multivariate analysis of *POR* variants with OS are presented in **Table 3 and Figure 1A–D**. The median OS of the *POR*-rs1057868 CC, CT, and TT group was 57, 84, and 87 months, respectively. In the univariate analysis, we found patients with the *POR*-rs1057868 CT genotype had better OS than patients with the CC genotype (CT vs. CC, hazard ratio [HR] = 0.73, 95% confidence interval [CI] = [0.56, 0.94], P = 0.014; TT vs. CC, HR = 0.65, 95% CI = [0.43, 0.99], P = 0.041). Furthermore, we found a greater significant difference in the merge model of *POR*-rs1057868 (CT/TT vs. CC, HR = 0.71, 95% CI = [0.56, 0.91], P=0.006). The median OS of the *POR*-rs1135612 AA, AG, and GG group was 81, 82, and 52 months, respectively. There was no significant difference among groups with different genotypes of *POR*-rs1135612 in the univariate analysis (AG vs. AA, GG vs. AA, and AG/GG vs. AA, all P > 0.05).

We also performed univariate analysis to investigate the association of clinicopathological factors with OS. As shown in **Figure 2**, the factors drinking (yes vs. no, P = 0.087), Child–Pugh (B vs. A, P = 0.067), AFP (≥400 ng/ml vs. < 400 ng/ml, P = 0.001), PVTT (vp1-4 vs. no, P < 0.001), BCLC (B vs. 0/A, P < 0.001; C vs. 0/A, P < 0.001), and antiviral therapy (yes vs. no, P = 0.015) with a P-value < 0.1 were entered into the multivariate analysis. As shown in **Table 3 and Figure 3,** the multivariate analysis, adjusted by drinking, Child–Pugh, AFP, PVTT, BCLC, and antiviral therapy, showed a similar result. Compared with the CC genotype, patients with the CT or TT genotype of *POR*-rs1057868 had a significant association with a better OS (CT vs. CC, HR = 0.70, 95% CI = [0.54, 0.90], P = 0.006; TT vs. CC, HR = 0.67, 95% CI = [0.45, 1.02], P = 0.062; CT/TT vs. CC, HR = 0.69, 95% CI = [0.54,0.88], P = 0.003). However, there was still no significant association between *POR*-rs1135612 and OS in the multivariate analysis (AG vs. AA, GG vs. AA, and AG/GG vs. AA, all P > 0.05).

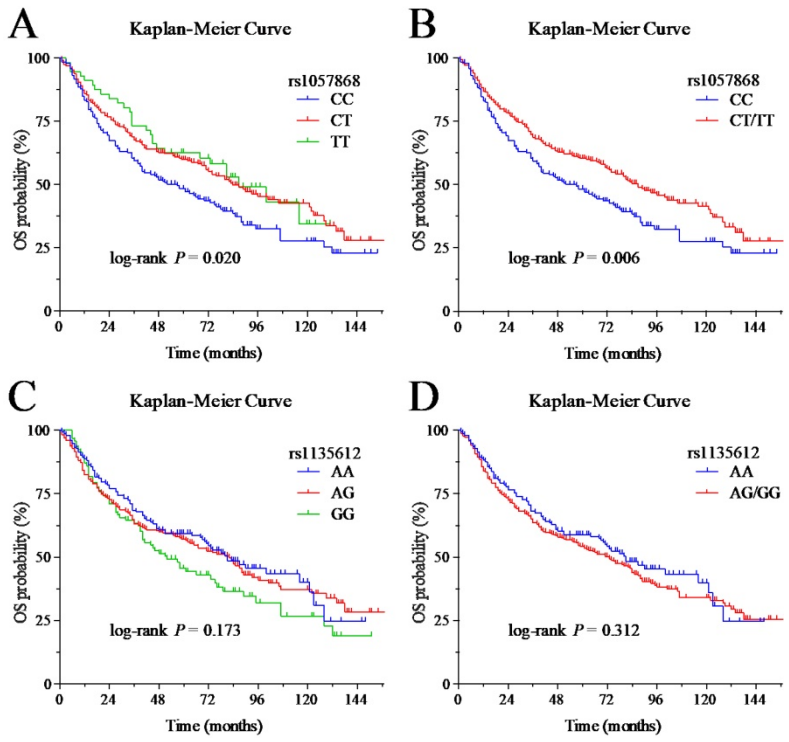


Figure 1. Kaplan-Meier curves for OS among different *POR* variants groups. (A) OS stratified by *POR*-rs1057868 CC, CT and TT genotypes; (B) OS stratified by *POR*-rs1057868 CC and CT/TT genotypes; (C) OS stratified by *POR*-rs1135612 AA, AG and GG genotypes; (D) OS stratified by *POR*- rs1135612 AA and AG/GG genotypes.

Association of *POR* variants with RFS

The result of univariate and multivariate analysis of *POR* variants with RFS was present in **Table 4 and Figure 4A–D**. The median RFS of the *POR*-rs1057868 CC, CT, and TT group was 13, 20, and 46 months, respectively. The median RFS of the *POR*-rs1135612 AA, AG, and GG group was 27, 14, and 16 months, respectively. In the univariate analysis, no significant difference in RFS was found among groups with different genotypes in *POR*-rs1057868 (CT vs. CC, CT/TT vs. CC, all P > 0.05) and *POR*-rs1135612 (AG vs. AA, GG vs. AA, and AG/GG vs. AA, all P > 0.05), except in *POR*-rs1057868 TT vs. CC (P = 0.024).

We also performed univariate analysis to investigate the association of clinicopathological factors with RFS. As shown in **Figure 2**, the factors smoking (yes vs. no, P = 0.052), Child–Pugh (B vs. A, P = 0.056), PVTT (vp1-4 vs. no, P < 0.001), and BCLC (B vs. 0/A, P = 0.001; C vs. 0/A,

$P < 0.001$) with a P-value < 0.1 were entered into the multivariate analysis. As shown in **Table 4,** adjusted by smoking, Child-Pugh, PVTT, and BCLC, there was still no significant difference in RFS among groups with different genotypes in *POR*-rs1057868 (CT vs. CC, TT vs. CC, and CT/TT vs. CC, all $P > 0.05$) and *POR*-rs1135612 (AG vs. AA, GG vs. AA, and AG/GG vs. AA, all $P > 0.05$) in the multivariate analysis.

Association of *POR* mRNA expression with OS

Furthermore, we also investigated the prognostic values of *POR* mRNA expression by using the 212 HBV-related HCC patients from the GSE14520 dataset. The results of univariate analysis of *POR* mRNA expression with OS are presented in **Table 5 and Figure 5A and B**. The median OS of the *POR* low- and high-expression group were not available because more than half of the patients survived up to the end of the follow-up. In the univariate analysis, we found patients with higher mRNA expression of *POR* in HBV-related HCC had better OS than the patients with lower expression (high vs. low: HR = 0.54, 95% CI = [0.35, 0.84], P =0.006). Time-dependent receiver operating characteristic (ROC) analysis substantiated that the *POR* mRNA expression showed a good performance in the HBV-related HCC OS prediction, as the area under curve (AUC) of the ROC curve was 0.600, 0.606, and 0.581 for 1-, 2-, and 5-year survival (**Figure 5C**), respectively. As shown in **Figure 6**, the clinicopathological factors (AFP > 300 ng/ml vs. ≤ 300 ng/ml, $P = 0.047$; BCLC: B/C vs. 0/A, $P < 0.001$; cirrhosis: yes vs. no, $P = 0.025$) with a P-value < 0.1 in the univariate analysis were entered into the multivariate analysis. Furthermore, as shown in **Table 5 and Figure 7,** in the Cox regression model, adjusted by AFP, BCLC, and cirrhosis, patients with high mRNA expression of *POR* in HCC also had better OS than the patients with low expression (high vs. low: HR = 0.61, 95% CI = [0.38,0.97], $P = 0.036$).

Table 3. The result of univariate and multivariate analysis of *POR* variants with OS.

Variable	N	Dead	MST (months)	Univariate analysis		Multivariate analysis*	
				HR(95%CI)	*P*-value	HR(95%CI)	*P*-value
rs1057868							
CC	191	120	57	1.00		1.00	
CT	228	121	84	0.73(0.56, 0.94)	0.014	0.70(0.54, 0.90)	0.006
TT	57	28	87	0.65(0.43, 0.99)	0.041	0.67(0.45, 1.02)	0.062
CT/TT	285	149	87	0.71(0.56, 0.91)	0.006	0.69(0.54, 0.88)	0.003
rs1135612							
AA	153	81	81	1.00		1.00	
AG	229	126	82	1.06(0.80, 1.41)	0.669	1.08(0.81, 1.43)	0.607
GG	94	62	52	1.35(0.97, 1.88)	0.077	1.31(0.94, 1.83)	0.106
AG/GG	323	188	71	1.14(0.88, 1.49)	0.312	1.15(0.88, 1.49)	0.302

Abbreviations: MST, median survival time; HR, hazard ratio; CI, confidence interval. Note: *Multivariate analysis was adjusted by drinking, Child-Pugh, AFP, PVTT, BCLC and antiviral therapy.

Table 4. The result of univariate and multivariate analysis of *POR* variants with RFS.

Variable	N[ε]	Recurrent	MRT (months)	Univariate analysis		Multivariate analysis*	
				HR(95%CI)	*P*-value	HR(95%CI)	*P*-value
rs1057868							
CC	134	100	13	1.00		1.00	
CT	169	125	20	0.91(0.70, 1.19)	0.503	0.95(0.73, 1.24)	0.708
TT	38	23	46	0.59(0.38, 0.94)	0.024	0.66(0.41, 1.05)	0.076
CT/TT	207	148	23	0.84(0.65, 1.08)	0.178	0.89(0.69, 1.15)	0.378
rs1135612							
AA	107	78	27	1.00		1.00	
AG	165	123	14	1.20(0.90, 1.60)	0.212	1.11(0.83, 1.48)	0.476
GG	69	47	16	0.95(0.66, 1.38)	0.786	0.91(0.63, 1.31)	0.597
AG/GG	234	170	15	1.12(0.85, 1.46)	0.428	1.05(0.80, 1.37)	0.751

Abbreviations: MRT, median recurrent time; HR, hazard ratio; CI, confidence interval. Notes: *Multivariate analysis was adjusted by smoking, Child-Pugh, PVTT, and BCLC; [ε] The information of RFS was unavailable in 135 patients.

Table 5. The results of univariate analysis of POR mRNA expression with OS.

Variable	N	Dead	MST (months)	Univariate analysis		Multivariate analysis*	
				HR(95%CI)	*P*-value	HR(95%CI)	*P*-value
POR expression							
Low	106	49	NA	1.00		1.00	
High	106	33	NA	0.54(0.35, 0.84)	0.006	0.61(0.38, 0.97)	0.036

Abbreviations: MST, median survival time; HR, hazard ratio; CI, confidence interval. Note: *Multivariate analysis was adjusted by AFP, BCLC and cirrhosis.

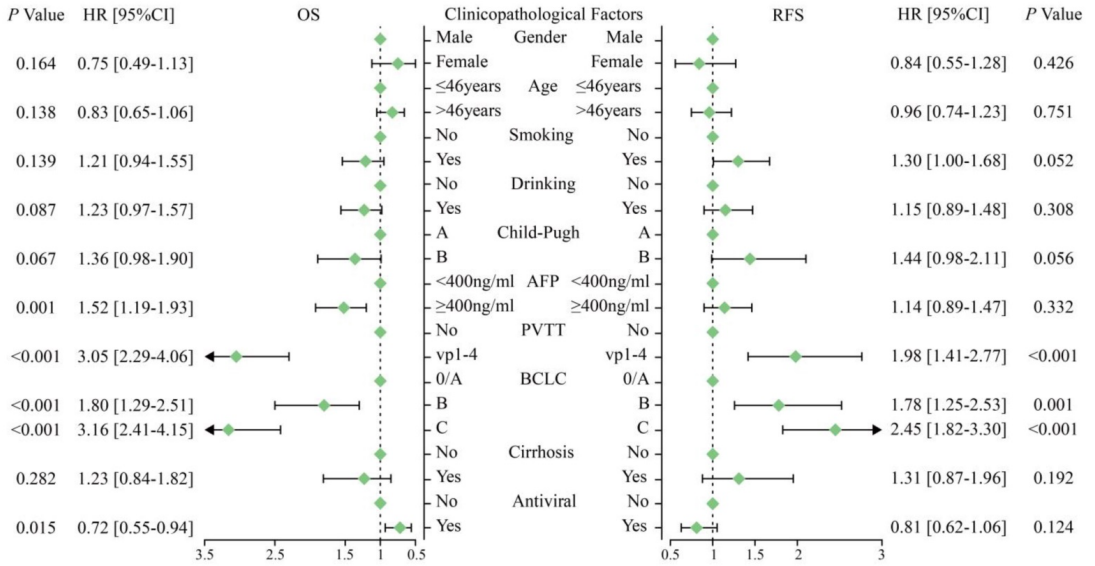


Figure 2. The result of univariate analysis of clinicopathological factors with OS and RFS in the cohort 1.

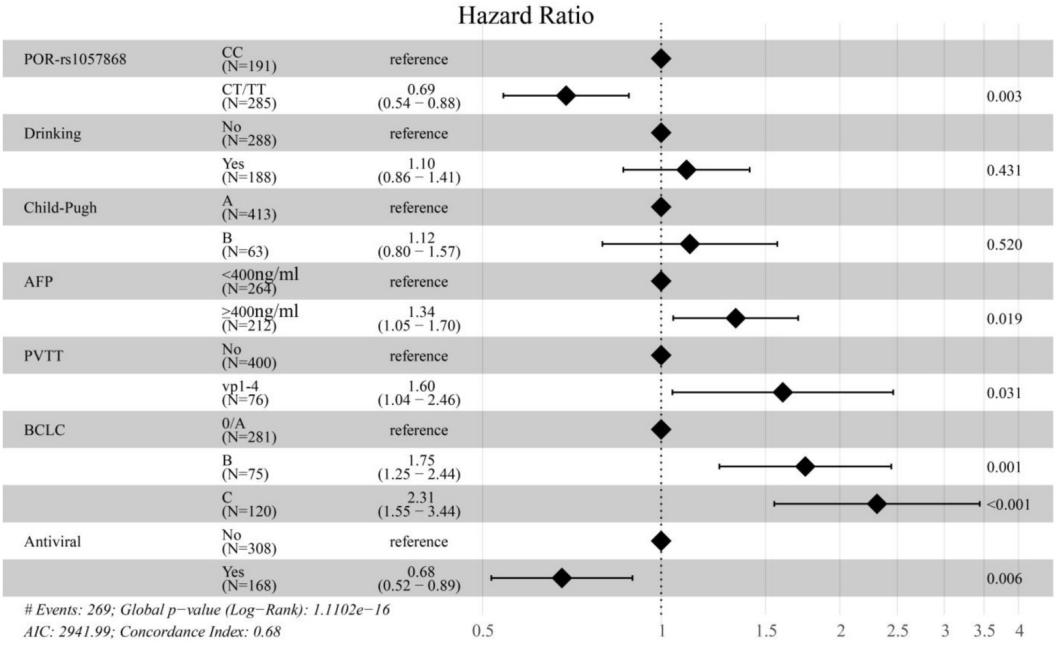


Figure 3. The result of multivariate analysis of *POR*-rs1057868 combined with clinicopathological factors with OS.

Association of *POR* mRNA expression with RFS

The results of the univariate analysis of *POR* mRNA expression with RFS are presented in **Table 6 and Figure 5D**. The median RFS of the *POR* low- and high-expression group was 28 and 51 months, respectively. In the univariate analysis, no significant difference in RFS was found between *POR* low- and high-expression groups (high vs. low: HR = 0.74, 95% CI = [0.52, 1.07], $P = 0.112$). As shown in **Figure 6**, the clinicopathological factors (gender: female vs. male, $P = 0.018$; BCLC: B/C vs. 0/A, $P < 0.001$; cirrhosis: yes vs. no, $P = 0.029$) with a P-value < 0.1 in the univariate analysis were entered into the multivariate analysis. Furthermore, as shown in **Table 6,** in the Cox regression model, adjusted by gender, BCLC, and cirrhosis, there was also no significant difference in RFS between *POR* low- and high-expression groups (high vs. low: HR = 0.78, 95% CI = [0.54, 1.14], $P = 0.201$).

Nomograms and stratified analysis

Multivariate Cox analysis suggested that *POR*-rs1057868 and mRNA expression were significantly associated with HBV-related HCC OS. To further investigate the prognostic values of *POR*-rs1057868 and mRNA expression in HBV-related HCC

prognosis, we performed a stratified analysis to assess prognosis values in different strata of HCC and developed two nomograms for individual prognostic evaluation based on *POR*-rs1057868 or mRNA expression combined with other clinical features.

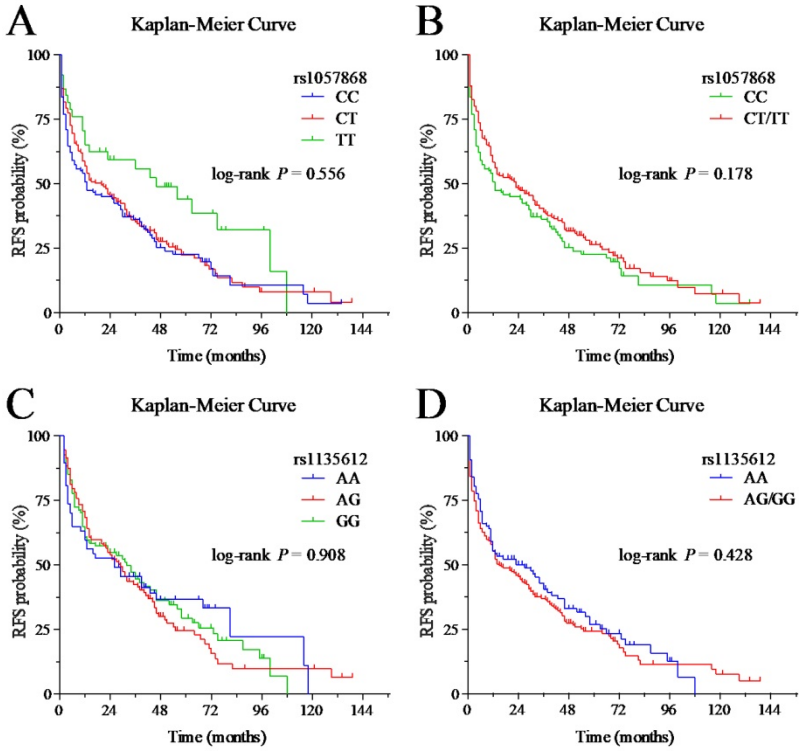


Figure 4. Kaplan-Meier curves for RFS among different *POR* variants groups. (A) RFS stratified by *POR*-rs1057868 CC, CT and TT genotypes; (B) RFS stratified by *POR*-rs1057868 CC and CT/TT genotypes; (C) RFS stratified by *POR*-rs1135612 AA, AG and GG genotypes; (D) RFS stratified by *POR*- rs1135612 AA and AG/GG genotypes.

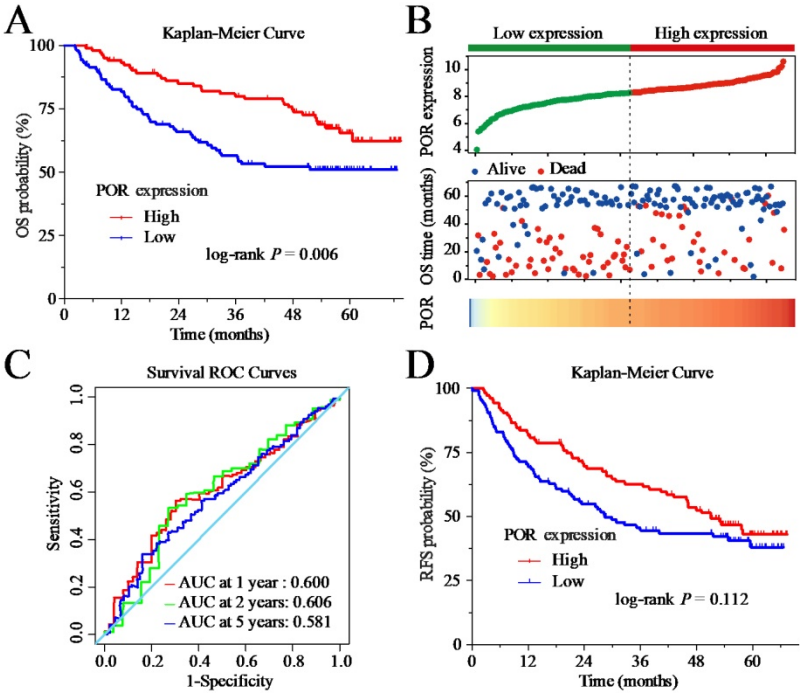


Figure 5. Prognosis values of POR mRNA expression in HBV-related HCC prognosis prediction. (A) OS stratified by POR mRNA expression levels; (B) From top to bottom are the POR mRNA expression, patients' survival status distribution, and POR mRNA expression heat map for low- and high-expression groups. (C) ROC curve for predicting OS in HBV-related HCC patients by POR mRNA expression; (D) RFS stratified by *POR* mRNA expression.

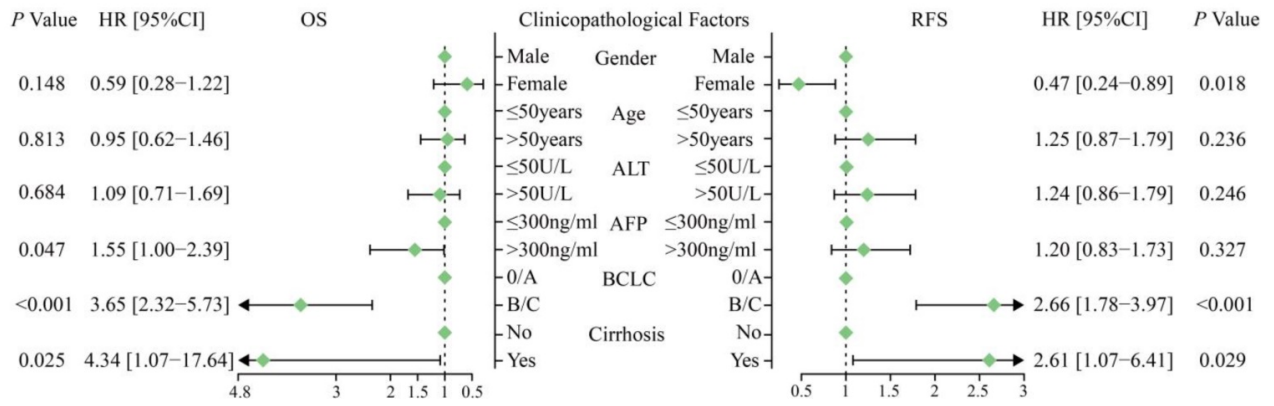


Figure 6. The result of univariate analysis of clinicopathological factors with OS and RFS in the cohort 2.

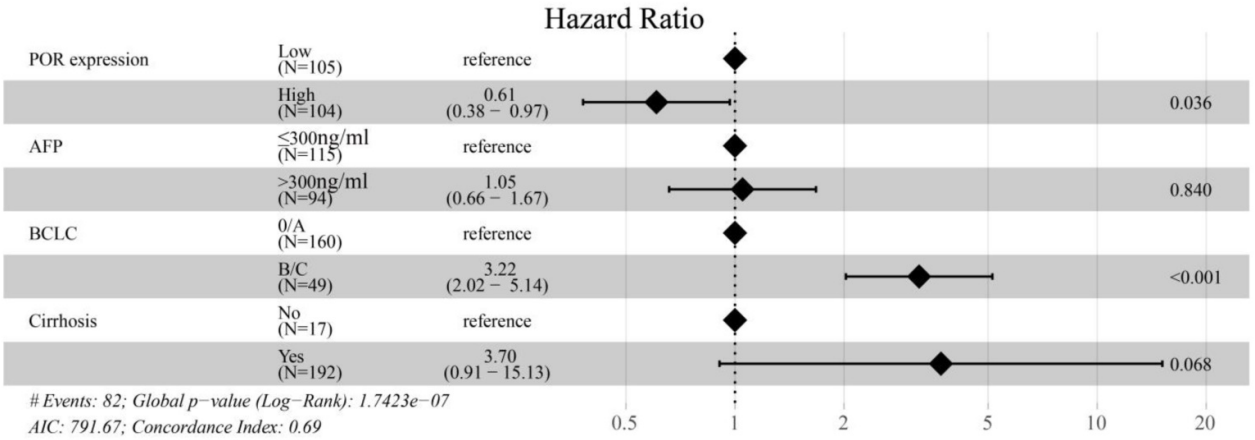


Figure 7. The result of multivariate analysis of *POR* mRNA expression combined with clinicopathological factors with OS.

Table 6. The results of univariate analysis of *POR* mRNA expression with RFS.

Variable	N	Recurrent	MRT (months)	Univariate analysis		Multivariate analysis*	
				HR(95%CI)	*P*-value	HR(95%CI)	*P*-value
POR expression							
Low	106	61	28	1.00		1.00	
High	106	55	51	0.74(0.52, 1.07)	0.112	0.78(0.54, 1.14)	0.201

Abbreviation: MRT, median recurrent time; HR, hazard ratio; CI, confidence interval. Note: *Multivariate analysis was adjusted by gender, BCLC, and cirrhosis.

The stratified analysis suggested that patients in both AFP values, without PVTT and antiviral therapy, with a T allele of *POR*-rs1057868 had a significantly reduced risk of death in HBV-related HCC compared with the CC genotypes (**Figure 8A**). We also performed a stratified analysis for *POR* mRNA expression in the GSE14520 cohort and suggested that high expression of *POR* had a significantly reduced risk of death of HBV-related HCC in patients with AFP ≤ 300 ng/ml and cirrhosis, compared with low expression (**Figure 8B**).

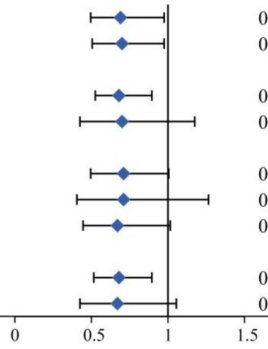
Nomograms base on *POR*-rs1057868 or mRNA expression combine with other clinical features are shown in **Figure 9A and B.** The c-index **(**95% CI) of the nomogram of *POR*-rs1057868 combined with AFP, PVTT, BCLC, and antiviral therapy was 0.67 (0.63 to 0.71). The c-index **(**95% CI) of the nomogram of *POR* mRNA expression combined with BCLC was 0.68 (0.62 to 0.74).

GSEA

In addition, we conducted a GSEA to identify gene sets that contributed to the HCC prognosis between *POR* high- and low-expression groups. The enrichment results of the high *POR* expression group are summarized in supplementary **Tables S3 and S4.** The enrichment results of the high *POR* expression group in the c2 gene set suggested that there were multiple gene sets were found to significantly correlate with liver cancer survival, recurrence, and development[27-31] (**Figure 10 A–F**). For the c5 gene set, we observed that high *POR* expression was significantly enriched in the metabolism of multiple substances in biological processes (**Figure 10 G–L**).

A

Subgroup	rs1057868 CT/TT Dead / N	MST	rs1057868 CC Dead / N	MST	CT/TT vs. CC	HR [95%CI]	*P* Value
AFP							
<400ng/ml	77/166	101	56/98	75		0.69 [0.49-0.98]	0.037
≥400ng/ml	72/119	69	64/93	38		0.70 [0.50-0.98]	0.039
PVTT							
No	116/245	100	90/155	73		0.68 [0.52-0.90]	0.007
vp1-4	33/40	24	30/36	15		0.70 [0.42-1.18]	0.183
BCLC							
0/A	70/171	123	55/110	95		0.71 [0.49-1.01]	0.054
B	27/46	71	22/29	47		0.71 [0.40-1.27]	0.249
C	52/68	32	43/52	24		0.67 [0.44-1.02]	0.062
Antiviral							
No	113/192	80	83/116	46		0.68 [0.51-0.90]	0.007
Yes	36/93	138	37/75	67		0.67 [0.42-1.06]	0.090



B

Subgroup	High expression Dead / N	MST	Low expression Dead / N	MST	High vs. Low	HR [95%CI]	*P* Value
AFP							
≤300ng/ml	21/75	NA	18/40	NA		0.52 [0.27-0.98]	0.043
>300ng/ml	12/29	NA	31/65	NA		0.71 [0.36-1.38]	0.311
BCLC							
0/A	22/89	NA	28/74	NA		0.57 [0.31-1.05]	0.069
B/C	11/17	37	21/32	18		0.63 [0.30-1.33]	0.229
Cirrhosis							
No	1/11	NA	1/6	NA		0.49 [0.03-7.94]	0.616
Yes	32/95	NA	48/100	52		0.61 [0.38-0.99]	0.044

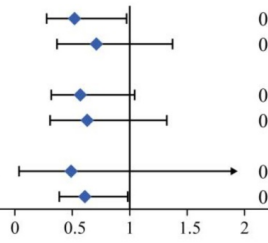
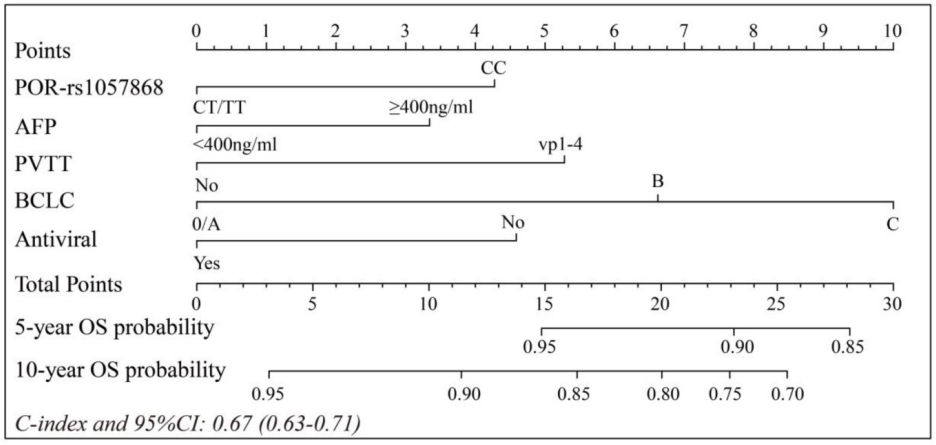


Figure 8. (A) Stratified analysis of *POR*-rs1057868 in different clinical features; (B) Stratified analysis of *POR* mRNA expression in different clinical features.

A



B

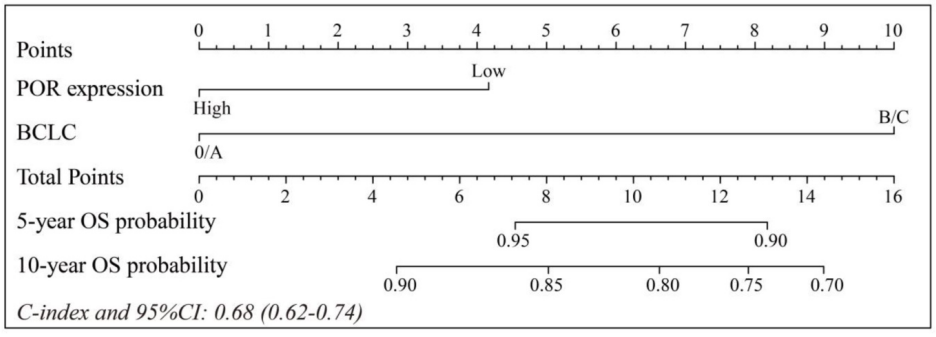


Figure 9. (A) Nomograms for predicting HBV-related HCC OS with *POR*-rs1057868 genotypes and clinical information; (B) Nomograms for predicting HBV-related HCC OS with *POR* mRNA expression and clinical information.

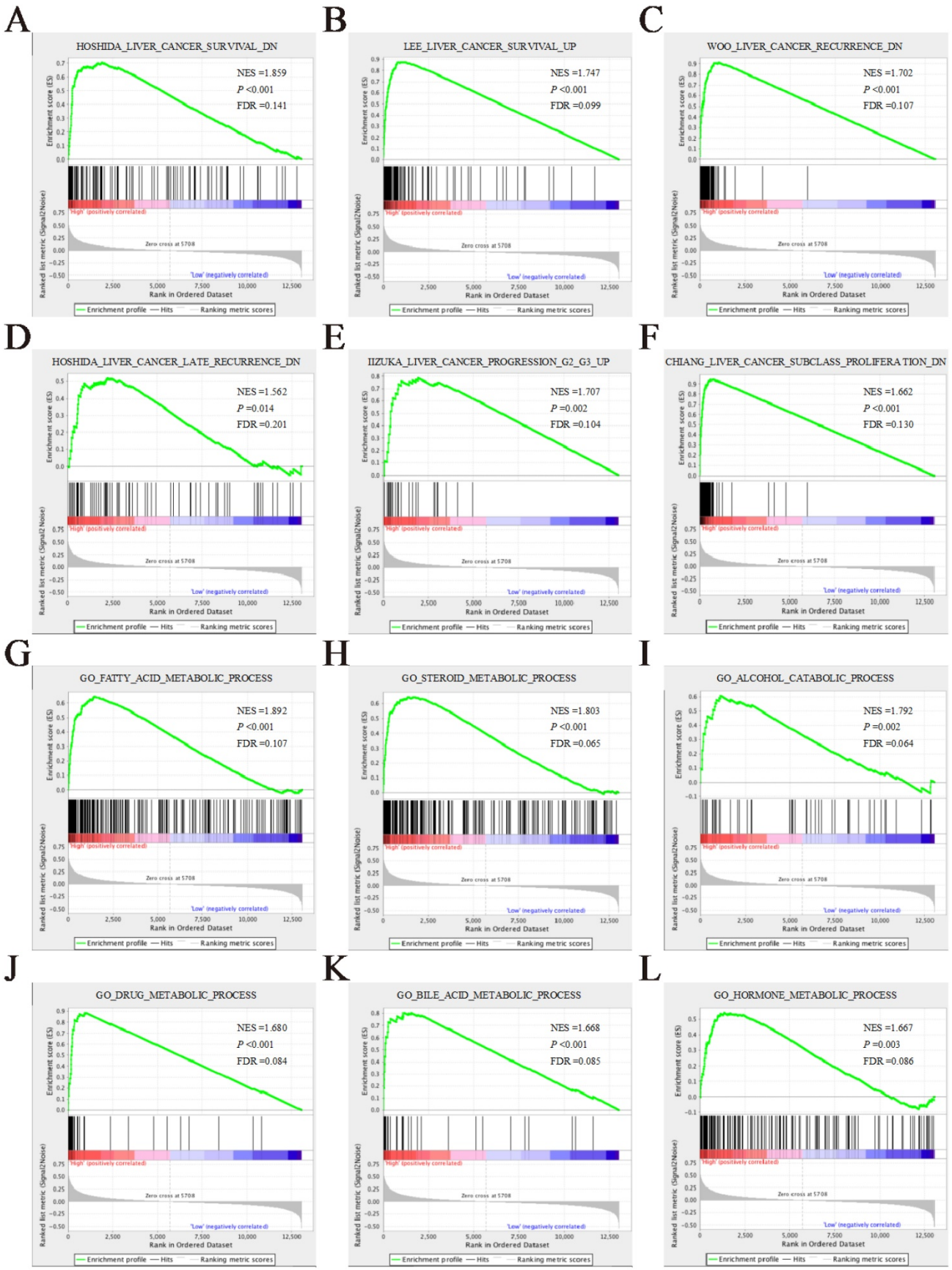


Figure 10. GSEA results of *POR* in GSE14520 HBV-related HCC patients. (A-F) GSEA results of c2 reference gene sets for high *POR* expression groups; (G–L) GSEA results of c5 reference gene sets for high *POR* expression groups.

Discussion

The human *POR* gene, located on chromosome 7q11.23, is highly polymorphic[13, 32]. To date, more than 50 *POR* SNPs have been reported[33]. In a study population of 476 HBV-related HCC patients from south China, we observed two SNPs, rs1057868 and rs1135612, in *POR* exons. The MAF of rs1057868 and rs1135612 was 35.9% and 43.8%, respectively, which was close to the MAF in the HapMap database (39.6% and 45.8%, respectively)[16]. *POR*-rs1057868 is a missense mutation (A503V), which has been identified as the most common *POR* variant in all racial/ethnic populations (about 28% of human alleles)[33]. It has been shown that the variant A503V is a conservative change in an unstructured loop of the FAD-binding domain, which may affect the activity of *POR*[34]. Hart et al. demonstrated that variant A503V was significantly related to POR activity ($P = 0.003$)[35]. As an obligate electron donor for CYP enzymes, POR plays a critical role in the metabolic process of CYPs. Many previous studies have reported that *POR*-rs1057868 was associated with the activities of CYPs. Agrawal et al. reported that *POR* A503V caused a 15% decrease in CYP1A2 activity, 13% increase in CYP2C19, and a 33%–49% decrease in CYP3A4 *in vitro*[36, 37]. Hylke et al. reported that *POR* A503V was associated with increased CYP3A5 activity in 298 patients[38]. Additionally, multiple studies observed that the A503V variant decreased cytochrome *c* reduction activity[33, 39]. Most of the previous studies suggested that *POR* variants were associated with certain genetic disorders, such as ambiguous genitalia, Antley–Bixler skeletal malformation syndrome, and congenital adrenal hyperplasia[40, 41]. Recently, a few studies have focused on the association between the polymorphisms in A503V and cancer risk. Xiao et al. reported that the *POR*-rs1057868 was correlated with the risk of bladder cancer in a Chinese population[16]. However, no study has investigated the association between *POR*-rs1057868 and the risk and prognosis of HCC. In the present study, we are first to report that *POR*-rs1057868 was significantly associated with OS in HBV-related HCC. The patients with rs1057868 CT or TT genotype had a better OS after hepatectomy compared to those with the CC genotype. However, no significant association was found between *POR*-rs1057868 and RFS. The other *POR* variant rs1135612 is a synonymous mutation (P130P). To date, no study has reported that *POR*-rs1135612 was associated with the activities of *POR* and CYPs or the risk and prognosis of cancer. In the present study, no significant association was found between *POR*-rs1135612 and OS and RFS in HBV-related HCC.

With the hypothesis that the mRNA expression level of *POR* might affect the activity of POR and in turn, the prognosis of HBV-related HCC, we also investigated the association between *POR* mRNA expression and the prognosis of HBV-related HCC using the GSE14520 dataset. We found that HBV-related HCC patients with a high-expression of *POR* have a better OS after hepatectomy than those with low-expression. GSEA is a powerful analytical method for interpreting gene expression data and identifying biological pathways in cancer[24]. In the present study, we also performed a GSEA to identify biological pathways that correlated with HBV-related HCC prognosis. We found that multiple gene sets were significantly correlated with liver cancer survival, recurrence, and HCC development and with the metabolism of multiple substances in biological processes. Therefore, the results of the GSEA suggested that the mRNA expression of *POR* might play an important role in the prognosis of HBV-related HCC.

In the present study, we found that variants *POR*-rs1057868 and mRNA expression were associated with the OS in HBV-related HCC. Nomograms are widely used as prognostic devices in oncology with the ability to generate an individual probability of a clinical event[26]. Considering OS might be affected by genetic and clinicopathological factors, we also developed two novel nomograms to predict OS by enrolling variants *POR*-rs1057868 and mRNA expression combined with clinicopathological factors. It might be useful in the clinic to identify high-risk patients.

This study has some limitations that should be pointed out. First, since the HCC specimens from our cohort were all collected before 2013, the mRNA of these HCC had been degraded. Therefore, we could not evaluate the association of *POR*-rs1057868 and mRNA expression with clinical outcome of HBV-related HCC in the same cohort population. Secondly, the patient's clinical information obtained from the GSE14520 dataset was limited, therefore some factors were missing and some cut-off factors were inconsistent with data from cohort 1. Thirdly, since this is a retrospective study, some postoperative reexaminations and treatment are not controllable. Some patients had not been examined for imaging up until death, and consequently, it was uncertain whether HCC had recurred. Therefore, the recurrence rate of this study was low. Fourth, due to the difficult in HCC tumor tissues, both the results of POR variants and mRNA expression level in HBV-related HCC prognosis were generated from single cohort, and lack of verification cohort. As well as, the present study is a clinical prognosis biomarker investigation

study and bioinformatics analysis study. Therefore, the present study was lack of *in vivo* and *in vitro* functional experiment verification.

Despite these limitations, we are first to report the prognostic application of *POR*-rs1057868 and mRNA expression in patients with HBV-related HCC. In addition, we investigated the potential mechanism of different *POR* mRNA expression levels in HCC prognosis through a GSEA approach. Once these results are confirmed, *POR* may have potential in the clinical application of prognostic monitoring, cancer management, and targeted therapy of HCC.

Conclusions

In summary, our study suggests that *POR*-rs1057868 (A503V) and mRNA expression were significantly associated with OS in HBV-related HCC patients and may serve as potential biomarkers for predicting OS in HBV-related HCC after hepatectomy. We also developed two novel nomograms consisting of traditional clinical parameters and *POR*-rs1057868 or mRNA expression to predict OS in HBV-related HCC. However, additional HBV-related HCC cohorts are needed to verify our results.

Abbreviations

POR: cytochrome p450 oxidoreductase; HCC: hepatocellular carcinoma; HBV: hepatitis B virus; HBsAg: hepatitis B virus surface antigen; PVTT: portal vein tumor thrombus; AFP: alpha-fetoprotein; ALT: alanine aminotransferase; BCLC: Barcelona Clinic Liver Cancer; SNP: single nucleotide polymorphisms; OS: overall survival; RFS: recurrence-free survival; MST: median survival time; MRT: median recurrent time; HR: hazard ratio; CI: confidence interval; MAF: minor allele frequency; HWE: Hardy–Weinberg equilibrium; FDR: false discovery rate; c-index: concordance index; ROC: receiver operating characteristic; AUC: area under curve; GSEA: gene set enrichment analysis.

Supplementary Material

Supplementary tables.
http://www.jcancer.org/v10p1453s1.xlsx

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Authors' Contributions

Ketuan Huang, Xiwen Liao and Tao Peng designed this manuscript; Ketuan Huang, Xiwen Liao, Chuangye Han, Xiangkun Wang, Tingdong Yu, Chengkun Yang, Xiaoguang Liu, Long Yu, Zhiwei Chen, Wei Qin, Guangzhi Zhu, Hao Su, Zhengqian Liu, Xianmin Zeng, Xin Zhou, Sicong Lu, Jianlv Huang, Yu Liang, Zhengtao Liu, Jianlong Deng, Xinping Ye, and Tao Peng conducted the study, collected and analyzed the data. Ketuan Huang wrote and revised the manuscript, Xiwen Liao contributed to the data interpretation, and Tao Peng guided the writing. All authors read and approved the final manuscript.

Ethical approval

The samples from human in this study were obtained in accordance with the ethical standards of the ethics committee of The First Affiliated Hospital of Guangxi Medical University.

Competing Interests

The authors have declared that no competing interest exists.

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