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ORIGINAL RESEARCH

Serum Osteopontin Enhances Hepatocellular Carcinoma Diagnosis and Predicts Anti-PD-L1 Immunotherapy Benefit

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Background: Osteopontin (OPN), a phosphorylated glycoprotein encoded by SPP1, critical in hepatic inflammation and fibrosis, requires further investigation for its role on hepatocellular carcinoma (HCC) and predictive value for anti-programmed cell death ligand 1 (anti-PD-L1) immunotherapy responses.

Methods: Publicly available datasets were utilized to explore OPN expression in HCC. A retrospective cohort study involving 316 participants, recruited from January 2015 to March 2017. Serum OPN levels were measured by enzyme-linked immunosorbent assay. Diagnostic performance was assessed using receiver operating characteristic (ROC) curves, a logistic regression model was developed for early HCC diagnosis. Prospective follow-up was conducted from 2017 to 2024 to evaluate overall survival (OS) and disease-free survival (DFS) using Kaplan-Meier analyses. The survival benefit of anti-PD-L1 immunotherapy for patients with OPN patterns was investigated.

Results: Serum OPN levels were significantly elevated in HCC compared to chronic liver disease and healthy individuals (both $p < 0.001$). The area under the curve (AUC) for OPN was 0.903, with 88.2% sensitivity and 83.3% specificity, significantly superior to AFP alone (AUC: 0.707). A combined diagnostic model integrating OPN with alpha-fetoprotein (AFP) and aspartate aminotransferase (AST) enhanced accuracy further (AUC: 0.941). High OPN levels indicated higher tumor burden and predicted worse clinical outcomes (mean OS: 49.1 vs 75.1 months; mean DFS: 37.7 vs 60.9 months, respectively; both log-rank $p < 0.001$). Anti-PD-L1 immunotherapy significantly prolonged survival (OS: 62.9 vs 38.0 months, p = 0.009; DFS: 48.7 vs 28.6 months, p = 0.033) in patients with OPN high pattern.

Conclusion: Serum OPN demonstrates standalone diagnostic value for HCC and enhances conventional biomarker panels when combined with AFP and AST. OPN high pattern identify patients likely to benefit from anti-PD-L1 immunotherapy, suggesting its dual utility as a diagnostic and predictive biomarker.

Keywords: hepatocellular carcinoma, osteopontin, diagnosis, prognosis, immunotherapy

Introduction

Liver cancer is one of the most prevalent cancers worldwide, ranking as the sixth most commonly diagnosed cancer globally with approximately 865,000 new cases and the third leading cause of cancer-related deaths with around 758,000 fatalities.[1] The incidence and mortality of liver cancer vary significantly across geographic regions, with East Asia, Southeast Asia, and North Africa being high-risk areas. The pathogenesis of liver cancer is influenced by multiple etiological factors, including hepatitis B or C virus (HBV/HCV) infections, chronic exposure to dietary aflatoxin-contaminated foods, excessive alcohol consumption, non-alcoholic fatty liver disease (NAFLD), inherited metabolic disorders, and tobacco use.[2] The risk of developing liver cancer also increases with age and is higher in males than

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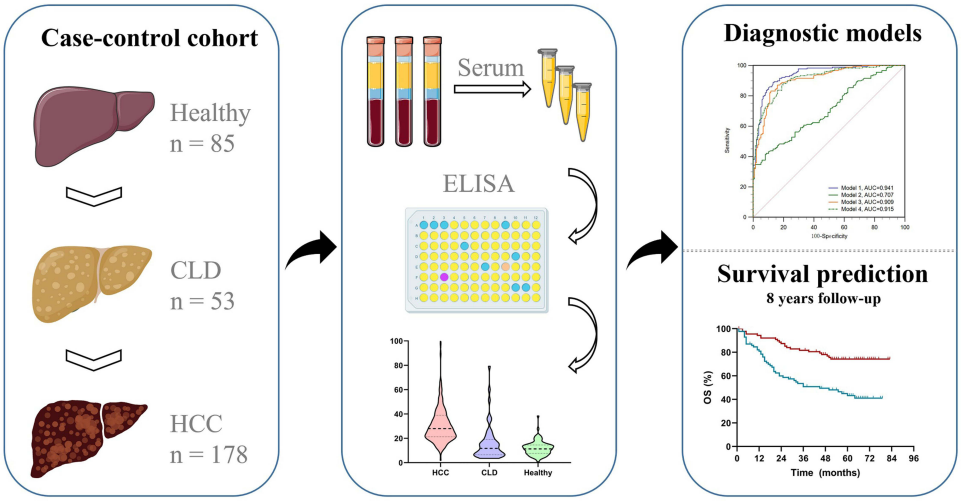
Graphical Abstract



females.[3] HCC, the most prevalent subtype of liver cancer, accounting for 90% of cases.[4] Due to the initial symptoms of HCC being insidious, many patients are in the advanced stage when diagnosed. Accordingly, many medical associations have advocated systematic screening and monitoring in high–risk populations to improve early–stage detection.[5] The current prognosis for advanced HCC remains poor, with mean overall survival ranging from 7.3 to 13.6 months.[6] Currently, AFP is used for screening and early diagnosis of HCC. However, studies have shown that the diagnostic sensitivity of AFP is far from satisfying at the cut–off point of 20 ng/mL.[7] Early-stage HCC often presents with normal or only slightly elevated AFP levels, making it challenging for early detection and intervention. The sensitivity and specificity of AFP can vary depending on ethnicity and age, further complicating its use as a universal diagnostic tool.[8] To improve patient outcomes, novel biomarkers for prognostic prediction, early detection, and accurate diagnosis are imperative.

OPN, encoded by the gene SPP1, is a sialic acid-rich, non-collagenous, chemokine-like, glycosylated phosphoprotein, involved in tumor proliferation, invasion, and metastasis.[9–12] A meta-analysis that included 14 case-control trials reveals that a combination of OPN and AFP improves the diagnostic accuracy of HCC.[13] Moreover, a recent study indicated that serum OPN can predict the response to atezolizumab plus bevacizumab in HCC patients, high OPN levels were associated with poor response.[14] These findings indicated that OPN may serve as a predictive biomarker for therapeutic response. However, it remains unknown whether HCC with different OPN patterns will achieve different benefits from anti-PD-L1 immunotherapy. Therefore, we conducted this comprehensive study to identify biomarkers that are complementary to serum AFP for HCC diagnosis and to investigate whether serum OPN pattern could predict anti-PD-L1 immunotherapy benefit.

Materials and Methods

Analysis of Public Databases

The Tumor Immune Estimation Resource (TIMER 2.0; http://timer.cistrome.org/) online database was utilized to compare SPP1 mRNA expression levels between HCC (LIHC) tissues and adjacent normal liver tissues. Differential expression analysis was performed using RNA-seq data from The Cancer Genome Atlas (TCGA) LIHC cohort. Immunohistochemistry (IHC) staining images of OPN in HCC and normal liver tissues were retrieved from the Human Protein Atlas (HPA; www.proteinatlas.org) online database. Protein expression levels were evaluated based on staining intensity (negative/weak/moderate/strong) and cellular localization. Proteomic data from the Clinical Proteomic Tumor Analysis Consortium (CPTAC; https://proteomics.cancer.gov/programs/cptac) online database was utilized to

quantify OPN protein abundance between HCC and normal tissues. To investigate the transcriptional landscape of hepatic monocyte-derived macrophages (MoMFs) expression in HCC, we analyzed single-cell RNA sequencing (scRNA-seq) datasets from the PanglaoDB database (https://panglaodb.se/); human hepatic macrophages subsets which contained 2768 cells were selected for single-cell analysis.

Study Design and Participants

The study protocol was approved by Sun Yat–sen University Cancer Center research ethics committee and complied with the standards of the Declaration of Helsinki and current ethical guidelines. Overall, this study involved 316 participants between January 2015 and March 2017, which were divided into a case group and a control group. The case group included 178 patients with HCC diagnosed by histological examination for the first hospitalization. The control group included 53 patients with chronic liver disease (CLD) and 85 healthy individuals. Groups were matched for age and sex as far as possible. Chronic liver disease includes chronic hepatitis, cirrhosis without HCC, hepatic cysts, and hepatic hemangioma. Healthy volunteers showed no abnormalities found in laboratory tests or imagological examinations. The HCC was staged using the Barcelona Clinic Liver Cancer (BCLC) staging system.[15] Here, we defined BCLC stage 0 and stage A as early-stage HCC, and the remaining stages as advanced-stage HCC.

The inclusion criteria were as follows: 1) HCC was diagnosed based on ultrasound, computed tomography, or magnetic resonance imaging along with blood test results, and was confirmed by histopathology; 2) before the physical examination, routine blood treatment of patients after admission and a biochemical examination were performed; 3) adequate clinicopathological data. The exclusion criteria were as follows: 1) recurrent or metastatic HCC; 2) the concurrent presence of any other tumors or prior treatments; 3) patients with incomplete clinical data.

End Points and Follow-up

All HCC patients were followed up by telephone and medical records after initial treatment. The endpoints of this study were OS and DFS. OS was defined as the time from diagnosis to death from any cause. DFS was defined as the time from diagnosis to disease recurrence or death from any cause. The follow-up evaluation was performed 1 month after initial treatment, then every 3 months in the first 2 years, and every 3 to 6 months subsequently. Patients were followed up until HCC recurrence, death, or February 1, 2024, whichever occurred first. Patients who dropped out during the follow-up period were considered as censored events.

Testing of Serum Samples

All participants involved have signed informed consent before collecting data and blood samples. All serum samples were collected before any treatments and rapidly frozen at −80°C until use, avoiding repeated freezing and thawing cycles. All serum test data from the laboratory were collected within 3 days prior to the initial treatment. Serum OPN level was measured using an enzyme–linked immunosorbent assay kit (R&D systems, Minneapolis, USA) according to the manufacturer's instructions. The value was set as equal to 121000 ng/mL when the serum AFP concentration exceeds 121000 ng/mL (upper limit of the clinical reportable range). All assays were conducted by two laboratory technicians blinded to clinical data and sample grouping.

Statistical Analysis

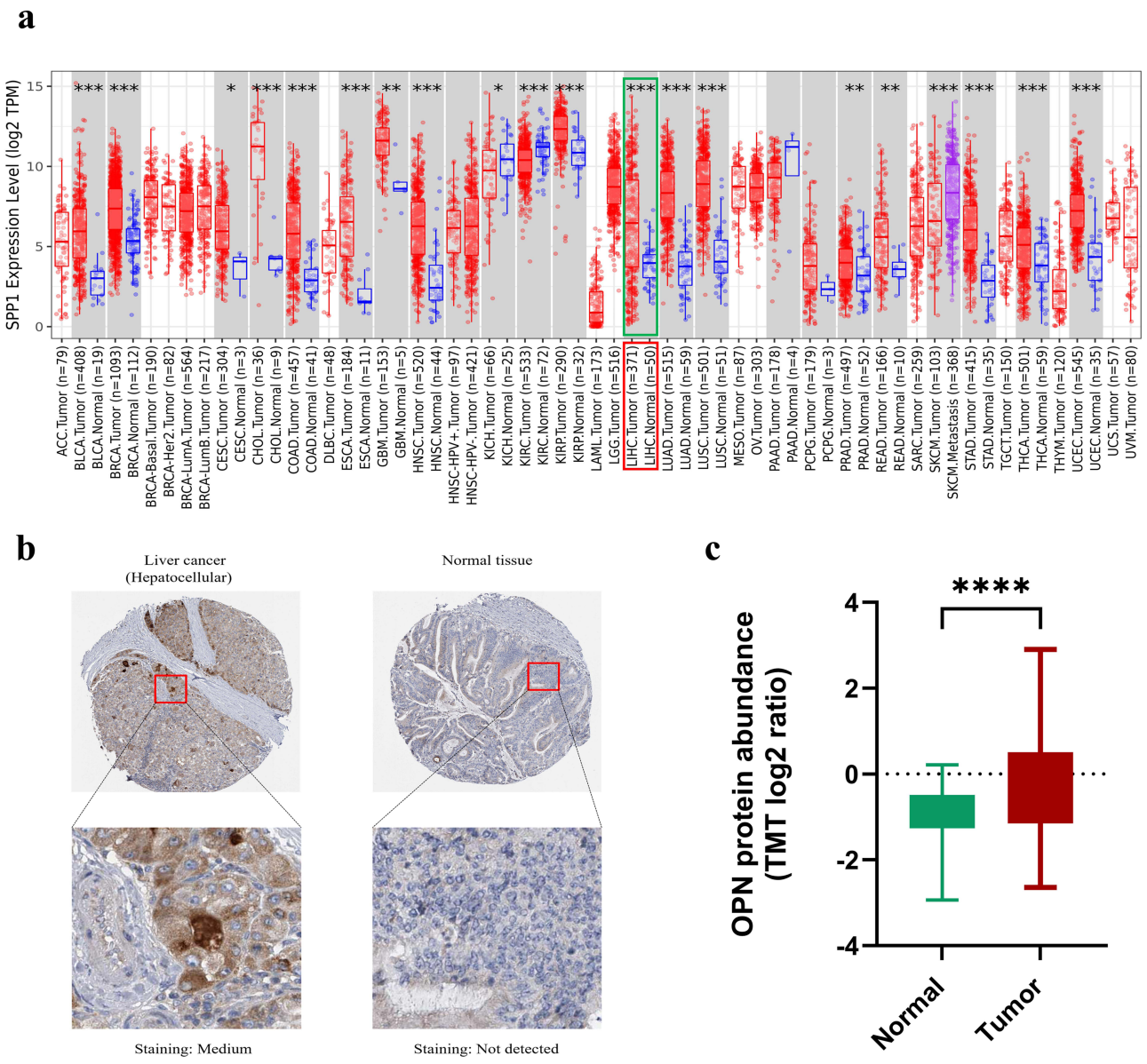
Continuous variables that follow a normal distribution are presented as the mean (standard deviation, SD), while those with a non-normal distribution are presented as the median (interquartile range, IQR). Categorical variables are presented as numbers and percentages. The Fisher's exact test or $\chi 2$ test was used for categorical variables, and Student's *t*-test or analysis of variance (ANOVA) was used for continuous variables. Nonparametric tests, including the Mann–Whitney U and Kruskal–Wallis tests, were used to compare serum biomarker levels and clinical characteristics among groups. A logistic regression model with backward elimination was used to assess the independent discriminative factors for HCC diagnosis. The ROC curve was used to compare the diagnostic performance and obtain the AUC, cutoff values, sensitivity, and specificity. Multivariate cox regression analysis was performed to determine the independent factors significantly associated with survival, while adjusting for potential confounders. A backward stepwise elimination

method, utilizing the "stepAIC" function from the MASS package in R, was then applied to iteratively remove non-significant variables based on the Akaike Information Criterion (AIC), resulting in a parsimonious final multivariate model. Kaplan-Meier analysis, with Log rank tests, was used to evaluate survival differences between groups. Analyses were performed with GraphPad Prism (version 8.0.2, GraphPad Software, Inc., San Diego, CA, USA), MedCalc (version 20.014, MedCalc Software Ltd), and R (version 4.1.0). A two–sided level of $p < 0.05$ was considered statistically significant.

Results

High SPP1 Expression in HCC

We primarily used the TIMER database to evaluate the mRNA expression level of SPP1 in different human cancer and normal tissues. The expression of SPP1 mRNA was significantly elevated in various types of cancer tissues, including liver HCC (LIHC) (Figure 1a). OPN, encoded by the gene SPP1, was found to be higher in HCC than that in adjacent



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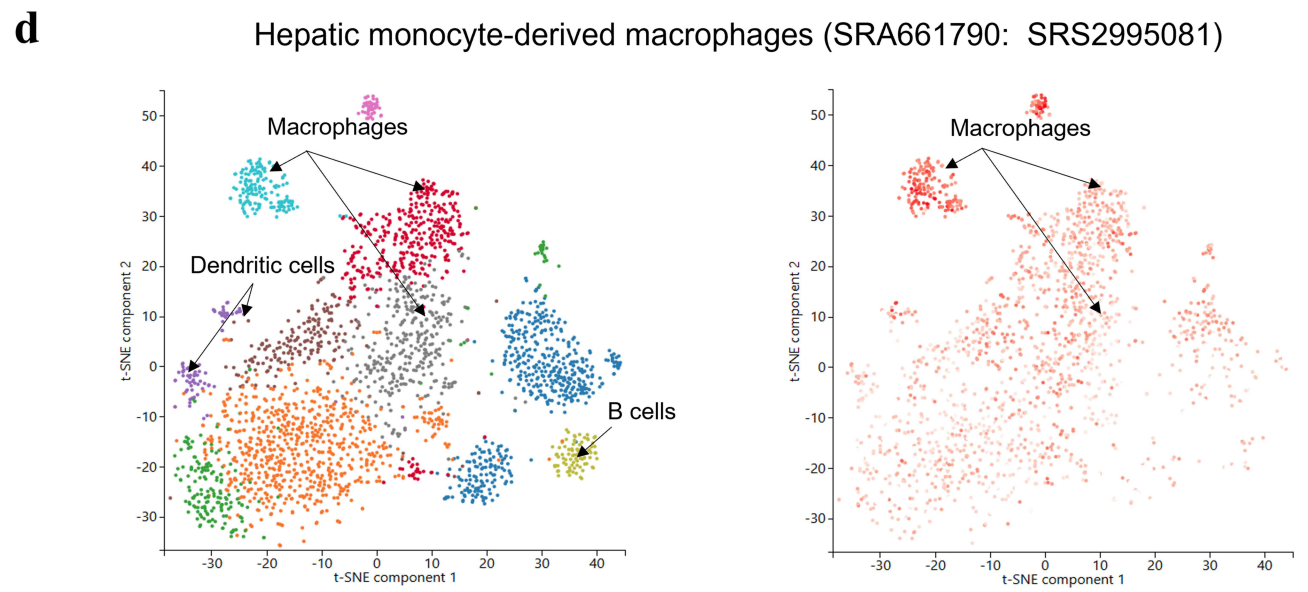


Figure 1 High OPN expression in HCC. (**a**) The mRNA level of SPP1 in different types of tumor tissues and normal tissues in TIMER database. (**b**) Representative images of OPN protein level in HCC detected by IHC staining in HPA database (×400 magnification). (**c**) Comparison of OPN protein expression between HCC and normal tissues in CPTAC database. A two-sided *p* value was calculated using a Student's *t*-test. The box plot indicates the median (center), 25th and 75th percentiles (box boundaries), and the minimum and maximum (whiskers). (**d**) Single-cell RNA sequencing localization analysis of SPP1. (Left) t-SNE plot of three main cell types (Macrophages, dendritic cells, and B cells) in hepatic monocyte-derived macrophages. (Right) SPP1 was enriched in macrophages clusters.

Notes: *$p < 0.1$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Abbreviations: SPP1, secreted phosphoprotein 1; TPM, transcripts per million; ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphoma; ESCA, esophageal carcinoma; GBM, glioblastoma multiforme; HNSC, head and neck squamous cell carcinoma; HPV, human papillomavirus; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM, thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma; TMT, tandem mass tags; OPN, osteopontin; SRA, sequence read archive; SRS, sequence read archive sample; t-SNE, t-distributed stochastic neighbor embedding.

tissues, according to IHC staining data from the HPA database (Figure 1b), which was reconfirmed by data from the CPTAC database (Figure 1c). PanglaoDB dataset was utilized to performed scRNA-seq localization analysis. Hepatic monocyte-derived macrophages were divided into three main cell types (macrophages, dendritic cells, and B cells), then SPP1 was set as overlay expression of gene, and we found that SPP1 was enriched in macrophages clusters according to single-cell analysis (Figure 1d). Together, these results imply that OPN contributes to the development of HCC through oncogenic effects.

Baseline Characteristics of Study Participants

A total of 316 participants were recruited, divided into HCC cohort and control cohort. The HCC cohort recruited 178 primary HCC patients, comprised of 152 males (85.4%) and 26 females (14.6%), with a mean age of 53.4 ± 11.0 years (Supplementary Table 1). The patients were classified as early-stage (stage 0 or A, 55.1%) and advanced-stage (stage B or C, 44.9%) according to BCLC staging system. Among them, there were 51.7% (n = 92) patients underwent hepatectomy during the entire treatment period. The control cohort comprised 53 CLD patients and 85 healthy participants, whose mean age was 48.2 ± 10.9 years and 41.4 ± 12.2 years, respectively.

Serum OPN is Elevated in HCC Patients

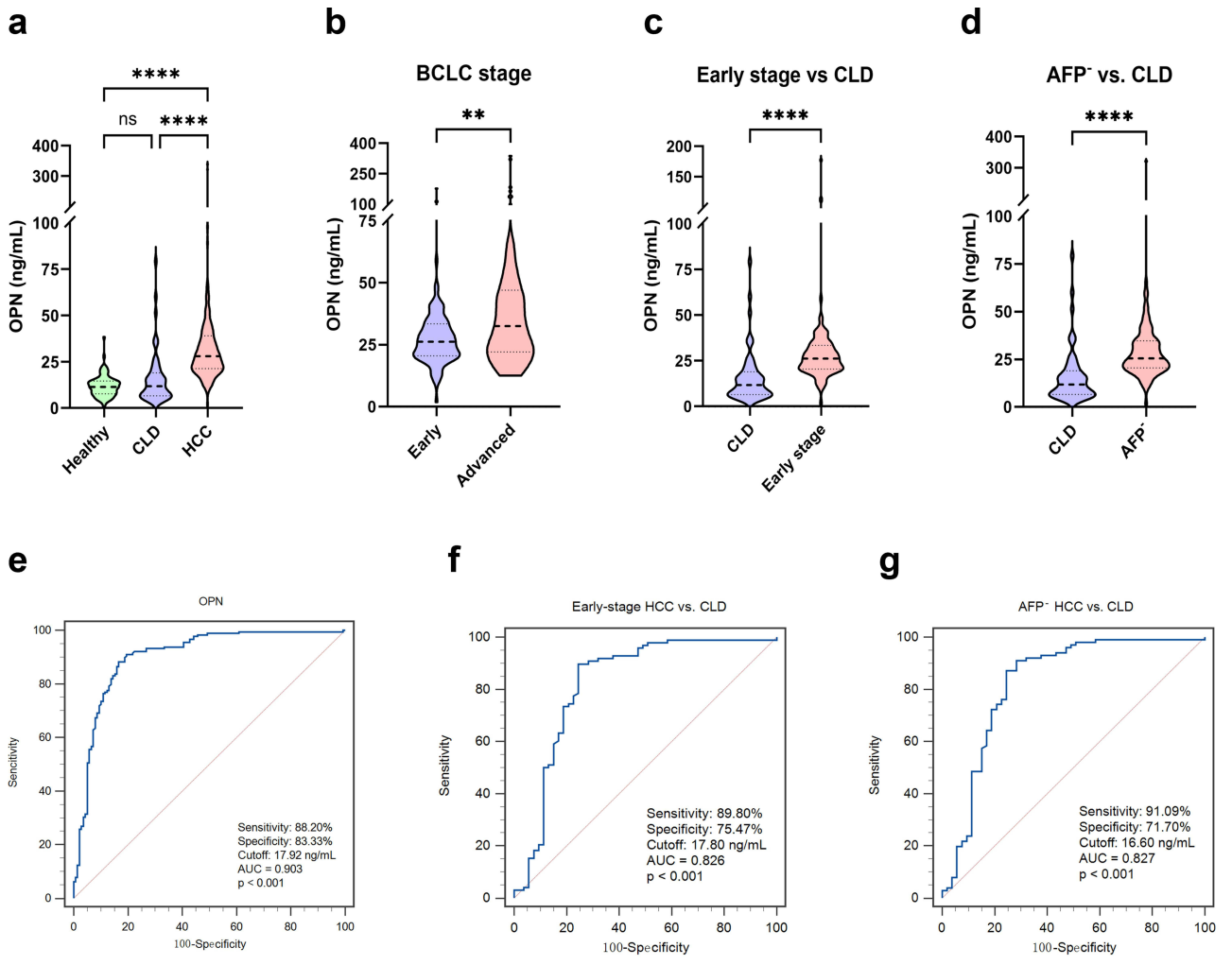
For clinical diagnostic application, we measured the level of serum OPN in HCC patients, CLD patients, and healthy participants before treatments. The median level of serum OPN in HCC patients was 28.02 (range: 21.37, 38.89) ng/mL, which was significantly higher than those in CLD patients (11.80; range: 6.74, 17.80; $p < 0.001$), and healthy participants (11.30; 7.86, 14.50; $p < 0.001$) (Figure 2a). There was no significant difference between CLD patients and healthy

participants. In the subgroup analysis, serum OPN levels were significantly higher in patients with advanced-stage HCC than those with early-stage, as expected (Figure 2b). Additionally, a higher serum OPN level also be observed in early-stage HCC (Figure 2c) and AFP-negative HCC (Figure 2d) patients compared to CLD patients.

ROC Analysis

Next, we conducted ROC analysis of OPN using serum data from 178 HCC patients and 138 controls (Figure 2e). The optimal cut-off value was determined as 17.92 ng/mL using the Youden index, with AUC of 0.903 (95% CI, 0.867–0.940), sensitivity of 88.2%, and specificity of 83.3%. OPN also showed a significant ability to distinguish early-stage HCC from CLD patients (AUC: 0.826; sensitivity: 89.80%; specificity: 75.47%) (Figure 2f), and to distinguish AFP-negative HCC from CLD patients (AUC: 0.827; sensitivity: 91.09%; specificity: 71.70%) (Figure 2g). We next performed logistic regression analysis to calculate the risk score, the waterfall plot was then performed to dichotomize the cases on the basis of their risk scores (Figure 2h–j). In this study, the cut-off value of AFP was set as 20 ng/mL according to clinical guidelines for HCC surveillance.[8] Consequently, a greater proportion of patients with HCC were positive for OPN than for AFP (88.20% versus 56.74%). The positive proportion of OPN combined with AFP increased to 95.51% in the whole HCC cohort. Furthermore, 87.13% of 101 AFP-negative HCC patients, and 89.80% of 98 early-stage HCC patients had positive OPN results (Figure 2k).

To identify the independent diagnostic factors of HCC, interference factors were eliminated and univariate analysis was performed with $p < 0.05$, followed by multivariate binary logistic regression analysis (Supplementary Table 2). Different models



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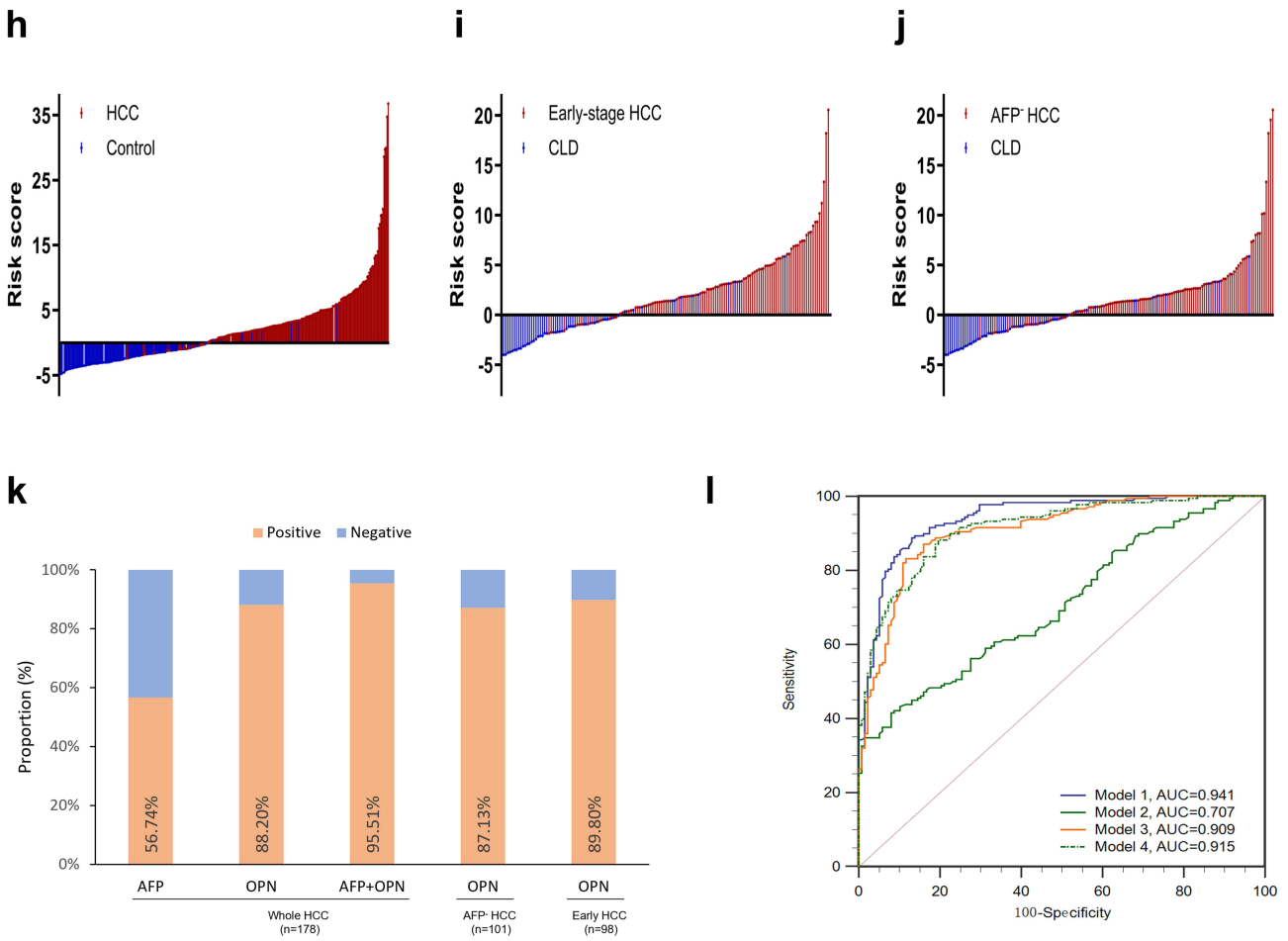

Figure 2 Diagnostic value of serum OPN for HCC. (**a**–**d**) Violin plots of serum OPN levels in healthy participants, CLD patients, and HCC patients (**a**), in early-stage HCC patients and advanced-stage HCC patients (**b**), in CLD patients and early-stage HCC patients (**c**), in CLD patients and AFP-negative HCC patients (**d**). (**e**–**g**) ROC curve analysis reveals the diagnostic potential of serum OPN in 178 HCC patients and 138 controls (**e**), in 98 early-stage HCC patients and 53 chronic liver disease patients (**f**), in 101 AFP-negative HCC patients and 53 chronic liver disease patients (**g**). (**h–j**) The waterfall plot illustrates the risk probability distribution between serum samples of patients with HCC (n = 178) and the controls (n = 138) in the whole cohort (**h**), patients with early-stage HCC (n = 98) and the chronic liver disease (n = 53) (**i**), patients with AFP-negative HCC (n = 101) and the chronic liver disease (n = 53) (**j**). (**k**) The rate of positive results for AFP, OPN, or both, in all patients with HCC, for OPN in AFP-negative HCC, and for OPN in early-stage HCC, respectively. (**l**) Comparison of ROC of four diagnostic models for distinguishing HCC from whole controls.
Notes: $^{**}p < 0.01$; $^{****}p < 0.0001$.
Abbreviations: ns, not significant; OPN, osteopontin; CLD, chronic liver disease; HCC, hepatocellular carcinoma; BCLC, Barcelona clinic liver cancer; AFP, alpha fetoprotein; AUC, area under the curve.

for diagnosing HCC were created by combining basic clinical information such as gender and age, along with common clinical tumor markers like AFP (Table 1), and ROC curves were then plotted (Figure 2L). The results suggested that models with OPN and/or AST (model 1, model 3, and model 4) had better diagnostic accuracy than that without OPN and AST (model 2) (AUC:

Table 1 Diagnostic Performance of Four Diagnostic Models

Models*	AUC	Sensitivity (%)	Specificity (%)	SE	95% CI
Model 1	0.941	88.76	86.96	0.0129	0.915–0.966
Model 2	0.707	41.57	92.03	0.0285	0.651–0.763
Model 3	0.909	83.15	88.41	0.0164	0.876–0.941
Model 4	0.915	88.20	80.43	0.0154	0.884–0.945

Note: *Model descriptions: Model 1 represents gender + age + OPN + AFP + AST; Model 2 represents gender + age + AFP; Model 3 represents gender + age + OPN + AFP; Model 4 represents gender + age + AST + AFP.
Abbreviations: AUC, area under the curve; SE, standard error; CI, confidence interval; OPN, osteopontin; AFP, alpha-fetoprotein; AST, aspartate aminotransferase.

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0.941, 0.909, and 0.915 vs 0.707). The formula of model 1 was: ln(p/1-p) = −7.722+0.885*gender+0.057*age+0.059*OPN +0.682*ln (AFP)+0.076*AST. Variables were coded as follows: sex (0 = female, 1 = male) and age (continuous integer).

Relationship Between Serum Markers and Clinicopathologic Characteristics of HCC

Given the high incidence of HCC resulting from hepatitis and cirrhosis in China, we sought to investigate the relationship between serum OPN levels and serum indicators of liver function and systemic inflammatory response, Spearman's rank correlation coefficient analyses were performed (Figure 3a). The results showed that serum OPN has weak positive correlation with AFP (spearman r = 0.23, $p = 0.002$), and DCP (Spearman r = 0.25, $p < 0.001$), as well as with liver function and inflammatory indicators such as ALT, AST, LDH, CRP, NEU, and FIB. Conversely, serum OPN levels demonstrated a weak negative correlation with ALB (Spearman r = −0.29, $p < 0.001$). Collectively, these results indicated that serum OPN level was correlated with liver function and systemic inflammation. We further evaluated the correlation between OPN and various clinicopathological features of HCC patients. Patients were stratified into high and low groups based on serum OPN median concentration. Our finding indicated that serum OPN was significantly correlated with hepatectomy, alcohol, tumor size, micro-vascular invasion (MVI), BCLC stage, and ALBI grade (Table 2). Together, the elevation of serum OPN levels was correlated with not only liver damage but also tumor progression.

Prognostic Value of Serum OPN in HCC Patients

To explore the independent prognostic factors in HCC patients, univariate and multivariate Cox proportional hazards regression analysis for OS and DFS were shown in Table 3. We identified an optimal cutoff value for OPN at 28.12 ng/mL by R function "surv_cutpoint", which was very close to the median level (28.02 ng/mL) observed in HCC patients (Figure 3b). Consequently, serum level of OPN was stratified into high and low groups based on 28 ng/mL. For OS, the multivariate analysis showed that tumor size ($p = 0.039$), Child-Pugh grade ($p = 0.005$), BCLC stage ($p = 0.028$), hepatectomy ($p = 0.040$), and serum OPN ($p = 0.001$) were independent prognostic parameters (Figure 3c). For DFS, the independent factors were Child-Pugh grade ($p = 0.031$), BCLC stage ($p < 0.001$), and serum OPN ($p = 0.002$) (Figure 3d). Besides, the Sankey plot showed that serum OPN levels of HCC patients were significantly correlated with AFP status, BCLC stage, and survival outcome (Figure 3e and f).

Overall, the median follow-up period was 74 months (95% CI, 72.34–75.66). At the time of last follow-up, there were 55 recurrences and 78 deaths. For the whole HCC cohort, the mean (SD) OS was 62.7 (2.9) months, and the mean (SD) DFS was 49.7 (3.0) months. As shown in Figure 4, patients with low levels of serum OPN had longer OS and DFS than those with high OPN level (mean OS, 75.1 months vs 49.1 months, log-rank $p < 0.001$; mean DFS, 60.9 months vs 37.7 months, log-rank $p < 0.001$) (Figure 4a and b). The 1-year, 3-year, and 5-year OS rates were 92.1% (95% CI, 86.7–97.9), 79.8% (95% CI, 71.9–88.6), and 71.4% (95% CI, 62.5–81.5) in the low OPN group and 78.2% (95% CI, 69.9–87.4), 47.3% (95% CI, 37.8–59.3), and 39.7% (95% CI, 30.5–51.8) in the high OPN group, respectively. During the same follow-up period, the DFS rates at 1-year, 3-year, and 5-year were 78.7% (95% CI, 70.6–87.7), 67.4% (95% CI, 58.3–77.9), and 57.8% (95% CI, 48.3–69.2) in the low OPN group and 63.2% (95% CI, 53.9–74.2), 36.2% (95% CI, 27.3–48.0), and 28.7% (95% CI, 20.5–40.2) in the high OPN group, respectively. Further subgroup analysis showed that patients with high OPN levels had shorter OS in subgroups regarding early-stage, AFP-negative, Child-Pugh grade A, hepatectomy, tumor size ≤5 cm, MVI absent, and ALBI grade 1 (all $p < 0.05$) (Figure 4c–i).

Serum OPN is a Predictor of Anti-PD-L1 Immunotherapy Benefit in HCC Patients

In the whole HCC cohort, 58 patients received anti-PD-L1 immunotherapy. To analyze the clinical benefit of immunotherapy in HCC patients with OPN high or low pattern, we compared patients' OS and DFS, respectively. For patients with OPN high pattern, anti-PD-L1 immunotherapy significantly improved the OS (mean OS: 62.9 vs 38.0 months, $p = 0.009$, HR = 0.467; Figure 5a) and DFS (mean DFS: 48.7 vs 28.6 months, $p = 0.033$, HR = 0.586; Figure 5b). However, patients with OPN low pattern did not derive significant survival benefits from anti-PD-L1 immunotherapy (mean OS: 67.2 vs 77.8 months, $p = 0.136$, HR = 1.840; mean DFS: 53.1 vs 63.3 months, $p = 0.347$, HR = 1.370) (Figure 5c and d). For patients received anti-PD-L1 immunotherapy, no significant differences were observed between OPN high and low

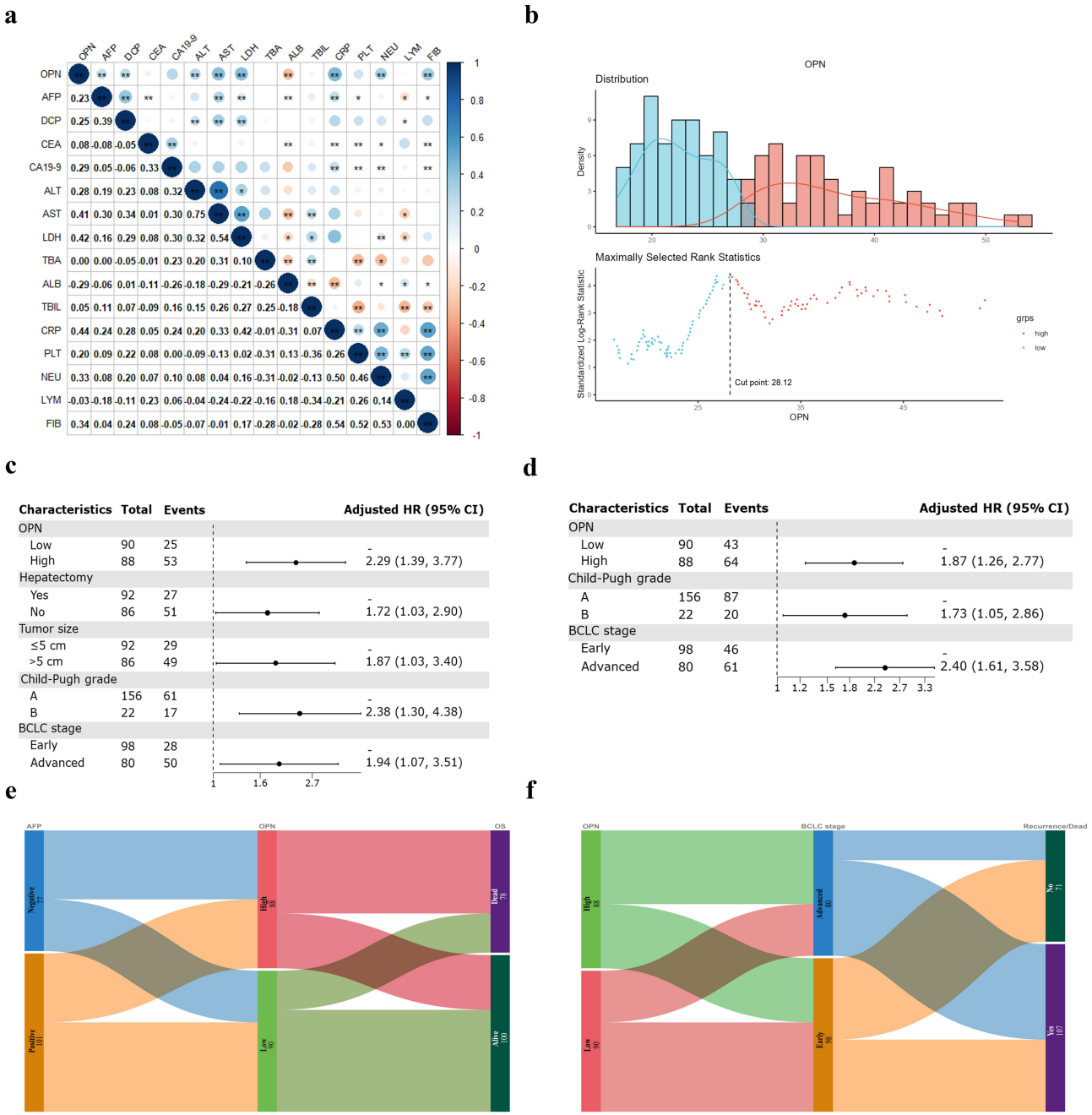

Figure 3 Serum OPN as an independent predictor for HCC survival. (**a**) The inter-relationship between each clinical parameters in HCC patients. Values denote the Pearson correlation coefficients; values closer to 1 indicate a better correlation (*p < 0.05; **p < 0.001). (**b**) Optimal cutoff value of serum OPN was determined by the "surv_cutpoint" function. (**c**) Forest plot of multivariate Cox regression analysis showing serum OPN level, hepatectomy, tumor size, Child-Pugh grade, and BCLC stage as independent prognostic factors for OS. (**d**) Forest plot of multivariate Cox regression analysis showing serum OPN level, Child-Pugh grade, and BCLC stage as independent prognostic factors for DFS. (**e**) Alluvial diagram showing the interrelationship between AFP status, OPN groups, and OS status in HCC. (**f**) Alluvial diagram showing the interrelationship between OPN groups, BCLC stage, and recurrence status in HCC patients.

Abbreviations: OPN, osteopontin; AFP, alpha fetoprotein; DCP, des-γ-carboxy-prothrombin; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19–9; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; TBA, total bile acids; ALB, albumin; TBIL, total bilirubin; CRP, C-reactive protein; PLT, platelet count; NEU, neutrophils; LYM, lymphocytes; FIB, fibrinogen; grps, groups; BCLC, Barcelona clinic liver cancer; HR, hazard ratio; CI, confidence interval; OS, overall survival.

pattern regarding OS (mean OS: 62.9 vs 67.2 months, p = 0.666, HR = 1.200; Figure 5e) and DFS (mean DFS: 42.0 vs 44.0 months, p = 0.570, HR = 1.220; Figure 5f). In contrast, OPN high pattern exhibited a significantly poorer OS and DFS in patients did not receive anti-PD-L1 immunotherapy (mean OS: 38.0 vs 77.8 months, p < 0.001, HR = 4.450; mean DFS: 28.6 vs 63.3 months, p < 0.001, HR = 2.860) (Figure 5g and h). Together, these results suggested that OPN

Table 2 Correlation Between Serum OPN and Clinicopathologic Factors in HCC Patients

Characteristic	Overall (n = 178)	OPN Group		*p*-value*
		High (n = 88)	Low (n = 90)	
AFP				0.073
≤ 20	101 (56.7%)	44 (50.0%)	57 (63.3%)	
> 20	77 (43.3%)	44 (50.0%)	33 (36.7%)	
DCP				0.447
≤ 40	47 (26.4%)	21 (23.9%)	21 (23.9%)	
> 40	131 (73.6%)	67 (76.1%)	67 (76.1%)	
Gender				0.717
Male	152 (85.4%)	76 (86.4%)	76 (84.4%)	
Female	26 (14.6%)	12 (13.6%)	14 (15.6%)	
Age				0.274
> 60	50 (28.1%)	28 (31.8%)	22 (24.4%)	
≤ 60	128 (71.9%)	60 (68.2%)	68 (75.6%)	
Hepatectomy				0.025
Yes	92 (51.7%)	38 (43.2%)	54 (60.0%)	
No	86 (48.3%)	50 (56.8%)	36 (40.0%)	
Cigarette				0.052
Yes	86 (48.3%)	49 (55.7%)	37 (41.1%)	
No	92 (51.7%)	39 (44.3%)	53 (58.9%)	
Alcohol				0.040
Yes	54 (30.3%)	33 (37.5%)	21 (23.3%)	
No	124 (69.7%)	55 (62.5%)	69 (76.7%)	
Family history				0.372
No	145 (81.5%)	74 (84.1%)	71 (78.9%)	
Yes	33 (18.5%)	14 (15.9%)	19 (21.1%)	
Ascites				0.568
Absent	162 (91.0%)	79 (89.8%)	83 (92.2%)	
Present	16 (9.0%)	9 (10.2%)	7 (7.8%)	
PHT				0.439
Present	46 (25.8%)	25 (28.4%)	21 (23.3%)	
Absent	132 (74.2%)	63 (71.6%)	69 (76.7%)	
Tumor number				0.136
Multiple	77 (43.3%)	43 (48.9%)	34 (37.8%)	
Single	101 (56.7%)	45 (51.1%)	56 (62.2%)	
Tumor size				<0.001
≤5 cm	92 (51.7%)	34 (38.6%)	58 (64.4%)	
>5 cm	86 (48.3%)	54 (61.4%)	32 (35.6%)	
MVI				0.009
Present	62 (34.8%)	39 (44.3%)	23 (25.6%)	
Absent	116 (65.2%)	49 (55.7%)	67 (74.4%)	
Child-Pugh grade				0.333
A	156 (87.6%)	75 (85.2%)	81 (90.0%)	
B	22 (12.4%)	13 (14.8%)	9 (10.0%)	
Cirrhosis				0.130
Absent	65 (36.5%)	37 (42.0%)	28 (31.1%)	
Present	113 (63.5%)	51 (58.0%)	62 (68.9%)	
BCLC stage				0.025
Advanced	80 (44.9%)	47 (53.4%)	33 (36.7%)	
Early	98 (55.1%)	41 (46.6%)	57 (63.3%)	

(*Continued*)

Table 2 (Continued).

Characteristic	Overall (n = 178)	OPN Group		*p*-value*
		High (n = 88)	Low (n = 90)	
HBsAg				0.467
Negative	23 (12.9%)	13 (14.8%)	10 (11.1%)	
Positive	155 (87.1%)	75 (85.2%)	80 (88.9%)	
ALBI grade				0.007
I	133 (74.7%)	58 (65.9%)	75 (83.3%)	
2–3	45 (25.3%)	30 (34.1%)	15 (16.7%)	

Note: *Pearson's Chi-squared test; Fisher's exact test.
Abbreviations: OPN, osteopontin; HCC, hepatocellular carcinoma; AFP, alpha fetoprotein; DCP, des-γ-carboxy-prothrombin; PHT, portal hypertension; MVI, microvascular invasion; BCLC, Barcelona clinic liver cancer; HBsAg, hepatitis B virus surface antigen; ALBI, albumin-bilirubin.

high pattern may predict enhanced anti-PD-L1 immunotherapy benefit, whereas low OPN subgroups showed no significant improvement. However, these findings require validation in larger prospective cohorts due to the limited sample size.

Discussion

Non-invasive markers with high sensitivity to discriminate HCC patients and non-HCC patients may be valuable tools for screening high-risk populations, rather than liver biopsy. Our results indicated that OPN exhibited superior diagnostic value for HCC better than that of AFP, especially for patients with AFP-negative status and early-stage HCC. However, Simao et al indicated that no significant differences in plasma OPN levels were observed between cirrhotic patients with and without HCC (AUC: 0.51; 95% CI, 0.39 to 0.63), showing that AFP was superior to OPN.[16] On the contrary, in a pilot study, Sufen et al identified OPN as a diagnostic biomarker with better performance in HCC than AFP, especially in AFP negative (< 20 ng/mL) HCC group.[17] This can be explained by the fact that OPN has a complex carcinogenesis, which may be affected by many factors such as ethnic variation and etiological differences.

AST is mainly derived from the mitochondria of hepatocytes, as levels increase with hepatocyte damage. In human tissues, both cytoplasmic and mitochondrial AST proteins consist of a compartment-specific homodimer encoded by two separate genes, GOT1 and GOT2, respectively.[18] According to current opinion, mitochondria play a fundamental role on all steps of oncogenesis, including malignant transformation, tumor progression, therapeutic responses, and immunosurveillance.[19] Our study validated that AST levels are strongly linked to the occurrence of HCC, regardless of hepatitis virus status, which is aligning with previous evidence.[20,21] Our study position OPN as a candidate first-line screening tool that could complement role of AFP in confirming diagnosis. Herein, we developed a multivariate logistic regression model incorporating sex, age, OPN, AFP, and AST, which demonstrated superior diagnostic performance compared to AFP alone. The model assigns weighted contributions to each variable based on regression coefficients, reflecting their independent diagnostic significance.

The mortality of HCC is mainly due to high recurrence rate even patients have undergone surgical resection.[22] Biomarkers that can predict recurrence of HCC are extremely beneficial for making clinical strategies and establishing follow-up procedures. Notably, beyond diagnostic accuracy, this study also demonstrated that OPN can serve as an independent prognostic factor on OS and DFS for HCC patients. However, the reasons why high OPN serum level are associated with tumor recurrence have not yet been clarified. Sun et al revealed that up-regulated expression of OPN is regulated by exosome S100A4 through activating the STAT3 signaling pathway, which predicted a poorer prognosis.[23,24] Similar results were also observed by Dong et al that OPN expression was regulated by genetic variation at locus −443 of the OPN promoter, which was significantly associated with overall survival and time to recurrence.[25] The current study revealed that elevated serum OPN levels were associated with larger tumor size, MVI, advanced BCLC stage, and ALBI

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Table 3 Univariate and Multivariate Cox Proportional Hazards Regression of Prognostic Factors on Overall Survival and Disease-Free Survival

Variables	Overall Survival				Disease-free Survival			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Gender (male vs female)	1.08 (0.57–2.04)	0.822			1.14 (0.66–1.96)	0.647		
Age (years; >60 vs ≤60)	0.97 (0.59–1.58)	0.896			0.82 (0.53–1.26)	0.363		
Cigarette (yes vs no)	1.17 (0.75–1.83)	0.485			1.02 (0.70–1.49)	0.927		
Alcohol (yes vs no)	1.27 (0.79–2.02)	0.322			1.00 (0.66–1.52)	0.993		
Family history of cancer (yes vs no)	0.69 (0.37–1.27)	0.234			0.73 (0.44–1.21)	0.227		
Ascites (yes vs no)	1.51 (0.73–3.14)	0.270			1.66 (0.91–3.02)	0.101		
PHT (present vs absent)	1.90 (1.20–3.03)	0.007			1.89 (1.27–2.83)	0.002		
No. of tumors (multiple vs single)	2.00 (1.28–3.13)	0.002			2.20 (1.50–3.24)	<0.001		
Tumor size (cm; >5 vs ≤5)	2.75 (1.74–4.37)	<0.001	1.87 (1.03–3.40)	0.039	2.11 (1.44–3.10)	<0.001		
MVI (present vs absent)	1.91 (1.22–3.00)	0.004			1.55 (1.05–2.29)	0.027		
Child-Pugh grade (B vs A)	3.04 (1.77–5.22)	<0.001	2.38 (1.30–4.38)	0.005	2.41 (1.48–3.94)	<0.001	1.73 (1.05–2.86)	0.031
Liver cirrhosis (present vs absence)	0.79 (0.50–1.25)	0.321			1.10 (0.74–1.64)	0.648		
BCLC stage (advanced vs early)	3.53 (2.21–5.63)	<0.001	1.94 (1.07–3.51)	0.028	2.81 (1.91–4.15)	<0.001	2.40 (1.61–3.58)	<0.001
Hepatectomy (no vs yes)	2.82 (1.77–4.50)	<0.001	1.72 (1.03–2.90)	0.040	1.99 (1.35–2.93)	<0.001		
HBsAg (positive vs negative)	0.77 (0.41–1.42)	0.402			0.83 (0.48–1.44)	0.518		
ALBI score (grade 1 vs grade 2/3)	2.31 (1.45–3.67)	<0.001			1.74 (1.16–2.61)	0.008		
OPN (ng/mL; >28 vs ≤28)	2.96 (1.84–4.78)	<0.001	2.29 (1.39–3.77)	0.001	2.14 (1.45–3.16)	<0.001	1.87 (1.26–2.77)	0.002
AFP (ng/mL; ≥20 vs <20)	0.73 (0.47–1.14)	0.166			1.05 (0.72–1.54)	0.798		
DCP (mAU/mL; >40 vs ≤40)	1.66 (0.96–2.88)	0.071			1.24 (0.80–1.92)	0.334		

Abbreviations: PHT, portal hypertension; No., number; MVI, microvascular invasion; BCLC, Barcelona clinic liver cancer; HBsAg, hepatitis B virus surface antigen; ALBI, albumin-bilirubin; OPN, osteopontin; AFP, α-fetoprotein; DCP, des-gamma-carboxy prothrombin; HR, hazard ratio; CI, confidence interval.

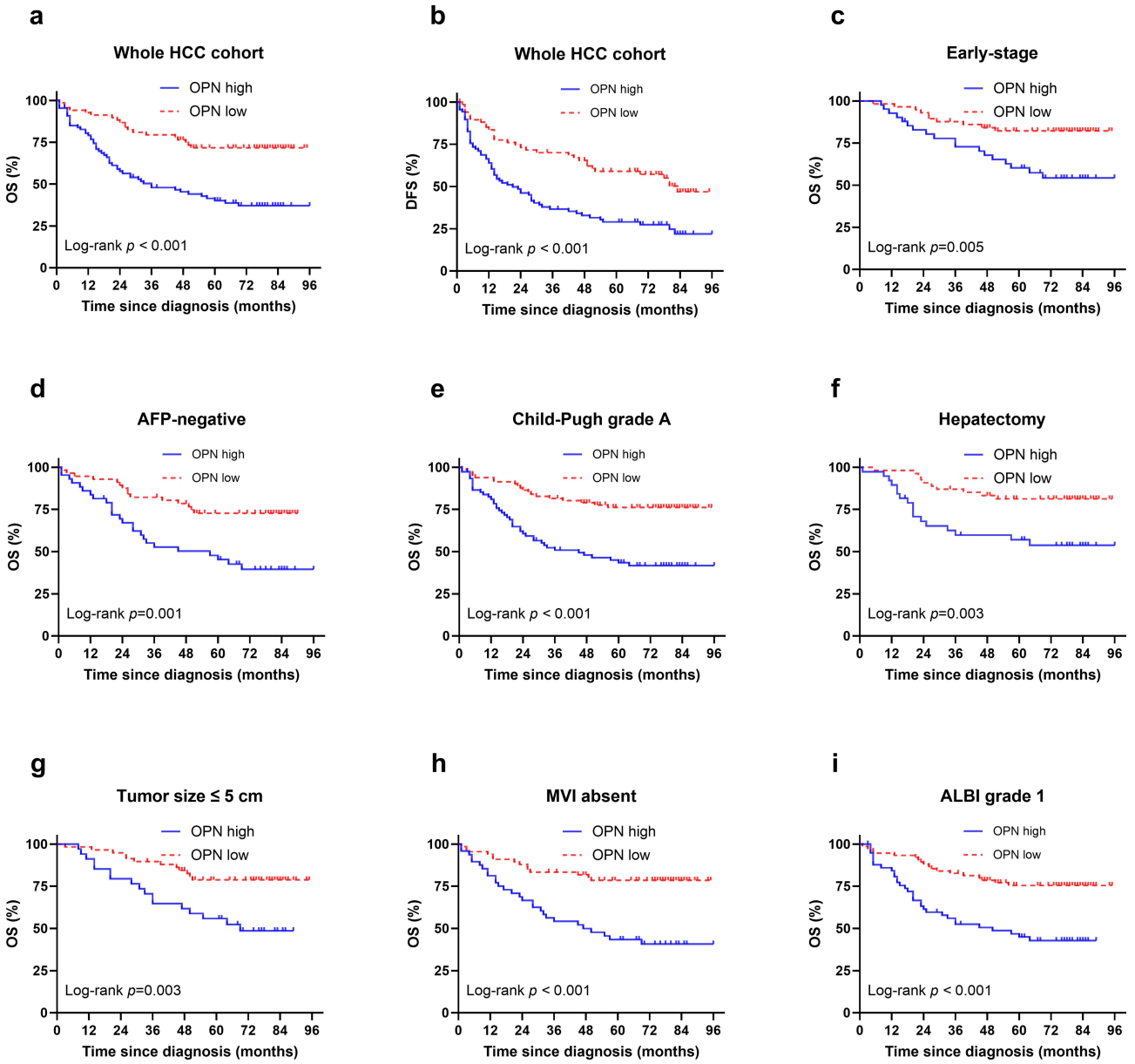

Figure 4 Prognostic value of OPN in patients with HCC. (**a** and **b**) In the whole HCC cohort, patients with low levels of serum OPN had longer overall survival (**a**) and disease-free survival (b). (**c**–**i**) The prognostic value of serum OPN in different subgroups regarding overall survival, including early-stage (**c**), AFP-negative (**d**), Child-Pugh grade A (**e**), hepatectomy (**f**), tumor size ≤ 5 cm (**g**), MVI absent (**h**), ALBI grade 1 (**i**).

Abbreviations: OS, overall survival; HCC, hepatocellular carcinoma; OPN, osteopontin; DFS, disease-free survival; AFP, alpha fetoprotein; MVI, microvascular invasion; ALBI, albumin-bilirubin.

grade 2–3, which confirms from a certain perspective that OPN is involved in regulating HCC metastasis through signaling pathway.

Immunotherapy, such as immune checkpoint inhibitors (ICIs), remains the most widely systemic drug for advanced HCC during the past decades.[26] Notably, the atezolizumab and bevacizumab combination has demonstrated superior OS rates compared to sorafenib, leading to its FDA approval.[27] The combination of atezolizumab and bevacizumab has shown better results than sorafenib in patients with advanced-stage HCC, establishing a new first-line median OS duration of 19 months.[28] However, the response to immunotherapy appears to be moderate, as evidenced by the results of the CheckMate 040 (clinical trial number: NCT01658878) and IMbrave150 trials (clinical trial number: NCT03434379).[28,29] The heterogeneity of HCC and the lack of robust biomarkers for predicting response to immunotherapy pose significant challenges in personalized treatment strategies.[30] There have been multiple other studies

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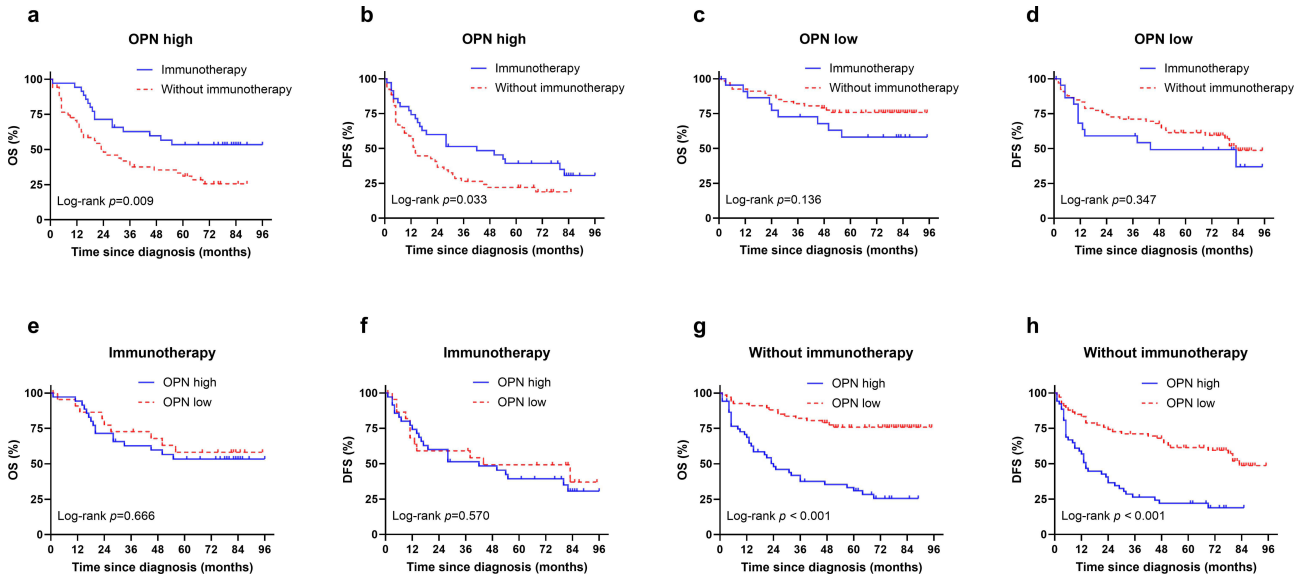

Figure 5 Serum OPN is a predictor of anti-PD-L1 immunotherapy benefit in patients with HCC. (**a** and **b**) Immunotherapy significantly prolonged the OS (**a**) and DFS (**b**) of HCC patients with high OPN levels. (**c** and **d**) HCC patients with low OPN levels did not derived a significant survival benefit from immunotherapy. (**e** and f) OS (**e**) and DFS (**f**) of OPN high and OPN low patients in the immunotherapy group were not significantly different. (**g** and **h**) OPN high patients exhibited a significantly shorter OS (**g**) and DFS (**h**) than OPN low patients in the group without immunotherapy.
Abbreviations: OS, overall survival; OPN, osteopontin; DFS, disease-free survival.

indicated that gut microbiome, macrophage-related gene signature affect the response to anti-PD-1 immunotherapy in HCC patients.[31,32] However, no optimal serum biomarkers are available clinically to predict responses to immunotherapy in HCC patients.[33] This current study indicated that serum OPN could be a predictor of immunotherapy benefit in HCC patients, that is, HCC patients with high OPN pattern indicated markedly improved survival and decrease death risk after anti-PD-L1 immunotherapy, whereas patients with low OPN pattern did not derive significant survival benefits from anti-PD-L1 immunotherapy. Notably, no significant differences were observed between OPN high and low pattern in patients who received anti-PD-L1 immunotherapy, and in contrast, OPN high pattern exhibited a significantly poorer OS and DFS in patients did not receive anti-PD-L1 immunotherapy. However, due to the limited cohort size, this finding needs validation in larger prospective studies. Collectively, our results suggest serum OPN may help stratify HCC patients for further investigation of anti-PD-L1 immunotherapy benefit, particularly in high-OPN subgroups. Prospective validation is required to confirm its predictive utility. This could lead to a more personalized approach to treatment, improving outcomes for patients with high OPN levels.

While our study provides valuable insights into the potential of OPN as a biomarker for HCC, several limitations should be acknowledged. First, as a secretory phosphoprotein, OPN elevation is not specific to HCC and may occur in other malignancies or inflammatory conditions.[34–36] Therefore, interpreting elevated OPN levels requires careful integration with clinical and imaging findings. Second, the etiology of HCC in this Chinese cohort (predominantly HBV-related) differs from Western populations where metabolic dysfunction-associated steatotic liver disease (MASLD) and HCV are major drivers, potentially limiting the generalizability of our findings. Third, the retrospective design of this study, despite incorporating prospective follow-up for survival outcomes, may introduce selection bias inherent to analyses of pre-existing cohorts. Fourth, the single-center design necessitates external validation in multi-institutional and ethnically diverse populations to establish standardized OPN cutoff thresholds for clinical use. Finally, while we identified OPN's prognostic significance, the molecular mechanisms underlying its role in HCC progression and immunotherapy resistance remain uncharacterized.

Despite above limitations, this study contributes novel perspectives to the field. Specifically, we demonstrated that serum OPN levels serve as an independent diagnostic and prognostic marker in treatment-naïve HCC patients, supported by the longest follow-up duration (8 years) reported to date in OPN-related HCC studies. Furthermore, this is the first study to systematically evaluate a combined diagnostic model integrating OPN with AFP and AST, two routinely used

biomarkers, achieving superior accuracy (AUC: 0.941) compared to AFP alone (AUC: 0.707). This approach aligns with clinical demands to enhance existing biomarker panels without additional diagnostic burdens. Additionally, our exploratory analysis of anti-PD-L1 immunotherapy outcomes suggests OPN's potential as a predictive biomarker for treatment benefit, laying groundwork for future mechanistic investigations.

Future research should prioritize three directions: First, prospective multi-center studies to validate OPN-guided risk stratification across diverse etiologies; second, functional experiments to elucidate OPN-mediated mechanisms; third, interventional trials assessing whether OPN-targeted therapies can synergize with immunotherapy in high-OPN subgroups. Clinically, patients with elevated OPN levels could be classified as higher-risk, prompting more aggressive therapy or increased monitoring to address their poorer prognosis.

Data Sharing Statement

The authenticity of this article was validated by uploading the key raw data onto the Research Data Deposit public platform (www.researchdata.org.cn) with the approval RDD number RDDB2023282509. All data included in this study are available from the corresponding author, Dr. Linfang Li, upon reasonable request.

Ethics Approval Statement

Our study was conducted in concordance with the principles of the Declaration of Helsinki, as approved by the Institutional Ethical Board of Sun Yat-sen University Cancer Center (Approval number: B2023-600-Y01).

Generative AI Statement

The authors declare that no Generative AI was used in the creation of this manuscript.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

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