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Original Article

Potential Role and Clinical Value of PPP2CA in Hepatocellular Carcinoma

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Abstract

Background and Aims: Protein phosphatase 2A (PP2A) is associated with many cancers. This study aimed to clarify whether PPP2CA, which encodes the alpha isoform of the catalytic subunit of PP2A, plays a role in hepatocellular carcinoma (HCC) and to identify the potential underlying molecular pathways. ***Methods:*** Based on bioinformatics, public databases and our in-house RNA-Seq database, we analyzed the clinical value and molecular mechanism of PPP2CA in HCC. ***Results:*** Data were analyzed from 2,545 patients with HCC and 1,993 controls without HCC indexed in The Cancer Genome Atlas database, the Gene Expression Omnibus database and our in-house RNA-Seq database. PPP2CA expression was significantly higher in HCC tissue than in non-cancerous tissues (standardized mean difference: 0.69, 95% confidence interval [CI]: 0.50–0.89). PPP2CA expression was able to differentiate HCC from non-HCC, with an area under the summary receiver operator characteristic curve of 0.79 (95% CI: 0.75–0.83). Immunohistochemistry of tissue sections confirmed that PPP2CA protein was up-regulated in HCC tissues. High PPP2CA expression in HCC patients was associated with shorter overall, progression-free and disease-free survival. Potential molecular pathways through which PPP2CA may be involved in HCC were determined using miRWalk 2.0 as well as analysis of Gene Ontology categories, Kyoto Encyclopedia of Genes and Genomes pathways, and protein-protein interaction networks. ***Conclusions:*** PPP2CA is up-regulated in HCC and higher expression correlates with worse prognosis. PPP2CA shows potential as a diagnostic marker for HCC. Future studies should examine whether PPP2CA contributes to HCC through the candidate microRNAs, pathways and hub genes identified in this study.

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Introduction

Many patients with hepatocellular carcinoma (HCC) are diagnosed when the disease is already advanced, resulting in poor prognosis. HCC involves the actions and interactions of numerous genes,[1,2] and increasingly powerful bioinformatics tools and datasets, such as the Gene Expression Omnibus (GEO) and the Cancer Genome Atlas (TCGA), have facilitated explorations of how specific proteins and pathways contribute to the disease. This may accelerate new discoveries about the disease and its treatment.

Protein phosphatase 2A (PP2A), a serine/threonine phosphatase highly conserved among eukaryotes, is associated with many cancers. The heterotrimeric enzyme comprises a backbone subunit A, regulatory subunit B, and catalytic subunit C.[3] The C subunit occurs as two isoforms, PPP2CA or PPP2CB, and the former is approximately 10 times more abundant than the latter.[4,5] Few studies have examined the potential involvement of either isozyme in HCC.

In the present study, bioinformatics and public databases were used to compare PPP2CA expression between HCC and non-cancerous tissues. The results were verified against our in-house RNA-Seq database and immunohistochemistry of our archived tissue samples. We compared survival between patients showing low or high PPP2CA expression, and we used various bioinformatics programs to explore molecular pathways through which the protein may

Keywords: Hepatocellular carcinoma; PPP2CA; Molecular mechanism; Diagnostic marker.

Abbreviations: DFS, disease-free survival; GEO, Gene Expression Omnibus; GEPIA2, Gene Expression Profiling Interactive Analysis; GO, Gene Ontology; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; KEGG, Kyoto Encyclopedia of Genes and Genomes; OS, overall survival; PFS, progression-free survival; PPI, protein-protein interaction; PP2A, Protein phosphatase 2A; sROC, Summary receiver operating characteristic; TCGA, The Cancer Genome Atlas.

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contribute to HCC.

Methods

Data collection

GEO database searching: We searched the GEO database (www.ncbi.nlm.gov/geo) through 30 September 2019 for microarrays related to HCC.[6] The search string was *(malignan* OR neoplas* OR cancer OR tumour OR tumor OR carcinoma) AND (hepatocellular OR hepatic OR liver).* The specific search strategy is shown (Supplementary Fig. 1). Array data were filtered according to pre-set inclusion and exclusion criteria. The inclusion conditions were as follows: (1) cancer and adjacent liver tissue samples obtained from HCC patients; (2) each dataset included HCC tissue diagnosed based on pathology as well as adjacent non-cancerous tissue (or healthy liver tissue); and (3) data on PPP2CA mRNA levels available. Data were excluded from: (1) HCC tissues of patients who had not been diagnosed based on pathology; (2) human blood, cell lines or animal models; (3) patients for whom only cancerous tissue or adjacent non-cancerous tissues were available; (4) HCC patients who had received chemotherapy, radiotherapy, interventional treatment or immunotherapy; (5) cholangiocarcinoma or mixed liver cancer; and (6) poor-quality samples.

TCGA data on 361 samples and 50 normal tissues: RNA-Seq data in the liver hepatocellular carcinoma (known as LIHC) database were downloaded on October 3, 2019 from the TCGA database (https://portal.gdc.cancer.gov/), hosted by the University of California Santa Cruz (https://xena.ucsc.edu/). Patients who had been diagnosed with fibrolamellar carcinoma or mixed liver cancer based on pathology were excluded.

The forest plot of subgroup meta-analysis for PPP2CA expression between HCC and control tissues, based on random-effect meta-analysis. The forest plot of PPP2CA expression for alcohol, hepatitis B virus (HBV), hepatitis C virus (HCV) and other subgroups in TCGA, GSE62232 and in-house RNA-Seq datasets.

Data extraction

Two researchers (JYL and XQ) checked and then extracted data on levels of PPP2CA mRNA from microarrays and the TCGA database, together with relevant clinicodemographic information. Considering that the levels of PPP2CA mRNA may be affected by etiology of HCC, all extracted data underwent second extraction. HCC was divided into HBV-related HCC, alcohol-related HCC, HCV-related HCC and other HCC. Datasets with samples greater than 50 and etiological information were extracted for subgroup analysis. Any disagreements were resolved by discussion, mediated by a third researcher (CLY).

Meta-analysis of PPP2CA mRNA levels

Data on PPP2CA mRNA levels were $\log_2$-transformed (X+0.001) and meta-analyzed using Stata/SE 15.1. The standardized mean difference and corresponding 95% confidence interval (CI) between HCC tissues and control tissues were determined. The robustness of the meta-analysis was assessed by performing sensitivity analysis. Heterogeneity was assessed using the chi-squared-based Q test and the I^2 test, and it was considered significant if $p<0.05$ or $I^2>50\%$. Risk of publication bias was assessed using Egger's and Begg's tests. PPP2CA mRNA data of different types of HCC were analyzed in the same way.

Clinical significance of PPP2CA in HCC

The expression of PPP2CA was imported into SPSS software (IBM Corp., Armonk, NY, USA) to calculate the number of true positives, true negatives, false positives, and false negatives. The potential diagnostic value of PPP2CA expression was assessed in terms of the area under a summary ROC curve (AUC) drawn using Stata/SE 15.1 (College Station, TX, USA). An AUC >0.7 was considered to indicate appreciable discriminatory ability.

Potential relationships between PPP2CA expression and clinicopathological parameters of HCC were assessed based on clinical information from the TCGA database. After selecting appropriate cut-off values, we compared the overall survival (OS) and progression-free survival (referred to as PFS) of patients showing high or low PPP2CA expression using the Kaplan-Meier plotter (http://kmplot.com/analysis/index.php?p=service).[7] OS and disease-free survival (DFS) were also examined based on Gene Expression Profiling Interactive Analysis (GEPIA2; http://gepia.cancer-pku.cn/).[8]

Exploration of potential pathways of PPP2CA involvement in HCC

We predicted microRNAs (miRNAs) that may target PPP2CA using miRWalk2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/genepub.html), a comprehensive atlas of predicted and validated miRNA-target interactions. Only miRNAs predicted by more than nine of the twelve algorithms in that software were considered in the present study.

Using GEPIA2, we explored genes whose expression may correlate with that of PPP2CA. Using DAVID (http://david.abcc.ncifcrf.gov/), we examined candidate co-expressed genes for enrichment in Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) biological processes, molecular functions and cellular components. The results of GO and KEGG analyses were depicted in bubble charts, plotted by R version 4.0.2. A protein-protein interaction (PPI) network was constructed using the online STRING database (https://string-db.org/). The expression of hub genes extracted from the PPI network was explored using data from the TCGA database.

Tissue samples and follow-up

To complement our analyses of data from public databases, we performed RNA sequencing and immunohistochemistry of samples of HCC tissues and matched non-tumor tissues from patients treated in the Department of Hepatobiliary Surgery of Guangxi Medical University Cancer Hospital between July 2017 and July 2019. All procedures involving patient samples were approved by the Ethics Committee of Guangxi Medical University Cancer Hospital, and written informed consent was obtained from all patients.

To be included in the study, samples had to be from patients who (1) underwent radical hepatocarcinoma resection, (2) had been definitively diagnosed with HCC based on postoperative pathology, (3) had not received any anticancer treatment before surgery, and (4) did not have any malignancies in addition to HCC.

All patients were followed up in the hospital for the first month after surgery. If there was no recurrence or death,

follow-up was repeated every 3 months during 2 years. Follow-up tests included physical examination, serum alpha-fetoprotein test, serum liver function test, abdominal ultrasonography and computed tomography/magnetic resonance imaging. DFS and OS were defined as the time from the day of surgery to the discovery of tumor recurrence or last follow-up, which was July 2020.

RNA-sequencing

Total RNA was extracted from HCC and matched non-tumor tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA purification, reverse transcription, library preparation and sequencing were performed by Wuxi NextCODE (Shanghai, China). RNA-Seq libraries were inspected for quality using FastQC, and the reads were compared against the reference human genome Hg19 using Salmon.[9]

Immunohistochemistry

Specimens of HCC and matched non-tumor tissues were fixed with 10% formalin solution, embedded in paraffin, and cut into sections approximately 4 μm thick. Sections were deparaffinized in xylene at 37°C, rinsed in a graded ethanol series, incubated in 10 mmol/L citrate buffer (pH 6.0) for antigen retrieval, rinsed in phosphate-buffered saline (PBS), incubated in 0.3% H_2O_2 for 10 min at room temperature to inhibit endogenous peroxidases, then rinsed again with PBS. Sections were incubated at 37°C for 1 h with anti-PPP2CA antibody (1:1,000) (ab106262; Abcam, Cambridge, UK), then thoroughly rinsed in PBS. Sections were incubated with MaxVision™/horseradish peroxidase at room temperature for 30 m, washed with PBS, stained with diaminobenzidene and hematoxylin. Sections were dehydrated through a graded ethanol series, allowed to dry, and sealed with neutral gum. In parallel, retinal tissue was processed as a positive control, while HCC tissue was processed with PBS instead of primary antibody to serve as a negative control.

Immunohistochemical staining was semi-quantified using a two-component score. First, staining intensity was assigned 0 points (uncolored), 1 point (light yellow), 2 points (yellow) or 3 points (dark yellow). Second, the percentage of total observed cells that were stained was assigned 1 point (≤25%), 2 points (26–50%), 3 points (51–75%) or 4 points (>75%). The two scores were multiplied together to obtain an overall score of 0–12 points. Overall scores of 0–4 were considered "low PPP2CA expression", while scores of 5–12 were considered "high expression". All sections were assessed independently by two senior pathologists blinded to tissue data. Disagreements were re-assessed.

Statistical analysis

Statistical analyses were carried out using SPSS 25.0 software. Parametric data were presented as mean±standard deviation. Differences between groups were assessed for significance using Student's *t*-test. Relationships between PPP2CA and clinicopathological parameters were analyzed using Student's *t*-test, chi-squared test or Fisher's exact test, as appropriate. The optimal cut-off for binary outcomes was obtained using the pROC package in R version 4.0.2, and patients were stratified by high or low PPP2CA expression based on RNA-Seq data. OS and DFS were analyzed using the Kaplan-Meier method, and curves were compared using the log-rank test in R. Differences associated with $p<0.05$ were considered significant.

Results

PPP2CA mRNA levels in HCC and non-HCC tissues based on GEO and TCGA data

After rigorous screening and evaluation by three researchers, 41 GEO datasets were included (Table 1). Among these, 35 datasets showed higher PPP2CA expression in the HCC group than in the non-HCC group, but only 22 datasets had statistical significance. In another six datasets with lower PPP2CA expression, only one dataset showed a significant difference between the HCC group and non-HCC group.

We collected data on 361 HCC tissues and 50 adjacent non-cancerous tissues from the TCGA database. PPP2CA expression tended to be higher in HCC tissues (11.388±0.360 vs. 11.328±0.306), but the difference was not significant ($p=0.265$).

Meta-analysis of PPP2CA expression based on TCGA, GEO and RNA-Seq databases

We performed a comprehensive meta-analysis based on TCGA, GEO and RNA-Seq data from 2,545 HCC cases and 1,993 non-cancerous cases. PPP2CA expression was higher in HCC tissues than in normal tissues (Fig. 1): the pooled standardized mean difference was 0.69 (95% CI: 0.50 to 0.89, $p<0.001$) based on the random-effects model, with $I^2=86.8\%$ ($p<0.001$). Risk of publication bias was low according to Begg's test ($p=0.530$) and Egger's test ($p=0.305$). Sensitivity analysis did not clearly identify individual studies that contributed to the heterogeneity (Supplementary Fig. 2), suggesting that the results were robust. Subgroup meta-analysis was conducted based on GSE62232, TCGA and RNA-Seq data. The subgroup meta-analysis results showed that PPP2CA expression was higher in HBV-related HCC tissues than normal tissues (Fig. 2A). However, in other subgroups, there were no statistical differences for PPP2CA expression between HCC tissues and normal tissues. The result indicated that PPP2CA may be closely related with HBV-related HCC. However, the heterogeneity of the HBV-related HCC subgroup was still high. After deleting the HBV-related samples of the TCGA dataset, the heterogeneity dropped to 0 (Fig. 2B), indicating that the heterogeneity came from the TCGA dataset.

Clinical usefulness of PPP2CA in HCC

Rates of true positives, false positives, false negatives, and true negatives were calculated for each study in the TCGA, GEO and RNA-Seq datasets (Table 1). PPP2CA showed strong diagnostic potential for HCC (AUC: 0.79, 95% CI: 0.75–0.83; Fig. 3).

In the TCGA dataset, PPP2CA expression was significantly associated with chronic HBV infection ($p=0.041$) but not other clinicopathologic parameters (Supplementary Table 2). Kaplan-Meier analysis indicated that PPP2CA expression above an optimal cut-off was associated with significantly shorter OS ($p<0.001$) and PFS ($p=0.039$). GEPIA2 analysis also indicated that high expression was associated with significantly shorter OS ($p=0.025$), and it tended to be associated with shorter DFS ($p=0.05$) (Fig. 4A–D). These results suggest that extremely high PPP2CA expression may have a

negative impact on HCC survival.

Validation of the clinical significance of PPP2CA using RNA-Seq data

PPP2CA expression was significantly higher in HCC tissues (17.655±6.247) than in adjacent tissues (12.309±2.916, $p<0.001$). Based on a cut-off of 17.664, patients with high PPP2CA expression showed significantly shorter OS ($p=0.0065$) and DFS ($p=0.0045$) than patients with low expression (Fig. 4E, F). Patients with high PPP2CA expression were significantly younger than those with low expression ($p=0.010$) and showed significantly higher prevalence of type 2 diabetes ($p=0.014$), HBV infection ($p=0.046$), gross vascular invasion ($p=0.013$), microvascular invasion ($p=0.019$) and tumor recurrence ($p=0.007$) (Table 1). In addition, patients with high expression showed significantly more advanced Barcelona Clinic Liver Cancer stage ($p=0.039$). Multivariate cox analysis showed that PPP2CA may not be an independent prognostic factor ($p>0.05$).

Validation of the clinical significance of PPP2CA using immunohistochemistry

Samples from 123 HCC patients were included in the immunohistochemistry analysis, comprising 104 men and 19 women with a median age of 51.3±11.2 years. PPP2CA was expressed mainly in the cytoplasm. It was expressed strongly in some HCC tissues but weakly in adjacent tissues. In 107 adjacent tissues, 9 were PPP2CA-negative, 79 weakly positive, 19 moderately positive and 0 strongly positive. We classified 88 cases (82.2%) as showing low PPP2CA expression, and 21 cases (17.8%) as showing high expression. In 123 HCC tissues, 45 were negative, 39 weakly positive, 28 moderately positive and 11 strongly positive (Fig. 5 and Supplementary Table 3). We classified 84 cases (68.3%) as showing low expression and 39 cases (31.7%) as showing high expression. PPP2CA expression was significantly higher in HCC tissues than in adjacent tissues ($\chi^2=5.905$, $p=0.015$; Table 2).

Prediction of miRNAs targeting PPP2CA

We predicted miRNAs that may target the PPP2CA mRNA using miRWalk 2.0. At least nine of the prediction algorithms in the suite identified the following candidate miRNAs: miR-139-5p, miR-141-3p, miR-548o-3p, and miR-200a-3p. We found that miR-548o-3p was expressed at significantly higher levels ($p=0.003$), while miR-139-5p and miR-200a-3p were expressed at significantly lower levels ($p<0.001$) in HCC tissues than in normal liver tissues (Supplementary Fig. 3). In contrast, levels of miR-141-3p did not differ significantly between HCC and normal liver tissues.

GO and KEGG pathway analysis

We analyzed the enrichment of GO and KEGG pathways among 200 genes co-expressed with the PPP2CA gene (Supplementary Fig. 4A–D). The most important GO biological process was vesicle-mediated transport from the endoplasmic reticulum to the Golgi. The most important cellular component was cytoplasm. The most important molecular function was nucleic acid binding. Of the 7 KEGG pathways identified, the most important was the spliceosome, followed by RNA transport.

PPI network of PPP2CA-associated genes

The results of the PPI network generated using the online STRING database showed relationships between PPP2CA and the top 150 selected co-expressed genes (Supplementary Fig. 4E). SKIV2L2, HNRNPK and ABCE1 were extracted as hub genes showing no fewer than 31 edges. The TCGA database showed that SKIV2L2 was significantly up-regulated and ABCE1 significantly down-regulated in HCC tissues compared with normal liver tissues ($p<0.001$). Expression of HNRNPK, in contrast, did not differ significantly between HCC and normal liver tissues ($p=0.079$; Supplementary Fig. 5).

Discussion

PPP2CA encodes the alpha isoform of the catalytic subunit of PP2A, which regulates PP2A activity by selecting PP2A regulatory subunits.[10–12] Down-regulation of PPP2CA leads to lower PP2A activity.[13] Lower levels of PPP2CA have been associated with gastric cancer, such as higher risk of gastric cancer among Chinese,[14] as well as with colorectal cancer and poor prognosis in that disease.[15] Down-regulation of PPP2CA has also been associated with anaplastic thyroid carcinoma (referred to as ATC), and its levels negatively correlate with those of miR-650, which targets PPP2CA in ATC cells.[16] Conversely, PPP2CA overexpression reduces the invasion and metastasis ability of prostate cancer cells.[17]

In other cancers, PPP2CA seems to be overexpressed rather than down-regulated. Up-regulation of PPP2CA in breast cancer is associated with poor prognosis,[18] and up-regulation in malignant osteosarcoma can promote cell proliferation and migration.[19] Thus, PPP2CA expression is altered in different ways in different cancers, suggesting multiple mechanisms of action. This may help explain why previous studies have not clarified whether PP2A promotes or suppresses HCC.[20,21] Another explanation is that many studies of the enzyme in HCC have not taken into account which C subunit isoforms are involved. Our preliminary data suggest that the expression levels of PPP2CA and PPP2CB may be inversely related (data not shown), highlighting the importance of clarifying the specific roles of each C subunit isoform in HCC.

Meta-analysis of 2,545 HCC tissues and 1,993 non-cancerous tissues in the GEO, TCGA and our in-house databases showed that PPP2CA expression was significantly higher in HCC tissues than in non-cancerous tissues. The heterogeneity of this pooled result was high, and sensitivity analysis did not identify any single study that accounted for most of it. In order to further explore the relationship between the expression of HCC with different etiologies and PPP2CA and the source of heterogeneity, we selected datasets with samples greater than 50, which contained specific etiology information of HCC for subgroup analysis. The results showed that the high expression of PPP2CA was related to HBV-related HCC, while it was not related with alcohol- or HCV-related HCC. PPP2CA expression may be related to HBV infection as we found here in our analysis of data and as other studies have reported,[22,23] and it may be related to hepatitis C virus infection,[24] but it may not be associated with alcoholic hepatitis. The subgroup meta-analysis showed that PPP2CA expression was not related to HCV-related HCC. It may also be due to the small sample size. This result needs to be further explored. In terms of heterogeneity, after the HBV-related TCGA dataset was excluded, the heterogeneity of the four subgroups was reduced to 0, while the overall heterogeneity was still high. Thus, we considered that the heterogeneity came from different etiology of HCC and HBV-related TCGA data.

Table 1. Relationships between PPP2CA expression based on RNA-Seq and clinicopathologic parameters of our in-house patient sample

Clinicopathologic parameter	n	PPP2CA expression		*p*
		Low (*n*=52)	High (*n*=64)	
PPP2CA expression	116	12.633.41	22.264.21	<0.001*
Age in years				0.010*
<60	85	32	53	
≥60	31	20	11	
Sex				0.337
Male	101	5	10	
Female	15	47	54	
Hypertension				0.319
Yes	18	10	8	
No	98	42	56	
Type 2 diabetes				0.014*
Yes	7	0	7	
No	109	52	57	
Barcelona Clinic Liver Cancer stage				0.039*
A	58	29	29	
B	32	17	15	
C	26	6	20	
Hepatitis B virus infection				0.046*
Yes	96	39	57	
No	20	13	7	
Hepatitis C virus infection				0.114
Yes	3	0	3	
No	113	52	61	
Alpha-fetoprotein, ng/mL				0.484
<400	71	30	41	
≥400	45	22	23	
Gross vascular invasion				0.013*
Yes	23	5	18	
No	93	47	46	
Cirrhosis				0.230
Yes	54	21	33	
No	62	31	31	
Pathology grade				0.191
I–II	59	30	29	
III–IV	56	21	35	
Microvascular invasion				0.019*
Yes	71	26	45	
No	44	24	18	
Recurrence				0.007*
yes	63	21	42	
no	53	31	22	

*$p<0.05$.

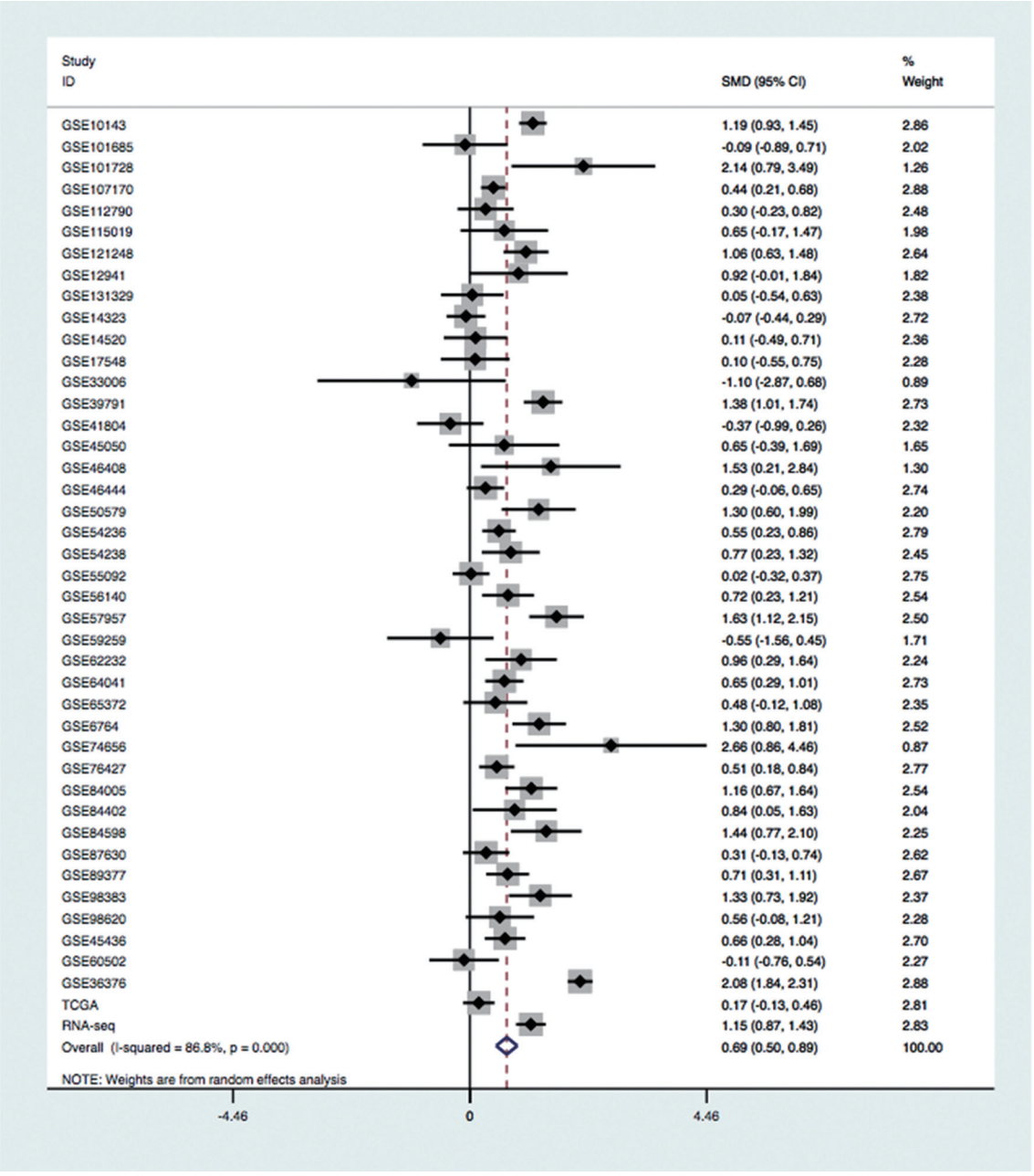

Fig. 1. Forest plot of the pooled standardized mean difference (SMD) for PPP2CA expression between HCC and control tissues, based on random-effect meta-analysis. HCC, hepatocellular carcinoma.

We found evidence that PPP2CA expression shows diagnostic potential in HCC. In addition, high PPP2CA expression in HCC was associated with poor OS, PFS and DFS. Nevertheless, our results should be interpreted cautiously because our follow-up was shorter than that in the TCGA database. We performed a multivariate analysis, which showed that the PPP2CA gene may not be an independent risk factor for the poor prognosis of HCC. Gong *et al*.[23] reported that PP2Ac expression plays a role in HCC tumorigenesis induced by HBV X protein, and PP2Ac protein overexpression is an independent predictor of poor OS of HCC patients. It can be seen that there are obvious differences between the two, which further proves the necessity of studying different subtypes of PP2Ac protein. We confirmed here with immunohistochemistry that PPP2CA protein is overexpressed in HCC tissues. These results may improve the diagnosis of HCC and prediction of prognosis. However, the most ideal biomarkers would be non-invasive. We look forward to seeing more ideal reports of PPP2CA in the peripheral blood of HCC patients in the future.

We identified miR-139-5p, miR-200a-3p, miR-548o-3p, and miR-141-3p as potential miRNAs targeting PPP2CA. The first two were expressed at lower levels in HCC tissues than in normal liver tissues, while the third showed the inverse pattern. Consistent with our results, other studies showed down-regulation of miR-139-5p in HCC samples.[25,26] Its low

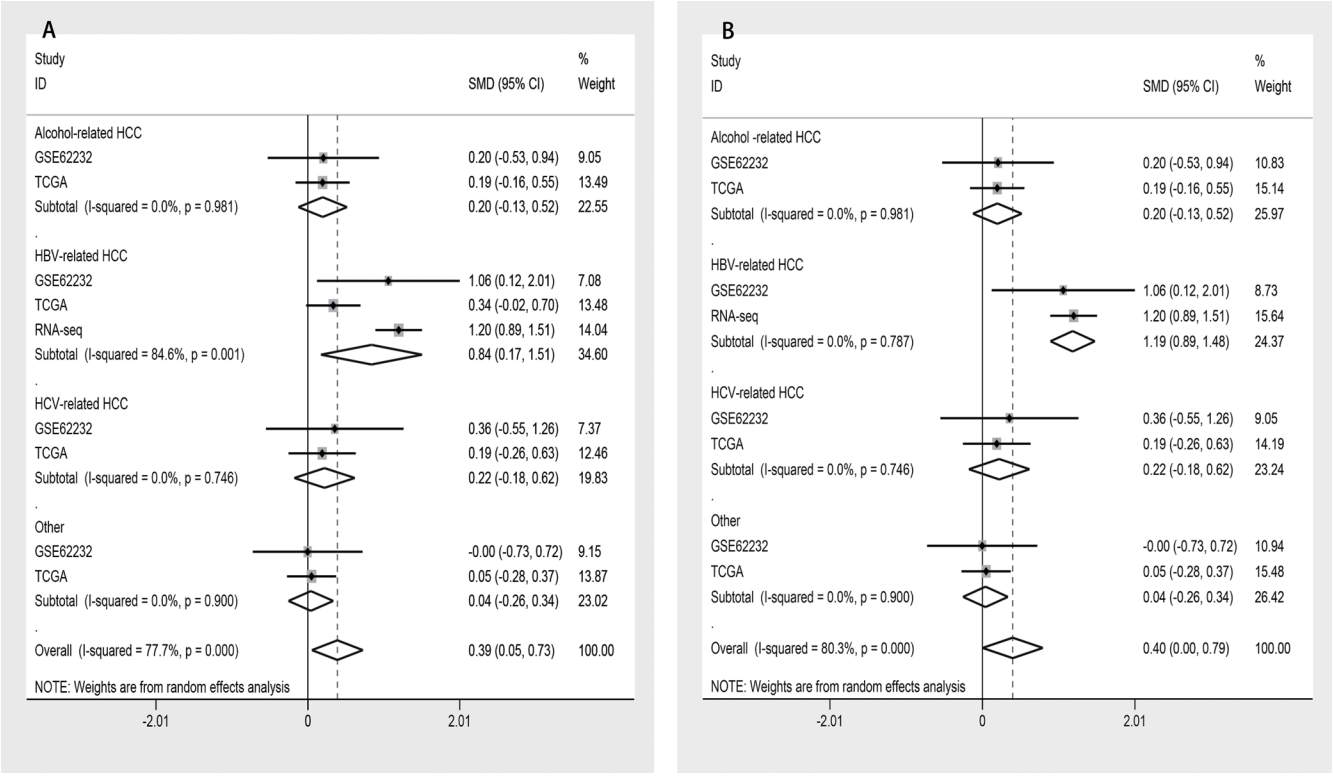

Fig. 2. The subgroup meta-analysis of PPP2CA expression levels between cancerous tissues and non-cancerous tissues in HCC. (A) Forest plot of the PPP2CA expression levels based on TCGA, GSE62232 and in-house RNA-Seq datasets. (B) Forest plot of the PPP2CA expression levels after eliminating HBV-related HCC considered to be sources of heterogeneity. HCC, hepatocellular carcinoma; TCGA, The Cancer Genome Atlas; HBV, hepatitis B virus.

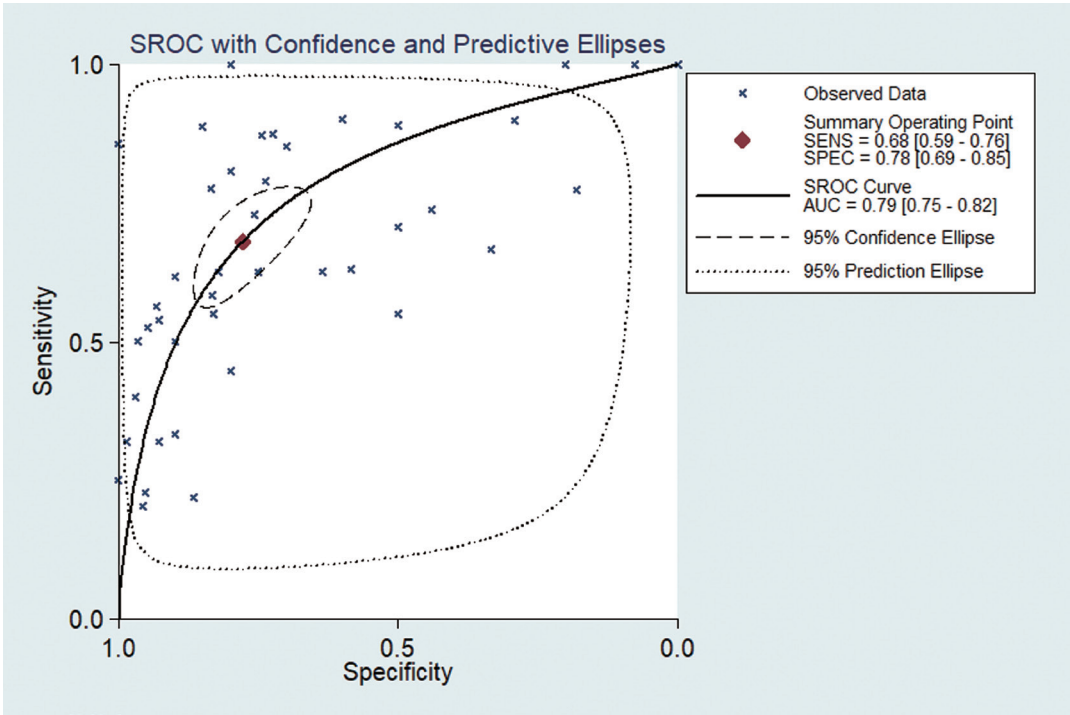

Fig. 3. Summary receiver operating characteristic (sROC) curve assessing the ability of PPP2CA expression to diagnose HCC, based on data from GEO microarrays and the TCGA database. The y-axis represents specificity, and the x-axis represents sensitivity. HCC, hepatocellular carcinoma; GEO, Gene Expression Omnibus; TCGA, The Cancer Genome Atlas.

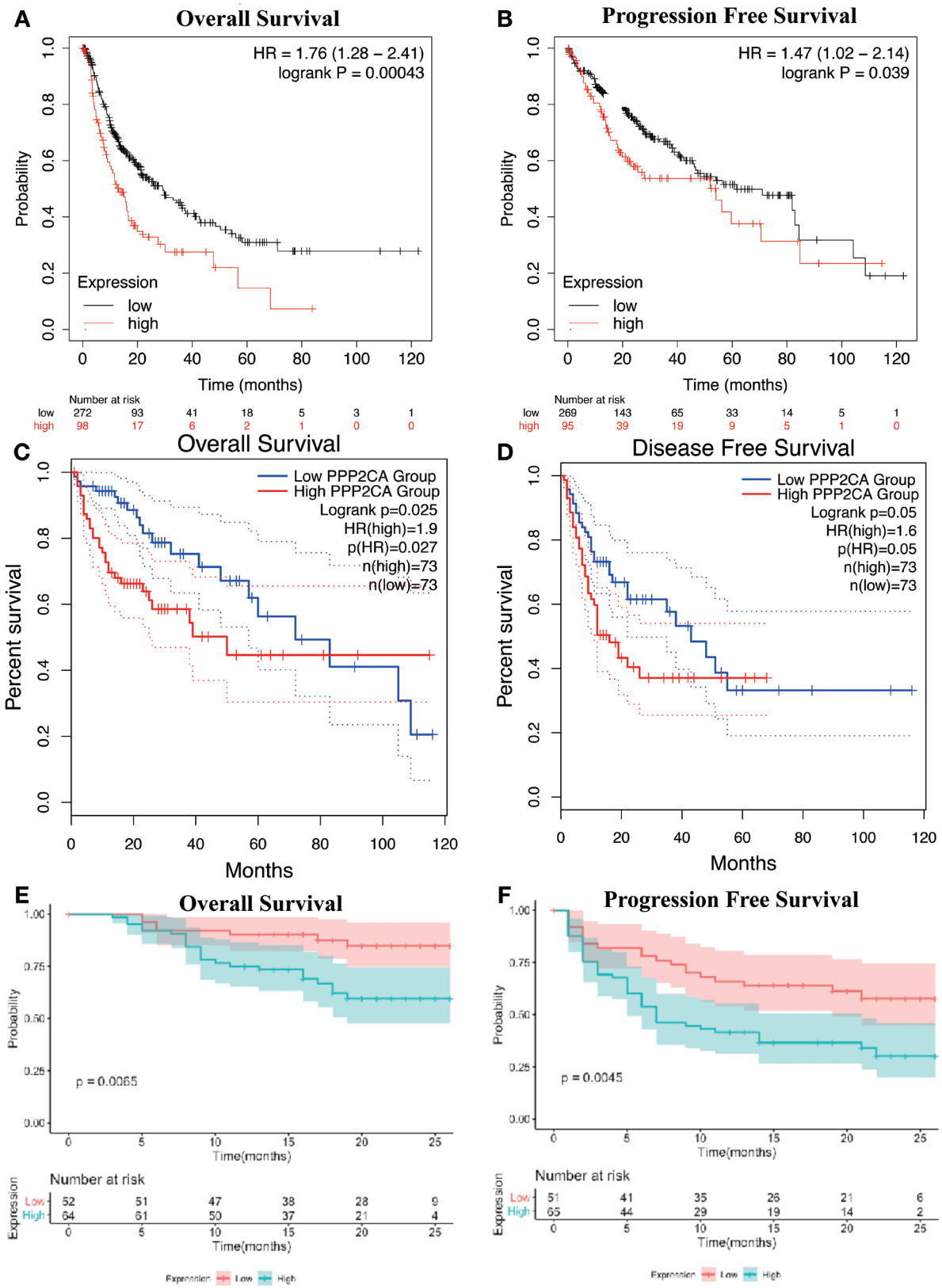

Fig. 4. Overall survival and progression-free survival of hepatocellular patients, stratified based on PPP2CA expression, according to the Kaplan-Meier plotter (A–B), GEPIA (C–D) and our in-house RNA-Seq database (E–F). GEPIA2, Gene Expression Profiling Interactive Analysis.

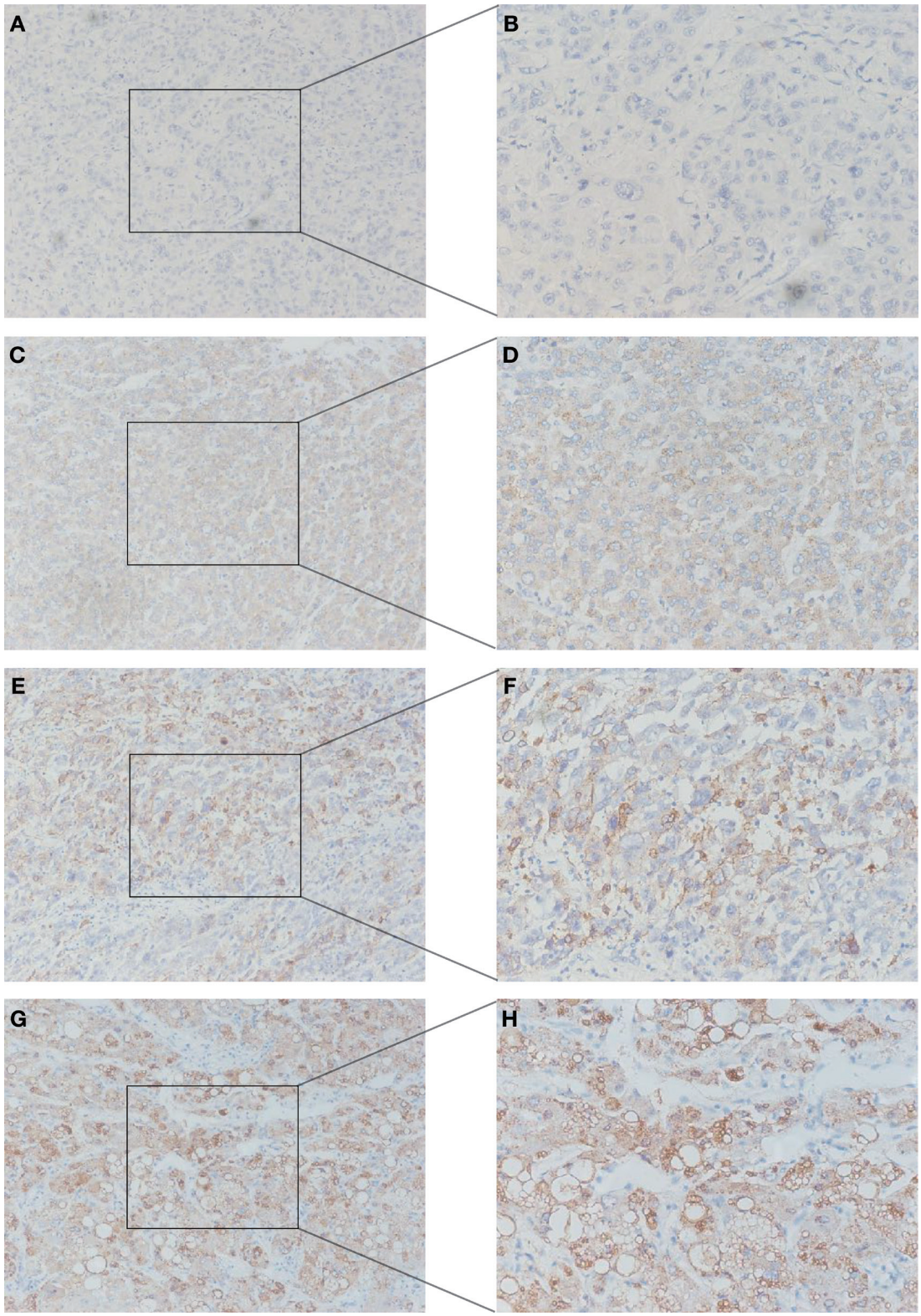

Fig. 5. Expression of PPP2CA in HCC tissues, showing primarily cytoplasmic localization. (A–B) A representative tissue slice classified as showing negative expression (0 points in overall score). Magnification, ×100 (A), ×200 (B). (C–D) A representative tissue slice classified as showing weakly positive expression (1–4 points in overall score). Magnification, ×100 (C), ×200 (D). (E–F) A representative tissue slice classified as showing moderately positive expression (5–8 points in overall score). Magnification, ×100 (E), ×200 (F). (G–H) A representative tissue slice classified as showing strongly positive expression (9–12 points in overall score). Magnification, ×100 (G), ×200 (H). HCC, hepatocellular carcinoma.

Table 2. PPP2CA expression in HCC and adjacent tissues

Group	Low expression, *n* (%)	High expression, *n* (%)	Total	χ^2	*p*
HCC tissue	84 (68.3%)	39 (31.7%)	123	5.905	0.015*
Adjacent tissue	88 (82.2%)	19 (17.8%)	107		
Total	172 (100%)	58 (100%)	230		

*$p<0.05$. HCC, hepatocellular carcinoma.

expression has been associated with poor prognosis,[25] and its anti-tumor effects in HCC have been attributed to an ability to target SPOCK1.[26] Also consistent with our results, another study found that miR-200a-3p was down-regulated in HCC tissues, and that circ-ZEB1.33 promoted HCC proliferation by sponging this miRNA.[27] Therefore, we speculate that low levels of miR-139-5p and miR-200a-3p allow higher PPP2CA expression, thereby promoting HCC. Future studies should test this hypothesis and examine whether and how miR-548o-3p is involved in HCC or other tumors. We also expect that the roles of these miRNAs can be deeply studied in the peripherals of HCC patients to prove whether they may serve as surrogate soluble biomarkers.

KEGG pathway analysis of the 200 genes most closely related to PPP2CA showed the most enriched pathways to be spliceosome and RNA transport. HCC involves up-regulation of the spliceosome pathway and its related genes,[28] and mutations in exportins that affect RNA transport pathways are associated with worse OS among patients.[29] We speculate that PPP2CA may affect HCC through the spliceosome and RNA transport. Our PPI network further identified SKIV2L2 as a hub gene up-regulated in HCC, consistent with a previous report that it is overexpressed in the disease and is a predictor of poor prognosis.[30] Future studies should clarify interactions among SKIV2L2 and the other two hub genes, ABCE1 and HNRNPK, in HCC.

In conclusion, our study shows that PPP2CA expression is significantly higher in HCC tissue than in adjacent tissue, and that it may work as a tumor promoter in HCC. Its high expression correlates with poor prognosis. It may also be a reliable diagnostic marker for HCC. Our study also identifies several miRNAs, key pathways and hub genes that may be related to HCC and therefore merit further study.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Designed the research, analyzed the data, and wrote the manuscript (ZMZ, BDX, YMZ), participated in data preparation, experiments, analysis of data, and figure preparation (CLY, XQ, JYL, XYC, XYH, JHZ, ST, XYL). All authors read and approved the manuscript for publication.

Data sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

[1] Rao CV, Asch AS, Yamada HY. Frequently mutated genes/pathways and genomic instability as prevention targets in liver cancer. Carcinogenesis 2017;38(1):2–11. doi:10.1093/carcin/bgw118.
[2] Lin Z, He R, Luo H, Lu C, Ning Z, Wu Y, *et al*. Integrin-beta5, a miR-185-targeted gene, promotes hepatocellular carcinoma tumorigenesis by regulating beta-catenin stability. J Exp Clin Cancer Res 2018;37(1):17. doi:10.1186/s13046-018-0691-9.
[3] Virshup DM, Shenolikar S. From promiscuity to precision: protein phosphatases get a makeover. Mol Cell 2009;33(5):537–545. doi:10.1016/j.molcel.2009.02.015.
[4] Gu P, Qi X, Zhou Y, Wang Y, Gao X. Generation of Ppp2Ca and Ppp2Cb conditional null alleles in mouse. Genesis 2012;50(5):429–436. doi:10.1002/dvg.20815.
[5] Khew-Goodall Y, Mayer RE, Maurer F, Stone SR, Hemmings BA. Structure and transcriptional regulation of protein phosphatase 2A catalytic subunit genes. Biochemistry 1991;30(1):89–97. doi:10.1021/bi00215a014.
[6] Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, *et al*. NCBI GEO: archive for functional genomics data sets—update. Nucleic Acids Res 2013;41(Database issue):D991–995. doi:10.1093/nar/gks1193.
[7] Nagy A, Lanczky A, Menyhart O, Gyorffy B. Validation of miRNA prognostic power in hepatocellular carcinoma using expression data of independent datasets. Sci Rep 2018;8(1):9227. doi:10.1038/s41598-018-27521-y.
[8] Tang Z, Li C, Kang B, Gao G, Li C, Zhang Z. GEPIA: a web server for cancer and normal gene expression profiling and interactive analyses. Nucleic Acids Res 2017;45(W1):W98–W102. doi:10.1093/nar/gkx247.
[9] Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. Nat Methods 2017;14(4):417–419. doi:10.1038/nmeth.4197.
[10] Longin S, Zwaenepoel K, Louis JV, Dilworth S, Goris J, Janssens V. Selection of protein phosphatase 2A regulatory subunits is mediated by the C terminus of the catalytic Subunit. J Biol Chem 2007;282(37):26971–26980. doi:10.1074/jbc.M704059200.
[11] Bryant JC, Westphal RS, Wadzinski BE. Methylated C-terminal leucine residue of PP2A catalytic subunit is important for binding of regulatory Balpha subunit. Biochem J 1999;339(Pt 2):241–246.
[12] Tolstykh T, Lee J, Vafai S, Stock JB. Carboxyl methylation regulates phosphoprotein phosphatase 2A by controlling the association of regulatory B subunits. EMBO J 2000;19(21):5682–5691. doi:10.1093/emboj/19.21.5682.
[13] Bhardwaj A, Singh S, Srivastava SK, Honkanen RE, Reed E, Singh AP. Modulation of protein phosphatase 2A activity alters androgen-independent growth of prostate cancer cells: therapeutic implications. Mol Cancer Ther 2011;10(5):720–731. doi:10.1158/1535-7163.MCT-10-1096.
[14] Huang T, He K, Mao Y, Zhu M, Yan C, Yu F, *et al*. Genetic variants in PPP2CA are associated with gastric cancer risk in a Chinese population. Sci Rep 2017;7(1):11499. doi:10.1038/s41598-017-12040-z.
[15] Yong L, YuFeng Z, Guang B. Association between PPP2CA expression and colorectal cancer prognosis tumor marker prognostic study. Int J Surg 2018;59:80–89. doi:10.1016/j.ijsu.2018.09.020.
[16] Orlandella FM, Mariniello RM, Iervolino PLC, Imperlini E, Mandola A, Verde A, *et al*. miR-650 promotes motility of anaplastic thyroid cancer cells by targeting PPP2CA. Endocrine 2019;65(3):582–594. doi:10.1007/s12020-019-01910-3.
[17] Bhardwaj A, Singh S, Srivastava SK, Arora S, Hyde SJ, Andrews J, *et al*. Restoration of PPP2CA expression reverses epithelial-to-mesenchymal transition and suppresses prostate tumour growth and metastasis in an orthotopic mouse model. Br J Cancer 2014;110(8):2000–2010. doi:10.1038/bjc.2014.141.
[18] Chen J, Liu C, Cen J, Liang T, Xue J, Zeng H, *et al*. KEGG-expressed genes and pathways in triple negative breast cancer: protocol for a systematic review and data mining. Medicine (Baltimore) 2020;99(18):e19986. doi:10.1097/MD.0000000000019986.
[19] Yang D, Okamura H, Morimoto H, Teramachi J, Haneji T. Protein phosphatase

2A Calpha regulates proliferation, migration, and metastasis of osteosarcoma cells. Lab Invest 2016;96(10):1050–1062. doi:10.1038/labinvest.2016.82.
[20] Yang C, Huang S, Zhang Z. Mechanism of action of protein phosphatase 2A in the promotion and inhibition of hepatocellular carcinoma. J Clin Hepatol 2019;35(5):1123–1128.
[21] Frau M, Simile MM, Tomasi ML, Demartis MI, Daino L, Seddaiu MA, *et al*. An expression signature of phenotypic resistance to hepatocellular carcinoma identified by cross-species gene expression analysis. Cell Oncol (Dordr) 2012;35(3):163–173. doi:10.1007/s13402-011-0067-z.
[22] Christen V, Duong F, Bernsmeier C, Sun D, Nassal M, Heim MH. Inhibition of alpha interferon signaling by hepatitis B virus. J Virol 2007;81(1):159–165. doi:10.1128/JVI.01292-06.
[23] Gong SJ, Feng XJ, Song WH, Chen JM, Wang SM, Xing DJ, *et al*. Upregulation of PP2Ac predicts poor prognosis and contributes to aggressiveness in hepatocellular carcinoma. Cancer Biol Ther 2016;17(2):151–162. doi:10.1080/15384047.2015.1121345.
[24] Duong FH, Christen V, Lin S, Heim MH. Hepatitis C virus-induced up-regulation of protein phosphatase 2A inhibits histone modification and DNA damage repair. Hepatology 2010;51(3):741–751. doi:10.1002/hep.23388.
[25] Wu J, Zhang T, Chen Y, Ha S. MiR-139-5p influences hepatocellular carcinoma cell invasion and proliferation capacities via decreasing SLITRK4 expression. Biosci Rep 2020;40(5):BSR20193295. doi:10.1042/BSR20193295.
[26] Li P, Xiao Z, Luo J, Zhang Y, Lin L. MiR-139-5p, miR-940 and miR-193a-5p inhibit the growth of hepatocellular carcinoma by targeting SPOCK1. J Cell Mol Med 2019;23(4):2475–2488. doi:10.1111/jcmm.14121.
[27] Gong Y, Mao J, Wu D, Wang X, Li L, Zhu L, *et al*. Circ-ZEB1.33 promotes the proliferation of human HCC by sponging miR-200a-3p and upregulating CDK6. Cancer Cell Int 2018;18:116. doi:10.1186/s12935-018-0602-3.
[28] Xu W, Huang H, Yu L, Cao L. Meta-analysis of gene expression profiles indicates genes in spliceosome pathway are up-regulated in hepatocellular carcinoma (HCC). Med Oncol 2015;32(4):96. doi:10.1007/s12032-014-0425-6.
[29] Chen L, Huang Y, Zhou L, Lian Y, Wang J, Chen D, *et al*. Prognostic roles of the transcriptional expression of exportins in hepatocellular carcinoma. Biosci Rep 2019;39(8):BSR20190827. doi:10.1042/BSR20190827.
[30] Yu L, Kim J, Jiang L, Feng B, Ying Y, Ji KY, *et al*. MTR4 drives liver tumorigenesis by promoting cancer metabolic switch through alternative splicing. Nat Commun 2020;11(1):708. doi:10.1038/s41467-020-14437-3.