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Impact of Protein Phosphatase Expressions on the Prognosis of Hepatocellular Carcinoma Patients

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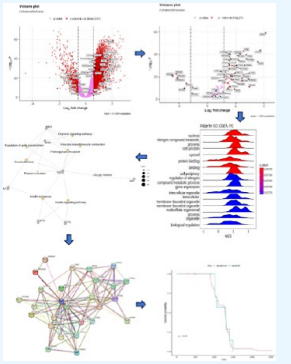
ABSTRACT: The study was conducted to unveil the significance of protein phosphatases in the prognosis of hepatocellular carcinoma (HCC) patients and its related molecular biological attributes as well as to discover novel potential biomarkers for therapeutic significance and diagnostic purposes that may benefit clinical practice. Analyzing a data set from 159 HCC patients using high-throughput phosphoproteomics, we examined the dysregulated expression of protein phosphatases. Employing bioinformatic and pathway analyses, we explored differentially expressed genes linked to protein phosphatases. A protein−protein interaction network was constructed using the search tool for the retrieval of interacting genes/proteins database. We quantified a total of 11,547 phosphorylation sites associated with 4043 phosphoproteins from HCC patients. Within this data set, we identified 105 identified phosphorylation sites associated with protein phosphatases; 28 genes were upregulated and 3 were downregulated in HCC. Enriched pathways using Gene Set Enrichment Analysis encompassed oocyte meiosis, proteoglycans in cancer, the oxytocin signaling pathway, the cGMP-PKG signaling pathway, the vascular smooth muscle, and the cAMP signaling pathway. The Kyoto encyclopedia of genes and genomes (KEGG) analysis highlighted pathways like mitogen-activated protein kinase, AMPK, and PI3K-Akt, indicating potential involvement in HCC progression. Notably, the PPI network identified hub genes, emphasizing their interconnections and potential roles in HCC. In our study, we found significantly upregulated levels of CDC25C, PPP1R13L, and PPP1CA, which emerge as promising avenues. This significant expression could serve as potent diagnostic and prognostic markers to enhance the effectiveness of HCC cancer treatment, offering efficiency and accuracy in patient assessment. The findings regarding protein phosphatases reveal their elevated expression in HCC, correlating with unfavorable prognosis. Moreover, the outcomes of gene ontology and KEGG pathway analyses suggest that protein phosphatases may influence liver cancer by engaging diverse targets and pathways, ultimately fostering the progression of HCC. These results underscore the substantial potential of protein phosphatases as key contributors to HCC's development and advancement. This insight holds promise for identifying therapeutic targets and charting research avenues to enhance the comprehension of the intricate molecular mechanisms underpinning HCC.



INTRODUCTION

Liver cancer is a prominent global health concern, ranking as the fourth leading cause of cancer-related fatalities. Hepatocellular carcinoma (HCC) constitutes approximately 85−90% of primary liver malignancies, with chronic infections by hepatitis B virus (HBV) and hepatitis C virus (HCV) being the principal contributors.[1] Additional factors such as alcohol misuse and metabolic syndrome also contribute. Although direct-acting antiviral therapy has proven effective in curing chronic HCV infection,[2] the current antiviral measures only manage to mitigate rather than fully eradicate HBV, impacting around 292,000,000 individuals globally.[3] Mass spectrometry (MS)-based proteomics plays a pivotal role in measuring global protein abundance and post-translational modifications, thereby offering insights that genomic analysis alone might not reveal. The amalgamation of sequencing and MS delivers a more holistic understanding, bridging the gap between cancer genotype and phenotype by means of functional proteomics and elucidation of signaling networks.

In a normal cell, one-third of proteins are regulated by phosphorylation, one of the most important post-translational modifications. This modification is vital for controlling numerous biological functions such as proliferation, cell division, apoptosis, and survival.[4] Proteins shift from a dephosphorylated to a phosphorylated state, and vice versa, under the specific control of protein phosphatases and kinases. Dephosphorylation is catalyzed by protein phosphatases, while the phosphorylation of these hydroxyl amino acid side chains is catalyzed by protein kinases.[5] Regarding the degree of phosphorylation between these phosphorylated amino acids, phosphoserine (pSer) predominates with 86.4% of the total phosphorylation, followed by phosphothreonine (pThr) at 11.8%, and then phosphotyrosine (pTyr) with 1.8%.[6] Researchers have identified many protein kinases, classifying

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them into protein tyrosine kinases and protein serine−threonine kinases. Protein serine/threonine phosphatases and protein tyrosine phosphatases (PTPs) constitute the two major classes of protein phosphatases.[7] The human genome sequence contains a total 25,000 proteins, with 518 total protein kinases (385 protein serine/threonine kinase, 90 protein tyrosine kinase, and 43 PTK-like protein) and 119 total protein phosphatases (21 protein serine/threonine phosphatase and 98 protein tyrosine-specific phosphatase).[8]

Protein phosphatases are a class of enzymes that play a crucial role in regulating various cellular processes by catalyzing the removal of phosphate groups from proteins. Phosphate groups are added to proteins through a process called phosphorylation, which is catalyzed by protein kinases. The balance between phosphorylation (addition of phosphate groups) and dephosphorylation (removal of phosphate groups) events is essential for maintaining proper cellular functions and signaling pathways. Protein phosphatases are responsible for reversing the effects of protein kinases, thereby controlling the activity and function of a wide range of target proteins. By dephosphorylating specific residues on target proteins, protein phosphatases can modulate cellular processes such as signal transduction, gene expression, cell cycle progression, and metabolism.

As of now, the US Food and Drug Administration (FDA) has granted approval for over two dozen small-molecule protein kinase inhibitors and six therapeutic antibodies targeting protein tyrosine kinases for clinical application, primarily focusing on targeted cancer treatments. Additionally, in conjunction with these FDA-endorsed medications, numerous other protein kinase inhibitors are undergoing evaluation in clinical trials.[9] A variety of small-molecule agents capable of modulating protein phosphatase activity, either by activation or inhibition, have been created, displaying therapeutic potential for diverse human ailments. For instance, immunosuppressive compounds like cyclosporine A and FK506, effectively employed in treating acute organ transplant rejection, rheumatoid arthritis, psoriasis, and Crohn's disease, target and inhibit protein serine/threonine phosphatase PP2B (also known as PP3 or calcineurin).[10] The precise mechanisms of action of these inhibitors, however, were only uncovered subsequent to their approval by the US FDA. These PP2B inhibitors do not obstruct the active site; instead, they bind and obstruct one of the substrate binding sites of PP2B.[11] Furthermore, a range of PTP inhibitors have been developed for therapeutic applications. These include diverse small-molecule inhibitors such as orthosteric inhibitors (which bind to the enzyme's active site), allosteric inhibitors (which bind outside the enzyme's active site), competitive inhibitors (which bind to the substrate binding site), and biologics.[12] Some of these inhibitors are currently undergoing clinical trials. For instance, small-molecule inhibitors targeting vascular endothelial PTP and protein tyrosine phosphatase 1B (PTP1B) are being tested for the treatment of conditions like diabetic macular edema and metastatic breast cancer, respectively.[7,13]

There are several families of protein phosphatases, each with distinct structures, functions, and substrate specificities. Some of the major families of protein phosphatases include the following.

Protein Phosphatase 1. Protein phosphatase 1 (PP1) is a ubiquitously expressed and a highly conserved serine/threonine phosphatase, approximately 38 kDa in size, which is involved in the regulation of many cellular processes, including glycogen metabolism, cell division, and protein synthesis. PP1 is a major player in cellular signal transduction, and its activity is essential for maintaining proper cell function, growth, and development. PP1 is a heterodimeric enzyme consisting of a catalytic subunit (PP1c) and a regulatory subunit. It is estimated that PP1 is accountable for over half of the total phosphoserine/threonine dephosphorylation reactions occurring within eukaryotic cells. The regulatory subunit is responsible for directing PP1 to specific substrates and locations within the cell. PP1 has multiple regulatory subunit isoforms, allowing for substrate specificity and versatile regulation.[14] Moreover, PP1 is involved in dephosphorylating a wide range of target proteins, including enzymes, transcription factors, and structural proteins. Its substrate specificity is conferred by the interactions between the regulatory subunit and the target protein. It plays a central role in regulating glycogen synthesis and degradation. It is a key enzyme in the regulation of glycogen phosphorylase and glycogen synthase.[15]

PP1 is critically involved in cell cycle progression, including entry into and exit from mitosis. It regulates key cell cycle checkpoints and ensures proper chromosome segregation during cell division. Furthermore, PP1 modulates the activity of various components involved in protein synthesis and translation, such as eukaryotic initiation factor 2, elongation factors, and ribosomal proteins. Also, it is involved in the regulation of various signaling pathways, including those mediated by protein kinases such as protein kinase A and protein kinase C.[16] It acts as a counter-regulator, reversing the effects of phosphorylation by kinases. PP1 is abundant in the nervous system, and dysregulation of PP1 activity in neurons has been linked to neurodegenerative diseases. In muscle cells, PP1 regulates the phosphorylation state of proteins involved in muscle contraction, such as myosin and troponin, thereby influencing muscle function.[4,10] Dysregulation of PP1 activity can lead to various diseases such as cancer, heart disease, memory loss, type 2 diabetes, and viral infections, suggesting a great therapeutic potential for PP1-directed drugs. Researchers continue to investigate the complex regulation and functions of PP1 to gain a deeper understanding of its roles in health and disease. The initial breakthrough came with the identification of a distinct phenotype in mice harboring a comprehensive deletion of protein tyrosine phosphatase PTP1B. Notably, these mice exhibited heightened sensitivity to insulin and demonstrated resistance to obesity. Subsequent to this discovery, both academic researchers and pharmaceutical enterprises embarked on a quest to identify inhibitors of PTP1B, aiming to develop treatments for type 2 diabetes and obesity.[4,17]

The information regarding PPP1 complexes in humans is currently limited. Although a few complexes have been characterized, our understanding of them remains incomplete. From a therapeutic perspective, the most encouraging strategy for the development of drugs targeting PP1 does not entail disruption of the catalytic site but rather focuses on distinct interaction sites within the PP1 holoenzymes. Compounds that bind to the catalytic site would indiscriminately affect all PP1 holoenzymes, potentially even impacting other closely related phosphatases like protein phosphatase 2A (PP2A) and PP2B holoenzymes. Conversely, substances that perturb specific PP1 holoenzymes would selectively alter PP1 activity toward a restricted set of substrates.[7,15]

Protein Phosphatase 2A. PP2A is a heterotrimeric enzyme complex that is widely distributed in eukaryotic cells. It plays roles in cell cycle regulation, signal transduction, and cellular differentiation. The PP2 family includes several isoforms, with PP2A being one of the most well-known and extensively studied

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members. PP2A is a major serine/threonine phosphatase that is involved in a wide range of cellular functions. PP2A is structurally a heterotrimeric complex with a dimeric core enzyme, consisting of a 55 kDa B regulatory subunit (PP2Ab), a 65 kDa scaffold subunit A (PP2Aa), and a 36 kDa catalytic subunit C (PP2Ac). PP2A exists in two forms, a dimer form (PP2AD) or a trimer complex (PP2AT).[18] Protein phosphatase 2B (PP2B or calcineurin) is involved in calcium signaling and immune responses as well as various cellular processes, including muscle contraction and synaptic plasticity. It is the master regulator of PP2A. PP2A-B is classified into four different families known as B (B55/PR55), B′ (B56/PR61), B″ (PR48/PR72/PR130), and B‴ (PR93/PR110). B55 has four different isoforms (α, β, γ, and δ), B56 has five isoforms (α, β, γ, δ, and ε), B″ contains PR72 (expressed in the skeletal muscle and heart) and PR130 (expressed in all tissues and is abundant in the muscle and heart), and B‴ is found by yeast two-hybrid screening.[19]

Protein phosphatase 2C (PP2C) is involved in stress responses, cell cycle regulation, and other cellular processes. It is most abundant in the brain and heart and expressed in almost all tissue. They are particularly important for regulating mitogen-activated protein kinase (MAPK) pathways. PP2Ac (37 kDa) exists in two isoforms (Cα and Cβ) and shares 40% identical sequences with PP2B, 50% amino acid sequences with PP1, and 86% identical in humans and yeast. Both isoforms share 97% sequence similarity and consist of 309 amino acids; Cβ is expressed in the nucleus and cytoplasm, and Cα is expressed in the plasma membrane. PP2Acβ is less abundant than PP2Acα because of the lower degree of mRNA translation and weak promoter activity. An interesting feature of PP2Ac is the C terminal tail is conserved (304TPDYEL309) and the tail binds to the regulatory and scaffold subunits (PR61γ).[4,18,19]

The activity and specificity of PP2A can be influenced by the presence and the binding of the other regulators against a particular substrate such as binding of α4 to PP2A is important to stabilize PP2Ac in its inactive conformation.[20] The two major modifications that modulate PP2A efficiency are methylation and phosphorylation. The phosphorylation on Tyr307 decreases PP2A activity by preventing its interaction with the regulatory subunit of PP2Ac and PP2A-PR55/PR61. In addition, PP2A undergoes carboxyl methylation on the carboxyl group of the C-terminal residue of Leu309. Leu-cine carboxyl methylTransferase1 (LCMT1), also known as PP2A-methyltransferase, is responsible for the methylation of PP2Ac; meanwhile, PP2A methyl Esterase is responsible for PP2Ac demethylation. Several investigators reported that the addition of a methyl group by LCMT1 at Leu309 increases the binding affinity of the core dimer (A and C subunits) and provides a specific activity to the holoenzyme.[21]

The diverse functions of the protein phosphatase 2 (PP2) family underscore their vital role in maintaining cellular function, regulating signaling pathways, and responding to various environmental cues. Abnormality of PP2 family phosphatases has been implicated in various disease states, including cancer, neurodegenerative disorders, and immune-related diseases. The role of PP2A, a member of the PP2 family, in HCC has been a subject of ongoing debate. Depending on the context, PP2A can function as either a tumor suppressor or a promoter. This controversy highlights the complex nature of its involvement in HCC. This information underscores the critical role of protein phosphatases in HCC, which has significant implications for clinical applications and ongoing research efforts. Researchers are actively exploring the specific roles and regulation of different PP2 family members to unravel their contributions to cellular and physiological processes.

Dual-Specificity Phosphatases. These phosphatases can target both serine/threonine and tyrosine residues, providing a unique mode of regulation. Dual-specificity phosphatases (DUSPs) are a subclass of protein phosphatases that possess the ability to dephosphorylate both serine/threonine and tyrosine residues on target proteins. Unlike traditional protein phosphatases, which are typically specific for either serine/threonine or tyrosine residues, they can target both serine/threonine and tyrosine phosphorylation sites on proteins.[22] DUSPs exhibit broader substrate specificity and play important roles in regulating various cellular processes. Examples of DUSPs include mitogen-activated protein kinase phosphatases (MKPs), which regulate MAPK pathways, and phosphatases that target the phosphatidylinositol 3-kinase (PI3K)/AKT pathway. Dual-specificity phosphatases (DUSPs) are subdivided into distinct subclasses, including MKP, Myotubularin, CDC14, Slingshot, phosphatase and tensin homologue (PTEN), phosphatases of regenerating liver-1 (PRL), and atypical DUSP subclasses. DUSPs are implicated in cellular responses to stressors such as DNA damage, oxidative stress, and cellular differentiation.[23] They can influence the activity of stress-activated protein kinases and transcription factors. Eccentricity of DUSPs has been linked to various diseases, including cancer and neurodegenerative disorders. Altered DUSP activity can contribute to aberrant cell signaling and cellular processes. They can participate in phosphorylation crosstalk, where the balance between phosphorylation and dephosphorylation at different residues on the same protein influences its function and interactions with other proteins. Because of their ability to dephosphorylate both serine/threonine and tyrosine residues, DUSPs play multifaceted roles in cellular regulation and are critical components of cellular signaling networks. Research on DUSPs continues to uncover their specific functions, regulation, and contributions to health and disease.

Tyrosine Phosphatases. Tyrosine phosphatases are a class of enzymes that specifically target tyrosine residues on various proteins, playing pivotal roles in crucial cellular processes such as growth factor signaling and cell adhesion. Tyrosine phosphorylation represents a critical post-translational modification involved in transmitting signals from the extracellular environment to the interior of the cell. These phosphatases help maintain a delicate balance between tyrosine phosphorylation and dephosphorylation, thereby modulating vital signal transduction pathways. They are integral components of signaling networks that regulate cell cycle progression, cell adhesion, and apoptosis. Several well-known tyrosine phosphatases include PTP1B, Src homology 2 (SH2) domain-containing phosphatase 2 (SHP2), CD45, Fas associated phosphatase-1, striatal enriched tyrosine phosphatases, MKP-1, PRL, and receptor protein tyrosine phosphatases, which exemplify this enzyme group's diversity. Additionally, the Class III PTP comprises three members: CDC25A, CDC25B, and CDC25C.[9,23] Both Class II and Class III PTPs possess catalytic cysteine residues. In the nervous system, tyrosine phosphatases play essential roles in synaptic plasticity, learning, and memory. They regulate the activity of tyrosine kinases, which are crucial in neuronal signaling pathways. However, dysregulation of tyrosine phosphorylation and tyrosine phosphatase activity has been linked to various diseases, including cancer, autoimmune disorders, and neurological diseases.

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Table 1. Identified Significant Phosphoproteins and Corresponding Phosphosites in Protein Phosphatases Linked to HCC (log FC > 0.58 or < −0.58; *P*-Value < 0.05) Using the LIMMA Package

no	gene	sites	log FC	*P*.value	adj.*P*.val	change	entrezid
1	PTPN1	S386	2.0534	3.26×10^{-60}	1.63×10^{-57}	up	5770
2	CDC25C	S216	1.8845	8.20×10^{-45}	2.02×10^{-43}	up	995
3	DUSP9	S16	1.7812	1.45×10^{-34}	1.18×10^{-33}	up	1852
4	PPP6R2	S796	1.4679	1.22×10^{-11}	2.26×10^{-11}	up	9701
5	PPP1R9A	T932	1.4261	3.28×10^{-19}	9.09×10^{-19}	up	55,607
6	PPP4R2	S218	1.3694	7.92×10^{-37}	8.11×10^{-36}	up	151,987
7	PTPN2	S327	1.3665	6.91×10^{-41}	1.05×10^{-39}	up	5771
8	PTPN1	S50	1.2697	2.45×10^{-43}	4.88×10^{-42}	up	5770
9	PTPN1	S15	1.2632	8.32×10^{-23}	2.91×10^{-22}	up	5770
10	PPP1R13L	Y132	1.2230	1.19×10^{-18}	3.19×10^{-18}	up	10,848
11	PTEN	S294	1.1957	4.34×10^{-17}	1.06×10^{-16}	up	5728
12	PPP1R12A	S422	1.1676	7.33×10^{-41}	1.11×10^{-39}	up	4659
13	PPP1CB	S311	1.0721	5.72×10^{-37}	5.95×10^{-36}	up	5500
14	CTDP1	S831; S833	1.0375	9.80×10^{-40}	1.28×10^{-38}	up	9150
15	PTPN12	S435	1.0204	5.02×10^{-31}	3.11×10^{-30}	up	5782
16	CTDP1	S474	0.9912	6.21×10^{-34}	4.81×10^{-33}	up	9150
17	PPM1E	S548	0.9577	3.57×10^{-10}	6.15×10^{-10}	up	22,843
18	PPP1R37	S576	0.9327	3.67×10^{-30}	2.14×10^{-29}	up	284,352
19	DUSP9	S189	0.9281	2.52×10^{-16}	5.91×10^{-16}	up	1852
20	PPP1CA	T320	0.8730	4.64×10^{-23}	1.65×10^{-22}	up	5499
21	PPP6R3	S471	0.8659	6.66×10^{-18}	1.70×10^{-17}	up	55,291
22	PPP6R3	S509	0.8576	5.96×10^{-31}	3.68×10^{-30}	up	55,291
23	PPP1R12A	S871	0.8432	1.23×10^{-21}	3.98×10^{-21}	up	4659
24	PPP6R3	T541	0.8406	9.56×10^{-19}	2.57×10^{-18}	up	55,291
25	PTPN12	S342	0.8231	6.91×10^{-22}	2.27×10^{-21}	up	5782
26	PTPN12	S603	0.8180	1.85×10^{-36}	1.81×10^{-35}	up	5782
27	CTDP1	S740	0.8151	1.45×10^{-40}	2.11×10^{-39}	up	9150
28	PPP1R15B	S508	0.8133	3.10×10^{-37}	3.31×10^{-36}	up	84,919
29	PTPN1	S352	0.7943	4.62×10^{-35}	3.98×10^{-34}	up	5770
30	PPP1R12A	S896	0.7905	2.75×10^{-12}	5.26×10^{-12}	up	4659
31	PPP1R2	S121; S122	0.7885	1.58×10^{-31}	1.02×10^{-30}	up	5504
32	PPP1R2	S101,S102	0.7885	1.58×10^{-31}	1.02×10^{-30}	up	5504
33	PPP1R12A	S862	0.7792	1.17×10^{-16}	2.81×10^{-16}	up	4659
34	PPM1E	S535	0.7776	1.13×10^{-12}	2.21×10^{-12}	up	22,843
35	PTPN14	S312	0.7588	4.98×10^{-28}	2.48×10^{-27}	up	5784
36	PPP1R10	S451	0.7584	2.13×10^{-11}	3.88×10^{-11}	up	5514
37	SSH2	S811	0.7415	6.14×10^{-20}	1.79×10^{-19}	up	85,464
38	PTPRF	S1299	0.7276	4.42×10^{-15}	9.71×10^{-15}	up	5792
39	PPP1R35	S52	0.7242	6.26×10^{-15}	1.36×10^{-14}	up	221,908
40	PPP1R18	S175	0.7212	1.93×10^{-15}	4.32×10^{-15}	up	170,954
41	PTPRO	S865	0.7122	1.03×10^{-10}	1.82×10^{-10}	up	5800
42	PTPN14	S642	0.6963	4.36×10^{-21}	1.36×10^{-20}	up	5784
43	PPP1R10	T256	0.6672	8.38×10^{-19}	2.26×10^{-18}	up	5514
44	PPP1R37	S544	0.6672	2.84×10^{-24}	1.09×10^{-23}	up	284,352
45	PPP2R5D	S573	0.6559	1.41×10^{-15}	3.18×10^{-15}	up	5528
46	CTDP1	S869	0.6087	9.88×10^{-21}	3.03×10^{-20}	up	9150
47	PTPN3	S228	0.6053	1.31×10^{-15}	2.97×10^{-15}	up	5774
48	PTPN13	S1063	−0.7467	3.29×10^{-15}	7.26×10^{-15}	down	5783
49	PTPN13	S240	−0.7924	6.35×10^{-11}	1.13×10^{-10}	down	5783
50	PPP1R12B	S900	−1.0679	1.13×10^{-19}	3.24×10^{-19}	down	4660
51	PPP1R12B	S43	−1.0679	1.13×10^{-19}	3.24×10^{-19}	down	4660
52	PPP1R3E	S33	−1.1039	3.37×10^{-23}	1.21×10^{-22}	down	90,673
53	PPP1R3E	S16	−1.2135	2.67×10^{-21}	8.44×10^{-21}	down	90,673

Abnormality of protein phosphorylation and dephosphorylation processes, including imbalances between protein kinases and phosphatases, can contribute to various diseases, including cancer, neurodegenerative disorders, and metabolic diseases. Protein phosphatases are therefore important targets for therapeutic interventions aimed at restoring proper cellular signaling and function. Research on phosphatases continues to uncover their roles in various physiological and pathological processes. In this study, we aimed to investigate the role of protein phosphatases in HCC by integrating clinical data and

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bioinformatics analyses. Our approach involves a retrospective analysis of 159 HCC patients who underwent initial surgery at the Department of Hepatobiliary Surgery. We conducted a comparative assessment of protein phosphatase expression levels between HCC tissues and non-HCC tissues. Additionally, we utilized bioinformatics tools and public databases to compare clinical data and patient survival outcomes based on low and high protein phosphatase expression. We also validated the differential protein phosphatase expression in HCC and non-HCC tissues, further corroborating the survival rates of patients with varying protein phosphatase expression levels. Furthermore, we employed diverse bioinformatics programs to elucidate potential pathways and targets associated with protein phosphatase involvement in HCC.

MATERIALS AND METHODS

Data Collection and Preprocessing. We gathered phosphoproteomic and proteomic data sets related to protein phosphatases from the publicly available Clinical Proteomic Tumor Analysis Consortium (CPTAC) database, which offers well-characterized, mass spectrometry-based discovery proteomics. Our study involved a comparative analysis of clinical data and patient survival outcomes based on low and high expression levels of protein phosphatases. This analysis relied on the utilization of bioinformatics tools and public databases. Furthermore, we investigated the differential expression of phosphatases between HCC tissues and non-HCC tissues. Subsequently, we validated the survival rates of patients with low or high protein phosphatase expression. To gain insights into potential pathways associated with these findings, we employed a variety of bioinformatics programs. It is worth noting that the three databases CPTAC, The Cancer Proteome Atlas, and The Cancer Genome Atlas (TCGA) are publicly accessible and open source. This study strictly adhered to the data access policies and publishing guidelines of these databases, obviating the need for local ethics committee approval.

Bioinformatics and Expression Analysis. The acquired data sets provide information on relative phosphorylation sites and total protein abundances across samples through TMT 11 labeling experiments. Each sample has undergone normalization, and we have computed relative abundances as log ratios (base 2) in comparison to the pooled reference samples. The data analysis was carried out using R version 4.2.2.

Identification of Differentially Expressed Phosphorylation Sites. We have conducted a paired *t*-test analysis using the LIMMA package in R/Bioconductor to identify differentially expressed phosphorylation sites between tumor and paired nontumor liver tissues. To address the issue of multiple hypothesis testing, we applied the Benjamini−Hochberg false discovery rate (FDR) correction to adjust the *p*-values. We established a significance threshold of adjusted *p*-values <0.05 and (FC ≥ 1.5 or ≤0.667) to determine statistical significance as the screening criteria for differentially expressed genes (DEGs) in CPTAC. Adjusted. *p*-values <0.05 were used in differential gene screening to control the false-positive rate. Our primary focus in this study centers on 31 unique protein phosphatases and their corresponding phosphosites, which exhibited significant differences between tumor and paired nontumor liver tissues, as detailed in Table 1. The heat and volcano maps were generated using the gplots package in R software.

Functional and Pathway Enrichment Analysis of Differentially Expressed Phosphorylation Sites. Our approach follows a systematic pipeline for data interpretation and visualization, enabling us to gain insights into the functional implications of the identified differentially expressed phosphorylation sites. Gene Ontology (GO) offers a framework for describing the functions of gene products across all organisms and for identifying characteristic biological properties within high-throughput transcriptome data from genomes. The combination of pathway enrichment analysis, visualization, and heatmaps helps us understand how these sites contribute to specific biological processes (BP), cellular component (CC) and molecular function (MF) such as the regulation of the pathway enrichment analysis. We performed pathway enrichment analysis using Gene Set Enrichment Analysis (GSEA) with Cluster Profiler R package. This analysis helps annotate and functionally interpret the differentially expressed phosphorylation sites by associating them with known pathways and biological functions.

Survival Analysis of the Hub Genes in HCC. The survival data were extracted from HCC patients within the CPTAC data set. Kaplan−Meier survival analysis and the log-rank test were employed to assess the correlation between potential candidates and HCC outcomes. These analyses were conducted using the survival and survminer packages. The low and high expression groups were defined based on a median cutoff of 50% expression. The expression levels of the identified phosphorylation sites and their associated proteins were visualized using the ggplot2 package. Additionally, the proteomics data were leveraged to further explore and analyze the phosphoproteomics results. To do so, we conducted survival analysis on the proteins and retained those whose expressions correlated with patient survival for subsequent analyses. All statistical computations and analyses were conducted using R software, version 4.2.2.

Prognostic Model Utilizing a 31-Gene Signature as an Independent Predictor for Overall Survival. We conducted univariate and multivariate Cox regression analyses to determine if the prognostic model could maintain its independence from other clinicopathological variables. These variables encompassed age, gender, tissue registration, pathological stage, T staging, and risk score for HCC patients. In these analyses, clinical features served as independent variables, while overall survival (OS) was considered the dependent variable for HR calculation, along with its 95% confidence interval and two-sided *P*-value.

Validation of the 31-Gene Signature across Multiple Databases. We conducted an analysis of the mRNA expression of the gene signature in HCC tissues and normal liver tissues using the online microarray database TCGA. The analysis was performed with the following threshold settings: *P* value < 0.01, fold change >2, and gene rank within the top 10%. Data sets used in the analysis were selected based on statistical differences in sample size, fold change, *t*-test results, analysis type, and *P*-values. Additionally, we compared protein expression levels related to the gene signature by retrieving immunohistochemical images from publicly available human protein maps (http://www.proteinatlas.org).[24] To further validate our findings, we obtained an independent HCC cohort from the International Cancer Genome Consortium, extracted the expression levels of the 31-gene signature, and conducted a comparative analysis between HCC and nontumor tissues using the Wilcoxon signed-rank test. Statistical significance was defined as a two-sided *P* values less than 0.05, indicating significant differences. We used the Human Protein Atlas (HPA) to observe the differences in key gene expression between HCC and nontumor tissues.

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Table 2. Summary of HCC Patient Information in the CPTAC Database

parameter	mean	coef	exp(coef)	se(coef)	z	*P* value
gender.x	NA	3.29×10^{-1}	1.39×10	3.35×10^{-1}	0.983	0.3258
age	53.8716	7.42×10^{-4}	1.00×10	1.09×10^{-2}	0.068	0.8396
PTT	12.0088	-2.70×10^{-3}	9.97×10^{-1}	6.45×10^{-2}	−0.042	0.9666
TB	12.0162	-4.73×10^{-2}	9.54×10^{-1}	3.02×10^{-2}	−1.566	0.1174
ALB	40.4865	3.16×10^{-2}	1.03×10	3.78×10^{-2}	0.836	0.4032
ALT	50.2162	-2.83×10^{-4}	1.00×10	2.70×10^{-3}	−0.105	0.9166
GGT	79.7095	-6.57×10^{-4}	9.99×10^{-1}	1.80×10^{-3}	−0.365	0.7154
AFP	6795.46	3.77×10^{-6}	1.00×10	9.35×10^{-6}	0.403	0.6869
medical history of liver cirrhosis	NA	-5.63×10^{-1}	5.69×10^{-1}	2.80×10^{-1}	−2.015	0.0439[a]
tumor size cm	6.56757	5.64×10^{-2}	1.06×10	4.03×10^{-2}	1.398	0.1622
number of tumors	1.64189	-4.40×10^{-2}	9.57×10^{-1}	1.11×10^{-1}	−0.396	0.6923
tumor differentiation	2.38514	-1.66×10^{-1}	8.47×10^{-1}	2.58×10^{-1}	−0.644	0.5193

[a]$P < 0.05$. HCC (hepatocellular carcinoma); AFP (alpha-fetoprotein); AST (aspartate aminotransferase); ALT (alanine aminotransferase); ALB (albumin) levels; PTT (partial thromboplastin time), GGT (gamma-glutamyl transferase), and TB (total bilirubin) levels.

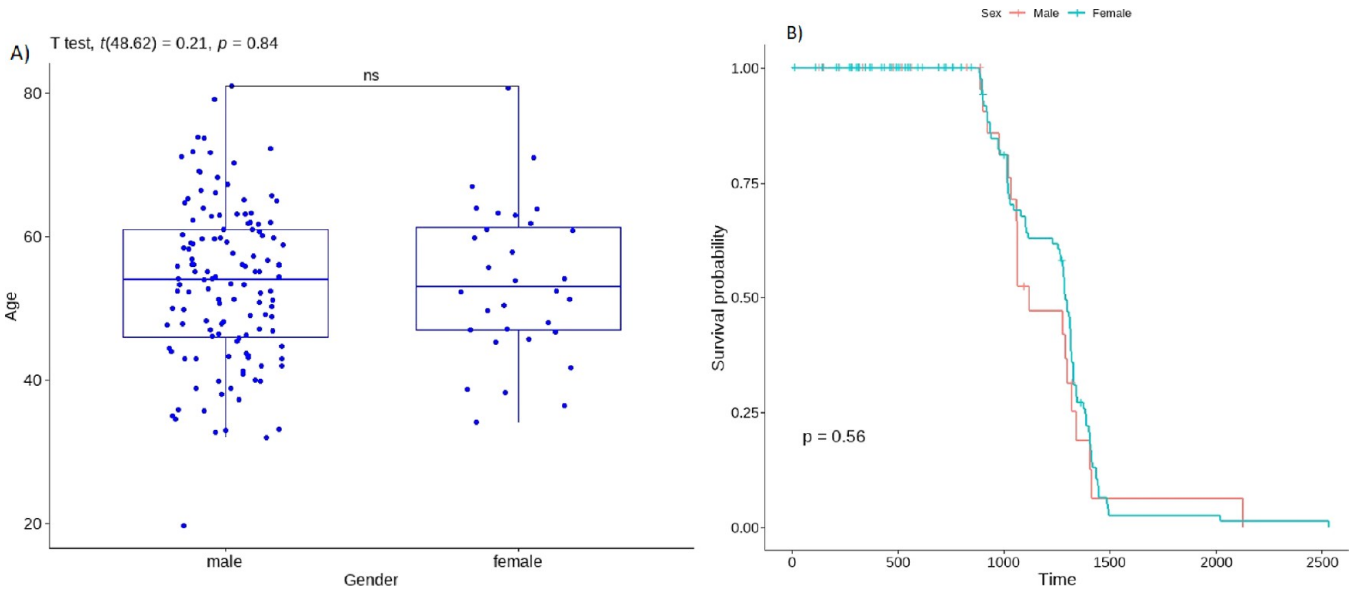

Figure 1. (A) Correlation between age and gender among HCC patients, revealing a corresponding *P*-value of 0.84. (B) Kaplan−Meier curve of OS analysis of two groups of HCC patients based on gender (*P*-value = 0.56).

Establishment and Evaluation of Nomograms for Predicting HCC Survival. Nomograms are effective tools for predicting the prognosis of cancer patients. They simplify complex statistical prediction models into easy-to-use charts, allowing for the assessment of an individual patient's probability of OS.[25] In this study, we incorporated all independent clinical and pathological prognostic factors identified through Cox regression analysis to create a nomogram. This nomogram can estimate the OS probabilities at 1, 3, and 5 years for HCC patients. To assess the nomogram's accuracy, we compared its predicted probabilities with observed actual probabilities using a calibration curve. If the curve overlaps with the reference line, it indicates that the model is accurate.

RESULTS

Patient Characteristics of CPTAC. We utilized the publicly available CPTAC database as our primary source for protein phosphatases from HCC samples. We downloaded data sets containing phosphoproteomics and proteomics data, along with clinical information, from 159 patients, which were selected for proteogenomic analysis and were treated at the Department of Hepatobiliary Surgery. The following clinical data were collected for analysis such as gender, age, family history of liver cancer, presence or absence of liver cirrhosis, alpha-fetoprotein (AFP) levels, tumor size, tumor number, total bilirubin (TBil) levels, PTT, GGT, TB, alanine amino-transferase (ALT) levels, and albumin (ALB) levels as shown in Table S1 (patient characteristics). All participants were followed up until the time of death or loss to follow-up as per the study protocol, and there were 9 observations deleted because of missing data, specifically because it was unclear whether the individuals were deceased or still surviving. This comprehensive data set allowed us to conduct a thorough investigation of the proteogenomic landscape in the context of protein phosphatases and related clinical information.

Clinical Application in HCC. Table 2 presents a comparison of general clinical data between the two participant groups. Among these parameters, there were no statistically significant differences (all $p > 0.05$) in gender, age, presence or absence of liver cirrhosis, AFP levels, tumor size, tumor number, TBil levels, PTT, GGT, TB, ALT levels, and ALB. However, statistically significant differences were found in the presence of a family history of liver cancer ($p < 0.05$). In Figure 1A, we depict the correlation between age and gender in HCC patients,

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Table 3. Summary of OS Analysis for Two Groups of HCC Patients Based on Gender[a]

sex	records	# max	# start	events	*r* mean	se(*r* mean)	median	0.95LCL	0.95UCL
female	31	31	31	19	1215.40	67.2272	1121	1058	1342
male	120	120	120	81	1240.60	28.4761	1290	1266	1319

[a]0.95LCL: 95% lower confidence limit; 0.95UCL: 95% upper confidence limit.

revealing a corresponding *P*-value of 0.84. Figure 1B presents a Kaplan−Meier survival curve, comparing OS between two groups categorized based on their median risk scores with a *P*-value of 0.56. The Summary of OS analysis for two groups of HCC patients based on gender is shown in Table 3. The nonsignificant *p*-value suggests that gender alone does not significantly influence prognosis in this data set. While there may be a slight difference in survival probability for females, this difference is not statistically significant.

Dysregulated Phosphosites Were Identified by Phosphoproteomics Analysis. We conducted a comprehensive assessment of phosphosite abundance across 159 pairs of tumor and adjacent nontumor samples. Our analysis encompassed a total of 11,547 phosphorylation sites associated with 4043 phosphoproteins. Remarkably, these sites were quantified in at least half of the samples. Among these, a noteworthy 9994 phosphorylation sites exhibited marked differential expression. To elaborate, we observed that 8198 sites experienced significant upregulation, while 1796 sites showed downregulation in tumor samples when compared to their corresponding nontumor counterparts as shown in the volcano plot in Figure 2. This differentiation was established using a

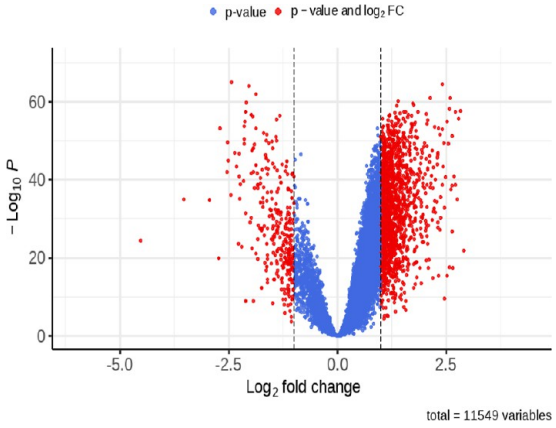

Figure 2. Volcano plot demonstrating DEGs in HCC samples. Downregulated genes are represented in red (left), while upregulated genes are shown in red (right).

paired two-sided Student's *t*-test, with Benjamini−Hochberg (BH)-adjusted *p*-values <0.05. Within this data set, we identified 110 instances of phosphorylation sites of protein phosphatases. Among these, 105 demonstrated significant changes between samples (with *p*-values <0.05) as illustrated in Table S2 (protein phosphatases). We undertook a thorough analysis of these 105 phosphorylation sites (31 unique genes) and their associated phosphoproteins to quantify the extent of upregulated and downregulated genes. For visualization and clarity, we employed R software to generate both volcano and heatmap plots. These graphical representations effectively convey our findings. Specifically, the results highlight that 28 genes were upregulated, 3 genes were downregulated, while the rest of genes did not experience statistically significant changes, as illustrated in the volcano plot in Figure 3. The identification of differentially expressed proteins between HCC and non-HCC samples in the CPTAC database was based on the criteria of |log 2FC| > 0.58 and a *P*-value <0.05, which was considered to be statistically significant.

Functional Annotation, Pathway Analysis, and Visualization. We conducted the Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis for the identified phosphoproteins (8198 upregulated and 1796 downregulated) using over-representation analysis (ORA) with Cluster Profiler using R. The most enriched pathways included spliceosome, carbon metabolism, cytochrome P450, cell cycle, and insulin resistance, as shown in Figure 4. Additionally, we performed GSEA for identified protein phosphatases and their DEGs, focusing on both GO and KEGG pathways. The GO analysis uncovered key BP, with prominence in nitrogen compound metabolic, protein dephosphorylation, regulation of the macromolecule, RNA metabolic process, protein modification process, and biological regulation. We examined the molecular functions of these genes and found that the most enriched functions included protein phosphatase regulator activity, phosphatase regulator activity, phosphoprotein phosphatase activity, kinase binding, molecular function, and protein binding. Additionally, our examination at the cellular biological level highlighted significant enrichments in nucleus, cell junction, cytosol, cell periphery, and intracellular organelle involving the 31 phosphoproteins, as illustrated in Figure 5 and in Table S3 (GO-GSEA). Among the 42 KEGG pathways identified, remarkable significance was observed in pathways such as oocyte meiosis, focal adhesion, oxytocin signaling, regulation of actin cytoskeleton, T cell receptor signaling, and insulin resistance pathways. Notably, our KEGG analysis highlighted substantial enrichment proteins in several pathways including MAPK, AMPK, PI3K-Akt, and proteoglycans in cancer. In our investigation, a functional GSEA of differentially expressed phosphoproteins using KEGG pathway resources yielded insightful outcomes. Among the top five enriched pathways, proteoglycans in cancer, the oxytocin signaling pathway, the cGMP-PKG signaling pathway, the vascular smooth muscle, and the cAMP signaling pathway emerged as significant contributors as visualized in Figure 6. The noteworthy prevalence of highly phosphorylated proteins within the regulation of actin cytoskeleton pathway in tumor samples aligns harmoniously with previous studies in liver cancer and other malignancies. This concurrence implies a plausible connection between the actin cytoskeleton and HCC. One of the challenges in enrichment analysis is the selection of pathways for further investigation. To address this, we introduced the 'pmcplot' function, which visualizes the number and proportion of publication trends based on query results from PubMed Central, as depicted in Figure 7.

Protein−Protein Interaction Network and Hub Gene Identification. To further investigate the relationships among differentially expressed proteins at the protein level, we utilized the online search tool for the retrieval of interacting genes/proteins (STRING) to construct a protein−protein interaction

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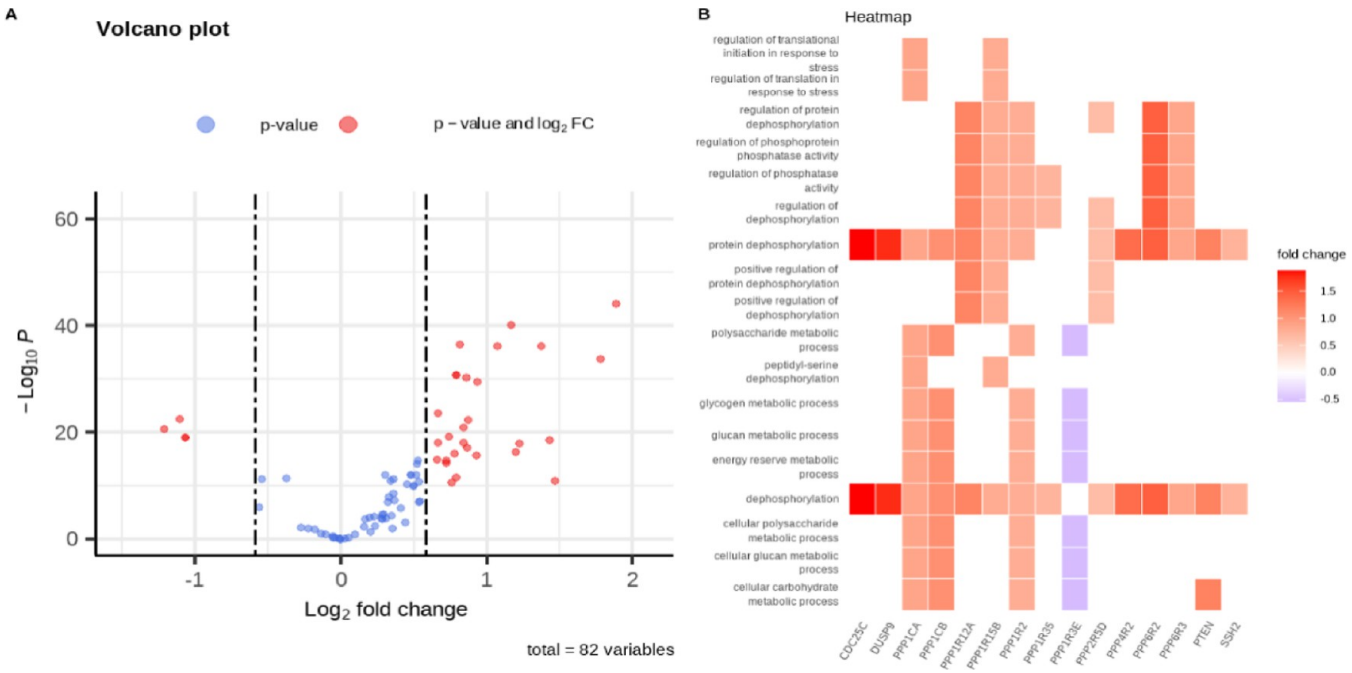

Figure 3. (A) Volcano plot and (B) heatmap depicting differential gene expression in HCC samples for 31 protein phosphatase genes and their corresponding phospohosites (|log 2FC| > 0.58 and a *P*-value <0.05). Genes with decreased expression are denoted in red (left), while those with increased expression are indicated in red (right) in the volcano plot.

(PPI) network. The interaction between proteins is essential to understanding the metabolic and molecular mechanisms of tumors. This network included 31 significant proteins and their phosphosites, with a confidence score of 200 at the protein level. As depicted in Figure 8, the PPI network consisted of 49 nodes, 382 edges (interactions), and 54 expected interactions. We constructed the PPI network for the DEGs using the STRING database and visualized it using R software version 4.2.2. Our analysis results identified PTPN1, CDC25C, DUSP9, PPP6R2, PPP4R2, PPP1R9A, PTPN2, PPP1R13L, PTEN, PPP1R12A, PPP1CB, CTDP1, PPM1E, PPP1R37, and PPP1CA as the top 15 highly connected hub genes with the highest scores as shown in Figure 9. These findings, along with their corresponding phosphosites, FDR, *p*-values, and enriched pathways extracted from the STRING network using R software, are presented in Table 4.

Kaplan−Meier Analysis and Time-dependent ROC Curves of a Gene Signature. We utilized the Kaplan−Meier survival curve to compare OS between two groups categorized based on their median risk scores, as listed in Table S4 (Kaplan−Meier Analysis) and represented in Figure 10. Additionally, we employed the area under the time-dependent ROC curve (AUC) to evaluate the prognostic capability of the 31-gene signature. A higher AUC value reflects superior model performance. We observed a substantial difference in OS between the high-risk and low-risk groups, with a *p*-value < 0.05. We found that the OS rate of individuals with high expression of PTP1B, CDC25C, PTPN12, PTPRF, PTPRO, PPP1R12A, PPP1R13L, PPP2R5D, PPP1R37, and PPP1CA was lower than that of those with low expression ($P < 0.01$). No significant correlation was found between the expression of the other genes ($P > 0.05$), as shown in Figure 11. Therefore, PTP1B, CDC25C, PTPN12, PTPRF, PTPRO, PPP1R12A, PPP1R13L, PPP2R5D, PPP1R37, and PPP1CA were identified as the key genes. Furthermore, elevated expression levels of PTP1B (HR = 1.7; $P = 0.0038$), CDC25C (HR = 2; $P = 6.8 \times 10^{-5}$), PTPN12 (HR = 1.5; $P = 0.029$), PTPRF (HR = 1.6; $P = 0.0057$), PTPRO (HR = 1.5; $P = 0.020$), PPP1R12A (HR = 1.5; $P = 0.019$), PPP1R13L (HR = 1.5; $P = 0.03$), PPP2R5D (HR = 1.6; $P = 0.0065$), PPP1R37 (HR = 1.5; $P = 0.028$), and PPP1CA (HR = 1.6; $P = 0.01$) were strongly associated with poorer survival in HCC patients. These findings suggest that higher expression levels of these 10 genes at diagnosis may serve as adverse prognostic markers, potentially leading to reduced OS in HCC patients.

In disease-free survival (DFS) analysis, gene expressions of HCC patients were categorized into low-expression and high-expression groups (cutoff-high at 50%, cutoff-low at 50%). As depicted in Figure 12, lower expression levels resulted in significantly higher survival probabilities ($P < 0.05$). Specifically, PTP1B (HR = 1.4; $P = 0.027$), CDC25C (HR = 2; $P = 5.0 \times 10^{-6}$), PPP2R5D (HR = 1.5; $P = 0.0069$), PPP1R13L (HR = 1.4; $P = 0.037$), PPP1R37 (HR = 1.6; $P = 0.016$), and CTDP1 (HR = 1.4; $P = 0.03$) were strongly associated with poorer survival in HCC patients. Additionally, PPP1R3E (HR = 1.4; $P = 0.028$) was associated with higher survival in HCC patients. Survival curve analysis for both OS and DFS demonstrated the effectiveness of these genes in predicting HCC patients' prognosis.

Immunohistochemistry Analysis. The HPA (http://www.proteinatlas.org/) is a website that provides immunohistochemistry-based expression data for 20 tumor tissues, 47 cell lines, 48 human normal tissues, and 12 blood cells. In our study, we utilized immunohistochemistry images to directly compare the protein expression of ten genes between normal and HCC tissues, as illustrated in Figure 13. Interestingly, we found that PPP1CA, PTPN2, PTEN, PPP6R3, PPP4R2, PPP2R5D, CTDP1, PPP1R13L, PPP1R3E, and PTPN13 proteins were not expressed in normal liver tissues but exhibited high to medium levels of expression in HCC tissues. Additionally, PPP1R9A, PPP1CB, and PTPRF were lowly expressed in normal tissues but highly expressed in tumor tissues. Notably,

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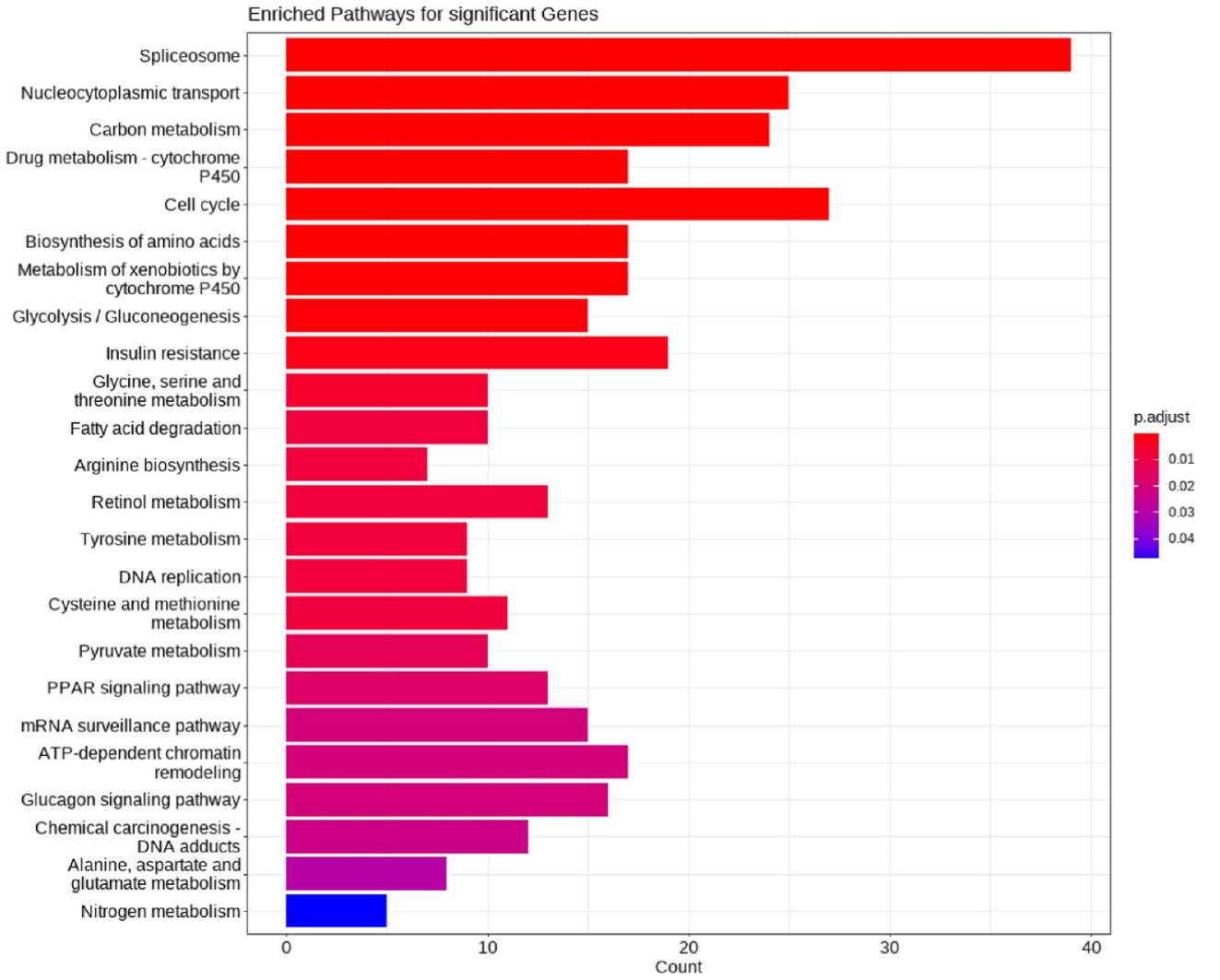

Figure 4. KEGG pathway enrichment using ORA of significant phosphoproteins in HCC from the CPTAC database correlated with genes in the high-risk group within the red module.

PPP1R12B and PPP1R12A were not detected in normal tissues, and there were no images available for them on HCC tissues.

DISCUSSION

HCC is the sixth most common cancer worldwide. In the past two decades, significant progress has been made in HCC treatment modalities, including surgical resection, ablation, liver transplantation, and targeted therapy. Despite these advancements, the 5 year survival rate for HCC remains suboptimal, with radiotherapy generally avoided because of its potential for liver toxicity. Liver transplantation is a potential option for HCC patients; however, its application faces limitations, including a shortage of available liver donors, low success rates beyond Milan criteria, and the influence of etiological factors, particularly the high relapse rate associated with HCV infection. HCC is characterized by a high mortality rate, yet the development of biomarkers for HCC treatment remains an ongoing challenge. Posttranslational modification of proteins alters protein functions and PPIs by modifying protein structures. Phosphorylation and dephosphorylation are reversible posttranslational modifications that generally regulate the activation and inhibition of intracellular signaling pathways. More than 70% of cellular proteins have been demonstrated to be regulated through phosphorylation and dephosphorylation. Protein phosphorylation carried out by kinases serves as a crucial regulatory mechanism in various cellular activities and has been implicated in HCC development and progression. However, despite their significance, fewer phosphatases, which govern dephosphorylation, have been identified compared to kinases. While about 518 kinases have been characterized in humans, only 137 phosphatases have been unveiled. Nonetheless, research has illuminated the critical roles that phosphatases play in liver disease and HCC development. Because of the complicated molecular mechanisms involved, HCC remains one of the most life-threatening malignancies national worldwide. Consequently, there is an urgent need for prognostic biomarkers to predict outcomes and formulate individualized treatment plans for HCC patients. Although some potential gene markers with predictive value for HCC patients have been identified with the advancement of gene sequencing technology, their numbers remain limited. To enhance HCC prognosis and patient care, there is an immediate requirement to identify additional biomarkers with higher prediction accuracy. In the present study, we did a comprehensive analysis of proteomics through CPTAC.

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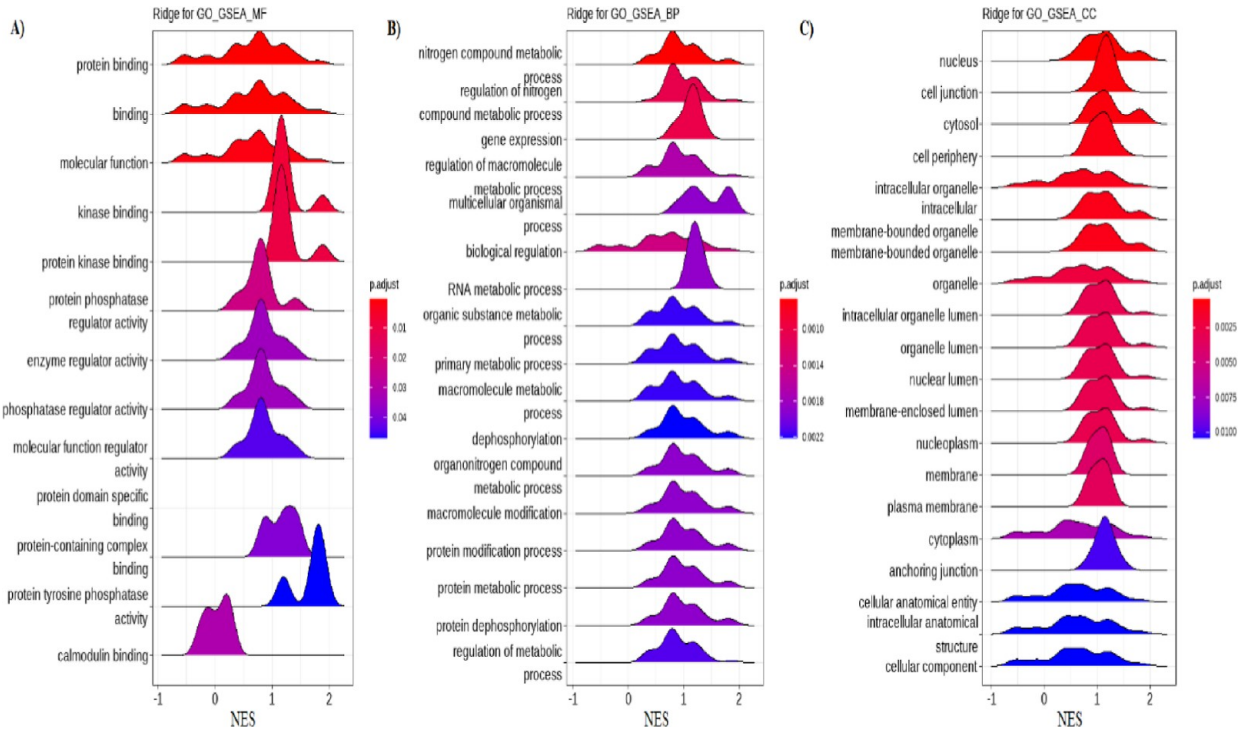

Figure 5. GO Analysis using GSEA of 31 significant genes correlated with genes in the high-risk group within the red module, revealing (A) significant MF, (B) significant BP, and (C) significant CC.

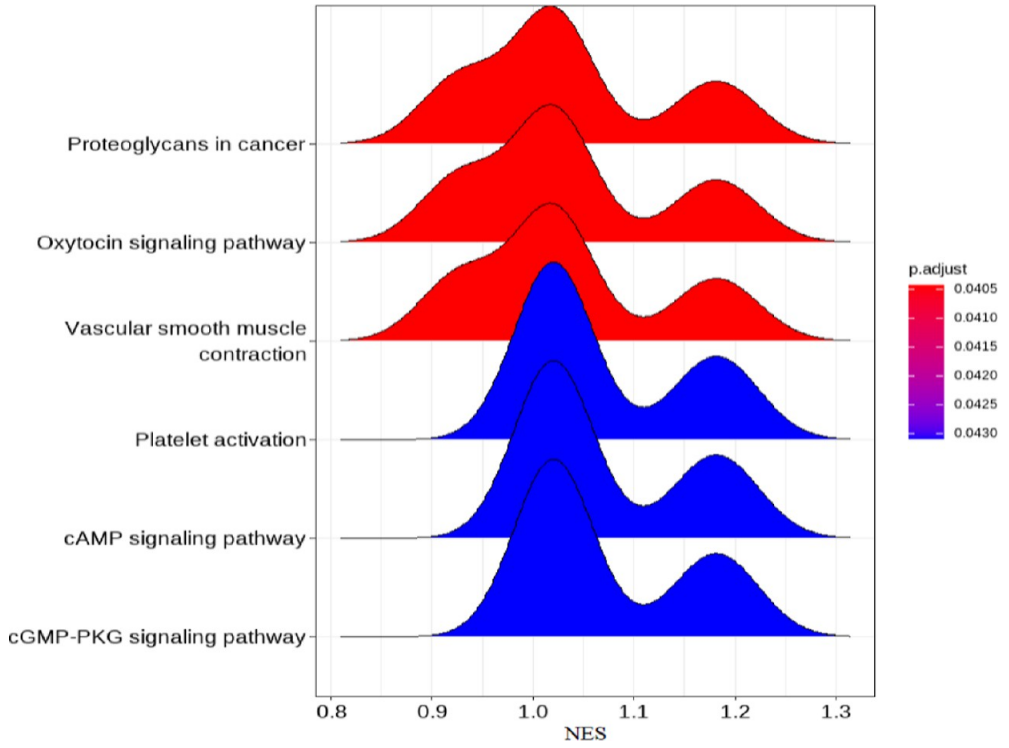

Figure 6. KEGG pathway enrichment using GSEA of 31 significant phosphoproteins (upregulated and downregulated) correlated with genes in the high-risk group within the red module.

We initially identified a total of 11,547 phosphorylation sites associated with 4043 phosphoproteins. Notably, these sites were quantified in over 50% of the samples. Among these, a substantial 9994 phosphorylation sites displayed significant differential expression. Specifically, 8198 sites showed substantial upregulation, while 1796 sites exhibited downregulation in tumor samples compared to their corresponding nontumor counterparts. This differentiation was established through a paired two-sided Student's *t*-test, with BH-adjusted *p*-values <0.05. Within this data set, we identified 110 phosphorylation sites. Among them, 105 exhibited significant changes between samples (with *p*-values <0.05). We performed an extensive

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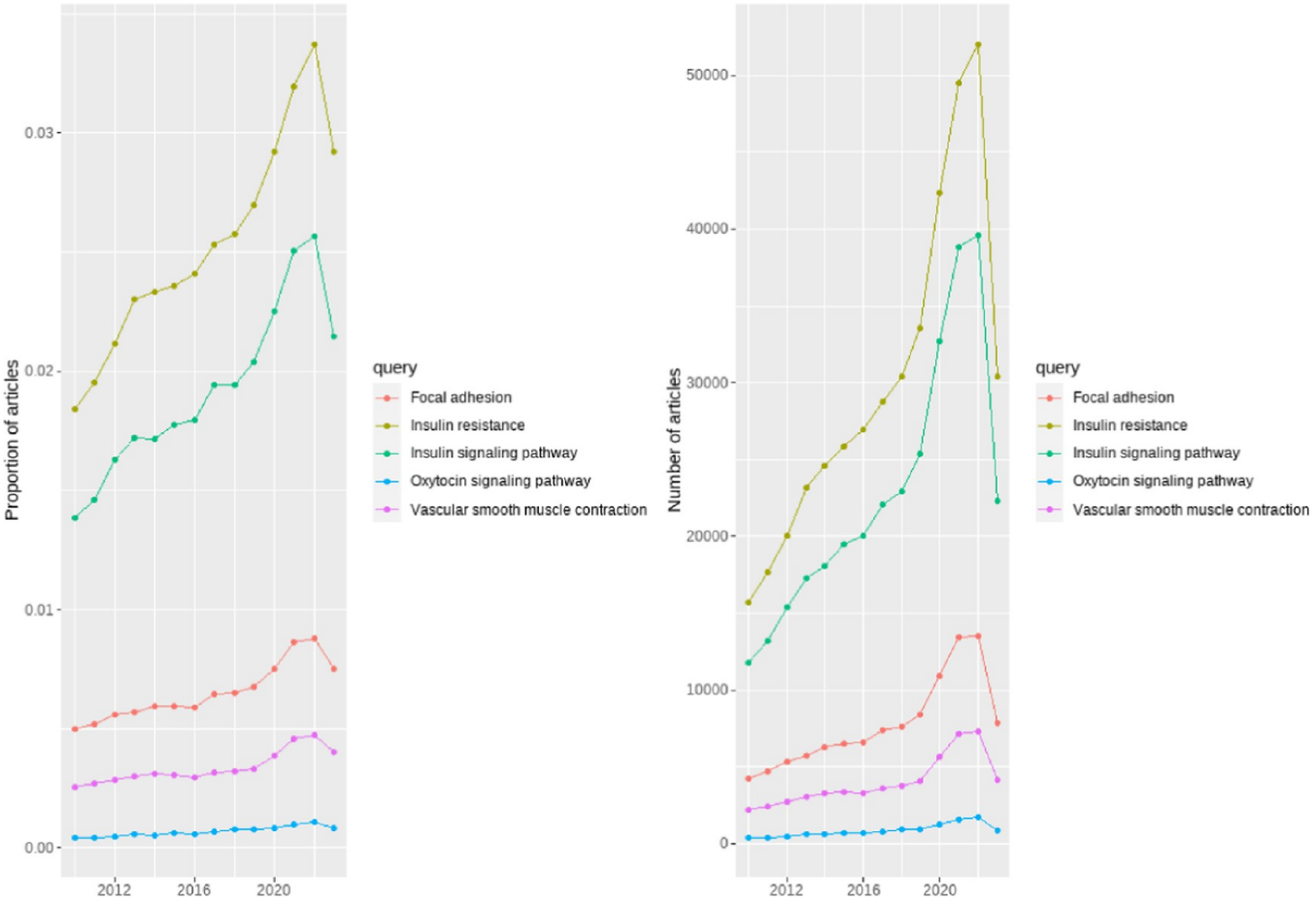

Figure 7. PmcPlot of enrichment analysis of 31 significant protein phosphatases.

analysis of these 105 protein phosphatases and their associated phosphosites to quantify the extent of upregulation and downregulation. Our findings revealed that 28 unique genes were upregulated, and 3 unique genes exhibited downregulation. We are eagerly anticipating further studies to delve into the functions of these proteins and their phosphorylation sites in HCC.

In this study, we conducted an in-depth investigation and discussion of the upregulated genes encoding protein phosphatases, including CDC25C, PPP1R12A, PPP1R15B, PPP1CB, PPP4R2, DUSP9, PPP1R2, PPP1CA, PPP1R9A, SSH2, PTPN2, PPP1R10, PPP1R13L, PTEN, PTPN1, CTDP1, PPM1E, PTPRO, PTPRF, and PPP2R5D. We also explored the downregulated genes PTPN13, PPP1R12B, and PPP1R3E, along with their quantified phosphorylation sites. Utilizing bioinformatics methods, we obtained gene expression levels and predicted potential therapeutic targets. Our goal was to determine whether these genes were associated with clinical outcomes and to assess the effects of altered expression on HCC biology. Taken together, the results of the phosphopeptide enrichment strategy established in this study could provide novel insights into phosphoproteins involved in liver cancer, and comprehensive analysis of the phosphoproteome in tumor cell lines can help us understand the cascade of signaling pathways mediated by phosphorylation in cancers.

Protein Tyrosine Phosphatase Family. The PTP family plays a crucial role in diverse cellular functions and has emerged as promising therapeutic targets, especially in chronic diseases like cancer. Researchers have developed numerous PTP inhibitors over the past two decades, aiming at specific PTP molecules for potential therapeutic use. However, previous reviews on HCC explored dual roles of PTPs, encompassing both oncogenic and tumor-suppressive functions. Huang et al. examined the involvement of diverse PTPs, including receptor PTPs, nonreceptor PTPs, and DUSPs, and their respective signaling pathways within HCC.[26] In contrast, another review specifically delved into the interplay between PTPs, inflammatory cytokines, and drug resistance in chemotherapy, elucidating how PTPs regulate inflammatory reactions and influence drug resistance, thus impacting the progression of HCC.[27] However, both reviews lacked thorough investigations into critical facets, such as PTP expression, identification of phosphorylation sites, PPIs, survival analysis associated with PTPs, and a comprehensive analysis of the phosphoproteome, especially concerning tyrosine in HCC samples. This current study is aimed at providing pragmatic insights to enhance researchers' understanding of PTP roles in cancer progression. By addressing these informational gaps, this research seeks to facilitate the discovery of additional therapeutic strategies for combating cancer. Several PTPs have been quantified in this study, and their detailed analysis will be presented.

PTP1B, Tyrosine-Protein Phosphatase Nonreceptor Type 1. PTP1B, tyrosine-protein phosphatase nonreceptor type 1 (PTPN1,) is related to phosphotyrosine phosphatases that attenuate insulin signaling by dephosphorylating the bisphosphorylated regulatory loop of the IR. The structure of PTP1B comprises an N-terminal catalytic domain, two proline-rich sequences, and a C-terminal hydrophobic region. PTP1B

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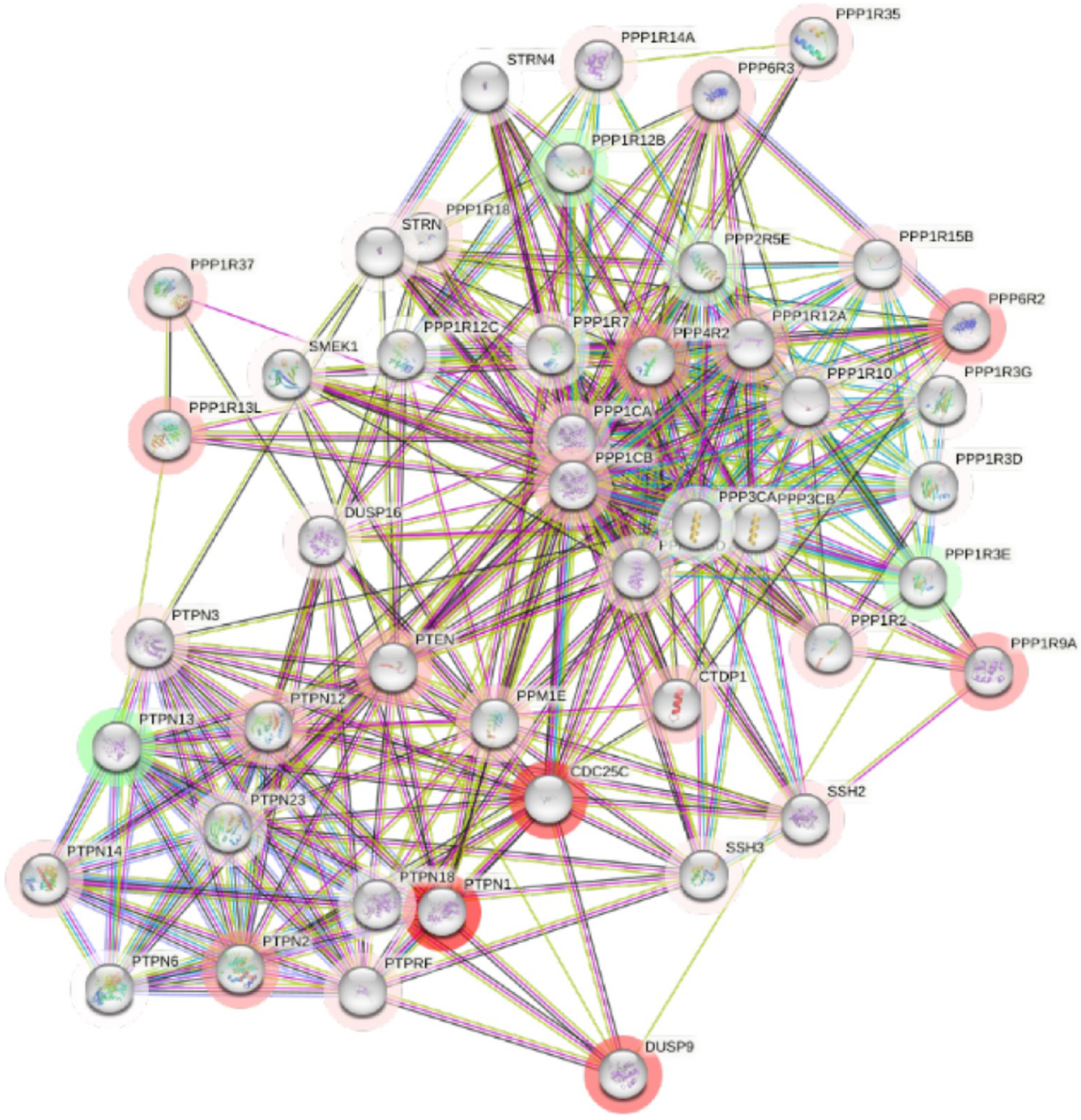

Figure 8. PPI of 31 significant phosphoproteins (upregulated proteins in red circles, downregulated proteins in green circles) with connection line thickness indicating betweenness, node color gradients representing log FC change, and green denoting coexpression nodes from the STRING database.

serves as a critical regulator in the pathogenesis of conditions such as diabetes and obesity.[28] Recent research has revealed that pituitary homeobox 1 (PITX1) acts as a tumor suppressor in hepatocarcinogenesis by activating the expression of p120RasGAP, also known as Ras p21 protein activator 1. This activation leads to the inactivation of Ras by converting GTP into GDP.[29] Downregulation of PITX1 mRNA and protein expression is frequently observed in HCC patient samples with poorer prognoses.[30] PTP1B can facilitate the proteasome-mediated degradation of PITX1 by directly dephosphorylating PITX1 at residues Y160, Y175, and Y179. The decline in PITX1 levels reduces p120RasGAP's transcriptional activity.[31] Through the PITX1-p120RasGAP signaling axis, PTP1B exhibits tumor-promoting effects in HCC. PTP1B exhibits a dual role in cancer, functioning as an oncogene through its interactions with several oncogenic substrates, including Src, extracellular signal-regulated kinase 1/2 (ERK1/2), p62dok, human epidermal growth factor receptor 2 (HER2), and p130Cas.[14,26] On the contrary, PTP1B can also act as a tumor suppressor by negatively regulating multiple oncogenic kinases, such as Bcr-Abl, JAK-STAT, and β-catenin.[31] We conducted an in-depth analysis and quantification of PTPN1 and its associated phosphorylation sites. In addition, we detected the phosphorylation sites (p-sites) of the identified phosphopeptides in our study. The results revealed that nearly all of the identified phosphopeptides contained four p-sites at positions S386, S50, S15, and S352. These phosphopeptides exhibited varying abundances, with corresponding log FC values of 2.05, 1.27, 1.26, and 0.80, respectively. Importantly, these p-sites are significantly overexpressed with a P-value $<4.35 \times 10^{-35}$. It is evident that phosphorylated serine (p-Ser) constituted a significant portion of the identified p-sites.

Tyrosine-Protein Phosphatase Nonreceptor Type 2. Tyrosine-protein phosphatase nonreceptor type 2 (PTPN2),

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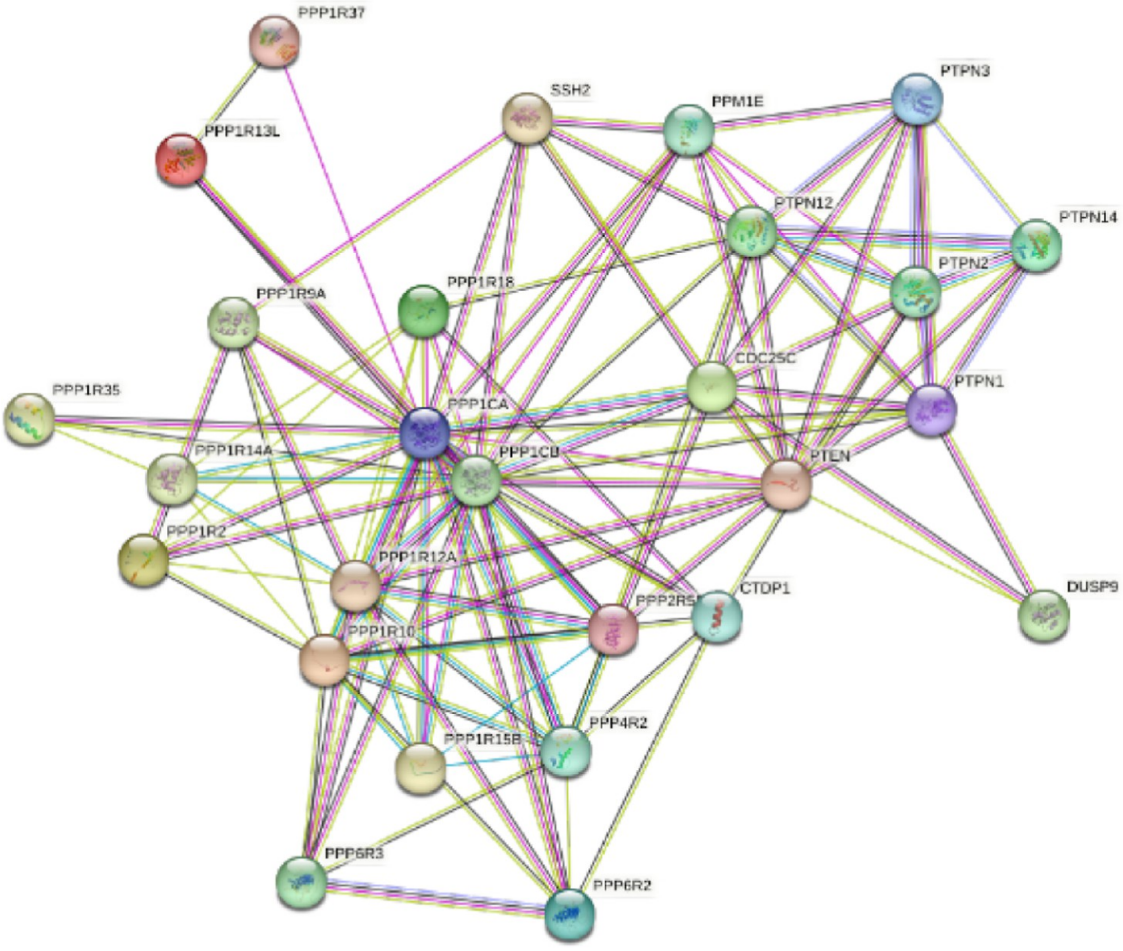

Figure 9. PPI of the top 15 significant genes related to HCC (threshold >200 scores).

also called TCPTP (T cell protein tyrosine phosphatase), belongs to the PTP family of signaling proteins. Its role in dephosphorylating tyrosine residues is crucial for regulating various cellular processes, including cell growth, development, differentiation, survival, and migration. PTPN2 gained significant attention when it was identified as a potential target for cancer immunotherapy through in vivo CRISPR library screening.[32] The diversity of PTPN2 effects in different types of tumors makes it a potential target for tumor immunotherapy. One of the key factors contributing to the interest in PTPN2 is the findings from genome-wide association studies. These studies revealed that loss-of-function single-nucleotide polymorphisms in the PTPN2 gene are associated with an increased risk of inflammatory bowel disease and various immune disorders such as Crohn's disease, Type 1 diabetes, rheumatoid arthritis, and celiac disease. In tumor cells, the deletion of PTPN2 enhances IFN-γ signaling and antigen presentation to T cells, leading to increased sensitivity to cytokine-induced growth arrest. This suggests that targeting PTPN2 could have therapeutic potential in enhancing the efficacy of immunotherapy.[33] Interestingly, PTPN2 levels are upregulated in certain human cancers that do not respond well to current immunotherapies. Deletion of PTPN2 in tumor cells has been shown to improve the effectiveness of immunotherapy by enhancing interferon-γ-mediated signaling and growth suppression. In the context of obesity, the oxidative hepatic environment can lead to the inactivation of PTPN2. This inactivation results in increased STAT1 and STAT3 signaling, promoting T cell recruitment and contributing to conditions like nonalcoholic steatohepatitis, fibrosis, and HCC. Notably, PTPN2 deletion in hepatocytes has been found to accelerate chemical carcinogen-induced HCC in mice,[33] with STAT3 signaling playing a significant role in this PTPN2-associated pathogenesis of HCC.[34] Furthermore, we detected the phosphorylation site (p-site) of the identified phosphopeptide in our study. The result indicated that this identified phosphopeptide was at S327, with corresponding log FC values of 1.37 and a *P*-value $<1.05 \times 10^{-39}$.

Tyrosine-Protein Phosphatase Nonreceptor Type 3. Tyrosine-protein phosphatase nonreceptor type 3 (PTPN3), also known as PTPH1, is a member of the nontransmembrane PTP family. While the exact mechanisms by which PTPN3 regulates tumor progression, particularly in liver cancer, are not fully understood, there are some notable findings. Furthermore, in our study, we identified the phosphorylation site (p-site) of the phosphopeptide as S228, with corresponding log FC values of 0.60 and a *P*-value $<1.31 \times 10^{-15}$. One significant discovery is related to the expression of PTPN3 in human hepatoma cells and its impact on HBV replication. It has been observed that the presence of PTPN3 in these cells leads to a significant reduction in HBV replication. Importantly, it has been found that the deletion of the FERM domain of PTPN3 impairs its ability to inhibit HBV replication.[35] This suggests that PTPN3 may play a role in suppressing liver cancer progression through its positive regulation of the TGF-β signaling pathway. Specifically, PTPN3 interacts with TβRI (TGF-β receptor type I) and promotes the

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Table 4. KEGG Pathways Identified from the STRING Network Analysis for the 31 Significant Genes Using R Programing

category	term	description	preferred names	number_of_Genes	number_of_genes_in_background	*P*_value	FDR
KEGG	hsa04931	insulin resistance	PPP1CA, PTPRF, PPP1R3D, PTPN1, PTEN, PPP1CB, PPP1R3E	7	107	1.31×10^{-8}	4.39×10^{-6}
KEGG	hsa04114	oocyte meiosis	CDC25C, PPP1CA, PPP2R5E, PPP3CB, PPP3CA, PPP1CB, PPP2R5D	7	120	2.77×10^{-8}	4.65×10^{-6}
KEGG	hsa04921	oxytocin signaling pathway	PPP1R12C, PPP1CA, PPP3CB, PPP3CA, PPP1CB, PPP1R12A, PPP1R12B	7	149	1.14×10^{-7}	1.28×10^{-5}
KEGG	hsa04270	vascular smooth muscle contraction	PPP1R12C, PPP1R14A, PPP1CA, PPP1CB, PPP1R12A, PPP1R12B	6	133	1.24×10^{-6}	8.38×10^{-5}
KEGG	hsa04728	dopaminergic synapse	PPP1CA, PPP2R5E, PPP3CB, PPP3CA, PPP1CB, PPP2R5D	6	128	9.98×10^{-7}	8.38×10^{-5}
KEGG	hsa04810	regulation of actin cytoskeleton	PPP1R12C, SSH3, PPP1CA, PPP1CB, PPP1R12A, SSH2, PPP1R12B	7	209	1.03×10^{-6}	8.38×10^{-5}
KEGG	hsa04910	insulin signaling pathway	PPP1CA, PTPRF, PPP1R3D, PTPN1, PPP1CB, PPP1R3E	6	133	1.24×10^{-6}	8.38×10^{-5}
KEGG	hsa04510	focal adhesion	PPP1R12C, PPP1CA, PTEN, PPP1CB, PPP1R12A, PPP1R12B	6	198	1.13×10^{-5}	0.00045
KEGG	hsa05205	proteoglycans in cancer	PPP1R12C, PPP1CA, PPP1CB, PPP1R12A, PTPN6, PPP1R12B	6	196	1.07×10^{-5}	0.00045
KEGG	hsa04720	long-term potentiation	PPP1CA, PPP3CB, PPP3CA, PPP1CB	4	64	2.49×10^{-5}	0.00084
KEGG	hsa05031	amphetamine addiction	PPP1CA, PPP3CB, PPP3CA, PPP1CB	4	66	2.79×10^{-5}	0.00085
KEGG	hsa04218	cellular senescence	PPP1CA, PTEN, PPP3CB, PPP3CA, PPP1CB	5	150	4.16×10^{-5}	0.0012
KEGG	hsa04022	cGMP-PKG signaling pathway	PPP1CA, PPP3CB, PPP3CA, PPP1CB, PPP1R12A	5	162	5.93×10^{-5}	0.0015
KEGG	hsa05235	PD-L1 expression and PD-1 checkpoint pathway in cancer	PTEN, PPP3CB, PPP3CA, PTPN6	4	88	8.17×10^{-5}	0.002
KEGG	hsa03015	mRNA surveillance pathway	PPP1CA, PPP2R5E, PPP1CB, PPP2R5D	4	93	1.00×10^{-4}	0.0022
KEGG	hsa04660	T cell receptor signaling pathway	PPP3CB, PPP3CA, PTPN6, PTPRC	4	101	0.00014	0.0029
KEGG	hsa04261	adrenergic signaling in cardiomyocytes	PPP1CA, PPP2R5E, PPP1CB, PPP2R5D	4	147	0.00055	0.0108
KEGG	hsa04520	adherens junction	PTPRF, PTPN1, PTPN6	3	67	0.00071	0.0133
KEGG	hsa04360	axon guidance	SSH3, PPP3CB, PPP3CA, SSH2	4	177	0.0011	0.019
KEGG	hsa04662	B cell receptor signaling pathway	PPP3CB, PPP3CA, PTPN6	3	78	0.0011	0.019
KEGG	hsa04922	glucagon signaling pathway	PPP3CB, PPP3CA, SMEK1	3	101	0.0022	0.0358
KEGG	hsa04071	sphingolipid signaling pathway	PPP2R5E, PTEN, PPP2R5D	3	116	0.0033	0.05

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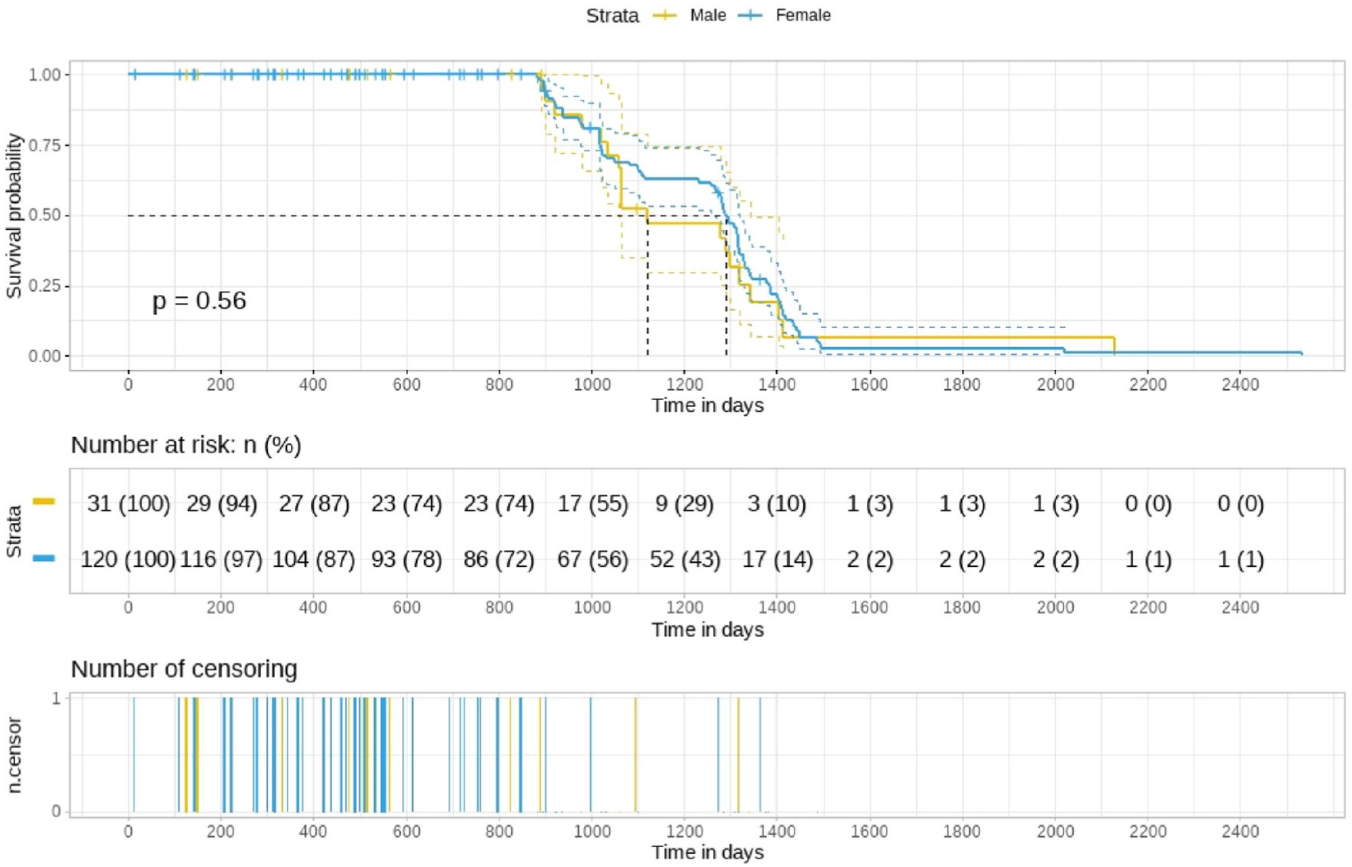

Figure 10. Kaplan−Meier OS plots, number at risk, and number of censorings in HCC generated through the R program. Dashed lines represent the upper and lower confidence intervals.

stability of TβRI. This interaction enhances the tumor-suppressive functions of TGF-β signaling. TGF-β is known to have antitumor properties, and its signaling pathway can inhibit the growth and progression of cancer cells.[35] Therefore, PTPN3 may act as a tumor suppressor in liver cancer by facilitating TGF-β-mediated anticancer effects. In summary, PTPN3 appears to achieve its tumor suppressor functions through its involvement in the TGF-β signaling pathway. This mechanism provides new insights into the pathogenesis of liver cancer and the potential role of PTPN3 in regulating this disease.

Tyrosine-Protein Phosphatase Nonreceptor Type 12. Tyrosine-protein phosphatase nonreceptor type 12 (PTPN12), also recognized as PTP−PEST, was initially isolated from human skeletal muscle. The structure of PTPN12 features a C-terminal PEST motif, which serves as a PPI domain. PTPN12 plays a pivotal role in the regulation of multiple oncogenic tyrosine kinases, including HER2 and EGFR, and is crucial for embryonic development.[36] Furthermore, PTPN12 is a key regulator of cell migration and cell−cell junctions, achieved through interactions with cytoskeletal and signaling proteins. In numerous human malignancies, PTPN12 has been characterized as a tumor suppressor. These malignancies include breast cancer, colon cancer, ovarian cancer, nasopharyngeal carcinoma, and esophageal squamous cell carcinoma.[26] Furthermore, we identified the phosphorylation sites (p-sites) of the phosphopeptides in the current study. The results showed that almost all of the identified phosphopeptides contained three p-sites at positions S435, S342, and S603. These phosphopeptides exhibited varying abundances, with corresponding log FC values of 1.02, 0.823, and 0.818, respectively. Notably, these p-sites showed significant overexpression, with a *P*-values 5.02×10^{-31}, 6.91×10^{-22}, and 1.85×10^{-36}, respectively. Additionally, PTPN12 plays a significant role in the development of HCC.

Tyrosine-Protein Phosphatase Nonreceptor Type 13. Tyrosine-protein phosphatase nonreceptor type 13 (PTPN13), also known as protein tyrosine phosphatase-Basophil (PTP-BAS), possesses a structural composition characterized by a catalytic PTP domain at its C-terminus, complemented by two significant structural domains: a region containing five PDZ domains and a FERM domain that binds to the plasma membrane and cytoskeletal elements. Cumulative evidence suggests that PTPN13 functions as a tumor suppressor in various human cancers, including colorectal cancer and breast cancer. In the context of HCC, PTPN13 expression is frequently downregulated or lost in clinical samples and HCC cell lines. Importantly, higher PTPN13 expression correlates positively with OS while demonstrating a negative correlation with the cumulative recurrence rate. Additionally, HCC cell lines exhibiting low PTPN13 expression exhibit a greater metastatic potential.[37] In this study, we report for the first time that PTPN13 was found to be downregulated in HCC samples, with log FC values of −0.747 and −0.7924 and corresponding *P*-values of 3.29×10^{-15} and 6.35×10^{-11}, respectively. These results identified the p-sites at S1063 and S240. Importantly, limited research has focused on PTPN13 in cancer patients, necessitating urgent data collection to determine critical information about this protein.

Protein Tyrosine Phosphatase Nonreceptor Type 14. Protein tyrosine phosphatase nonreceptor type 14 (PTPN14) is a nonreceptor protein tyrosine phosphatase involved in

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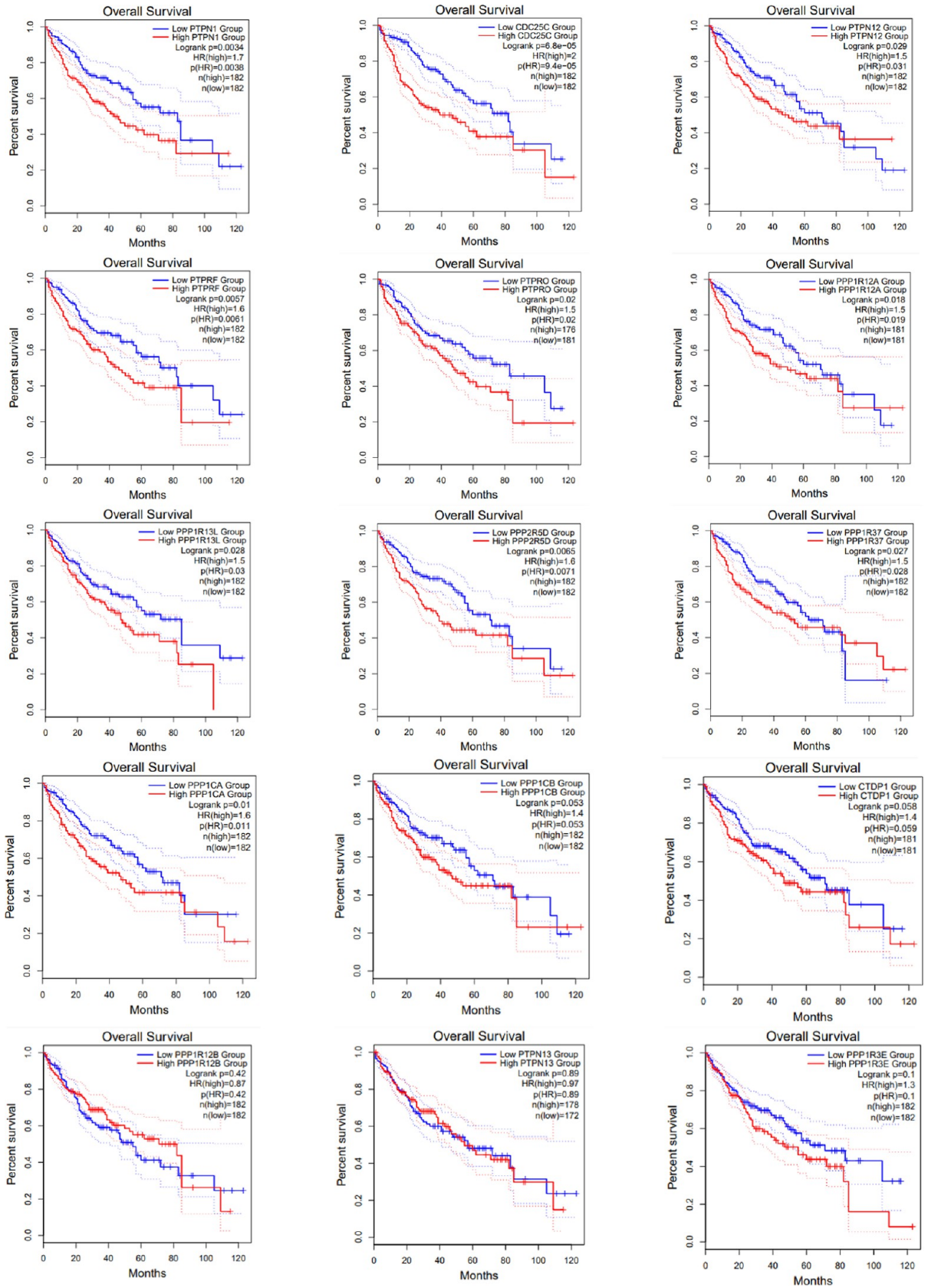

Figure 11. Kaplan−Meier OS plot analysis demonstrates that a group with elevated levels of 10 proteins experienced a worse prognosis than the group with lower levels ($P < 0.05$). Dashed lines represent the upper and lower confidence intervals. The last 3 graphs depict downregulated proteins ($P > 0.05$).

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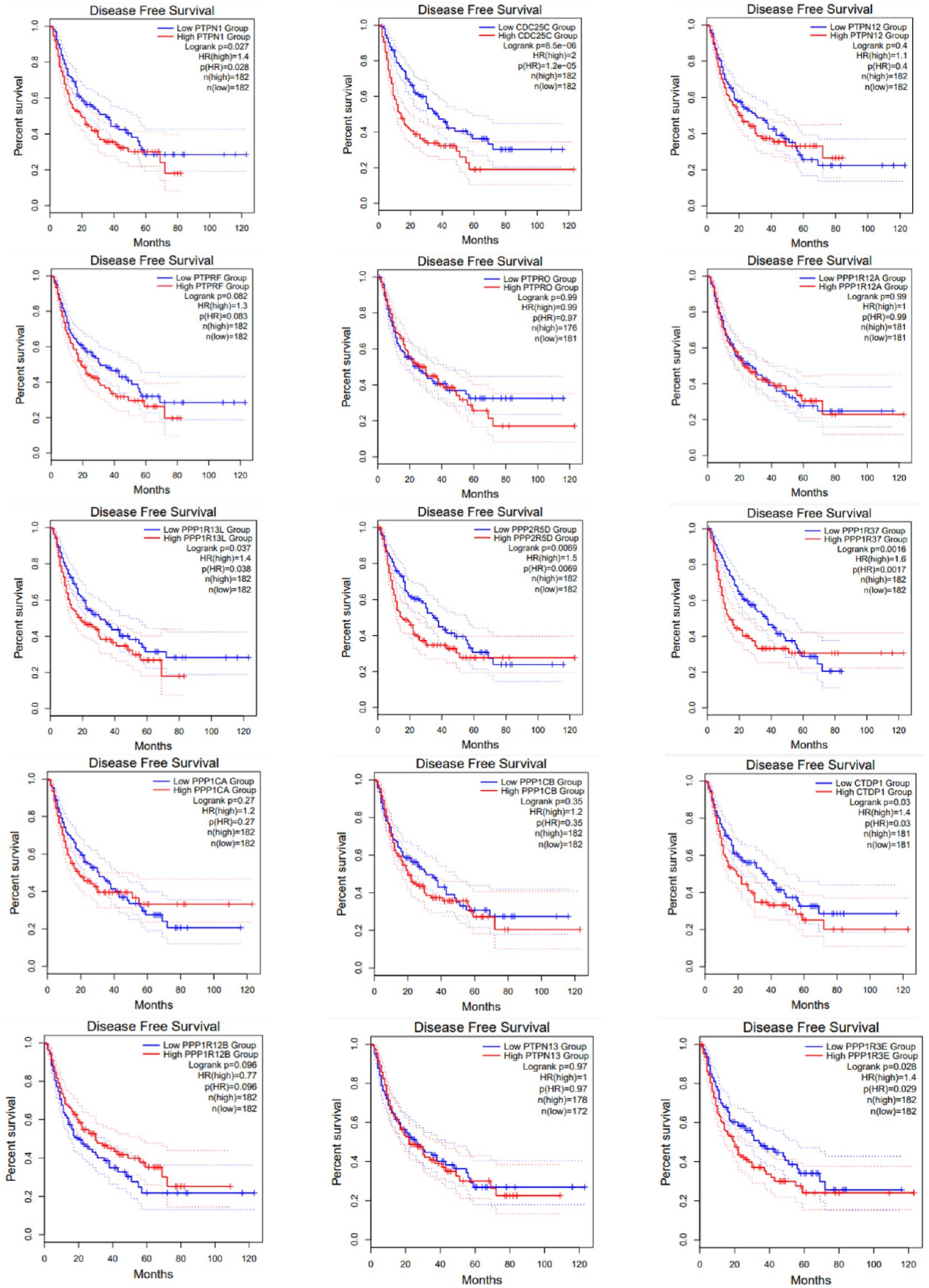

Figure 12. Kaplan−Meier DFS plot analysis demonstrates that a group with elevated levels of 10 proteins experienced a worse prognosis than the group with lower levels ($P < 0.05$). Dashed lines represent the upper and lower confidence intervals.

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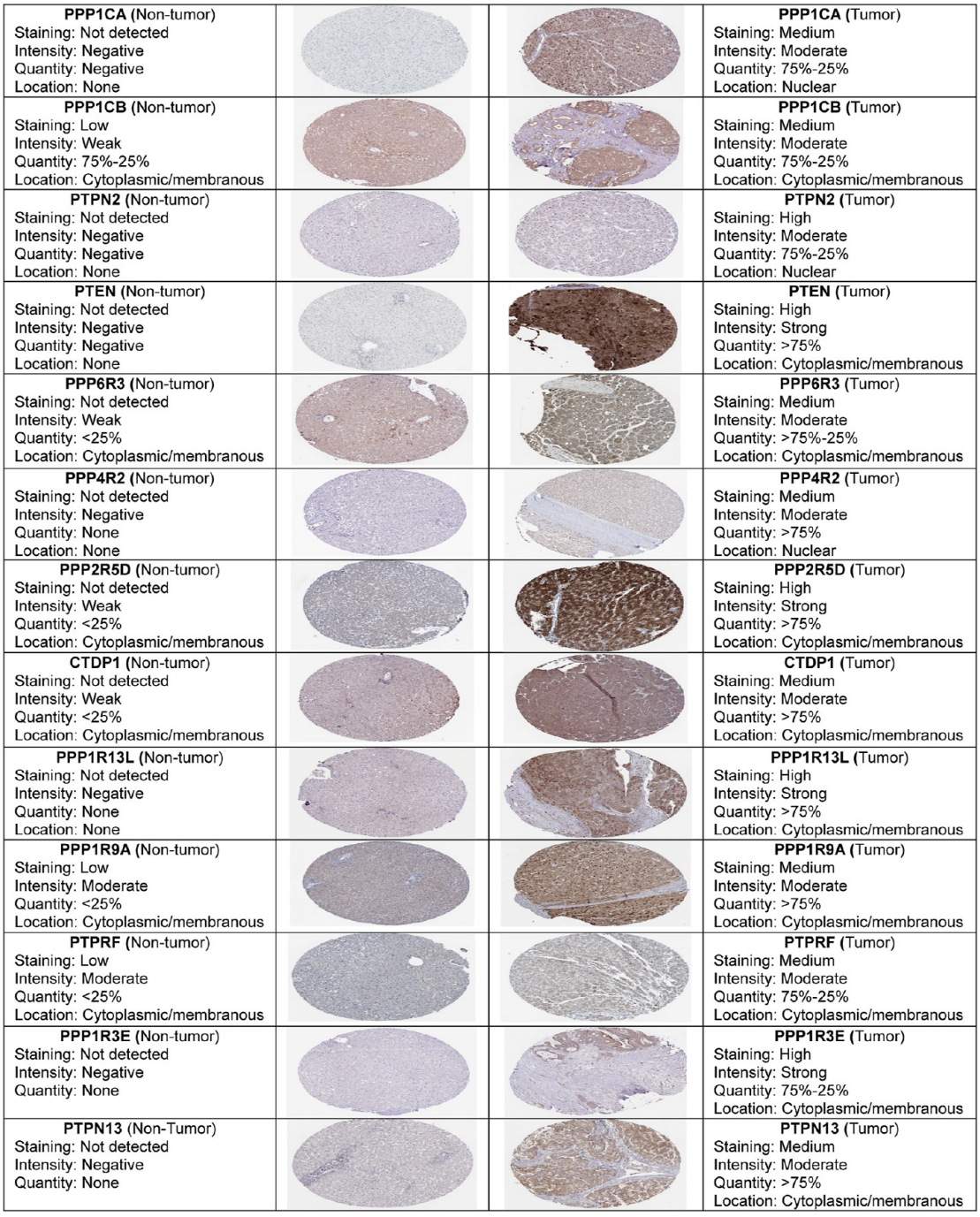

Figure 13. Immunohistochemistry images of 10 hub genes in normal liver tissues and HCC derived from the HPA database (http://www.proteinatlas.org/).

regulating various cellular processes, including cell adhesion, growth, differentiation, and cancer progression. It can be localized in both the cytoplasm and nucleus. Mutations in PTPN14 have been identified in various human malignancies, such as breast cancer, colon cancer, and skin basal cell carcinoma, implicating PTPN14 as a potential tumor and metastasis suppressor.[38] Several potential substrates of PTPN14, including β-catenin, p130Cas, RIN1, PRKCD, and YAP, are all associated with tumor progression and metastasis.[39] In HCC, it was observed that NPM1 exhibited high expression levels, promoting cell proliferation, while ATF5 had an inhibitory effect on proliferation.[40] These findings establish a mechanistic connection between increased NPM1 expression and decreased ATF5 in HCC, indicating that NPM1's regulation of ATF5 contributes to cell proliferation in HCC. Our mechanistic investigations suggest that NPM1 influences PTPN14 localization and modulates YAP by retaining PTPN14 within the nucleus during hypoxic conditions in HCC. Collectively, these findings propose a potential therapeutic approach to counteract hypoxia-induced sorafenib resistance in HCC by disrupting the NPM1/PTPN14/YAP axis. Our findings revealed that PTPN14 was upregulated in HCC, with log FC values of 0.759 and 0.696 and corresponding *P*-

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values of 5.0×10^{-28} and 4.36×10^{-21}, respectively. The identified p-sites were S312 and S642.

Protein Tyrosine Phosphatase Receptor Type O. Protein tyrosine phosphatase receptor type O (PTPRO), also known as glomerular epithelial protein 1 (GLEPP1), was initially discovered in the human renal glomerulus. PTPRO's structural composition includes a single intracellular catalytic domain responsible for catalyzing the dephosphorylation of tyrosine peptides as well as a transmembrane domain. PTPRO is recognized as a tumor suppressor in multiple human cancers, including chronic lymphocytic leukemia, lung cancer, and breast cancer.[26] Additionally, in our study, we identified the upregulated phosphorylation site (p-site) of the phosphopeptide as S865, with corresponding log FC values of 0.712 and a *P*-value $<1.03 \times 10^{-10}$. In HCC, PTPRO expression is significantly diminished in tumor specimens compared to adjacent tissues, often attributed to hypermethylation of the PTPRO promoter. In a rat model of HCC induced by a folate/methyl-deficient diet, PTPRO mRNA levels were markedly reduced, and the gene was found to be hypermethylated immediately upstream of the transcription start site, as revealed in a genome-wide screen for hypermethylated genes.[41] Notably, PTPRO gene methylation was absent in livers of animals on a normal diet. The restoration of PTPRO mRNA expression was achieved through treatment with 5-azacytidine after transplantation of hepatoma cells. Similar findings were observed in human HCC samples, where the CpG island of PTPRO exhibited significant hypermethylation. Functional studies demonstrated that PTPRO overexpression inhibited cell proliferation and promoted apoptosis in HCC cell lines. Conversely, tumor number and size were enhanced in PTPRO knockout mice.[42] Multiple signaling pathways contribute to PTPRO's tumor suppressor role in HCC. PTPRO negatively correlates with STAT3 activity in HCC tissues, indicating its potential role in suppressing HCC by controlling STAT3 activation. PTPRO mediates STAT3 Y705 dephosphorylation through janus kinase 2 and S727 dephosphorylation via PI3K signaling.[42] Furthermore, PTPRO's interaction with Toll-like receptor 4 (TLR4) plays a protective role against hepatocellular tumorigenesis and progression. PTPRO expression positively correlates with TLR4 expression in HCC specimens. TLR4 activity and expression increased in PTPRO-overexpressing HCC cells following lipopolysaccharide stimulation. Studies unveiled that PTPRO regulates TLR4 through nuclear transcription factor-κB (NF-κB) activation, with increased phosphorylation levels of IκBα and NF-κB/P65 in PTPRO-overexpressing HCC cells. Additionally, a truncated isoform of PTPRO, known as PTPROt, emerged as a critical player in antitumor immunity within the HCC microenvironment in a mouse model. PTPROt deficiency attenuated T cell-mediated antitumor immunity and markedly promoted HCC growth in mice.[43]

Protein Tyrosine Phosphatase Receptor Type F. Protein tyrosine phosphatase receptor type F (PTPRF), also known as leukocyte common antigen-related, is characterized by its structural composition, comprising an extracellular domain housing three immunoglobulin domains and eight fibronectin type III domains, a single transmembrane region, and two tandem cytoplasmic catalytic domains, D1 and D2. In the context of HCC, PTPRF demonstrates significant clinical relevance. It is frequently downregulated in HCC patients, while its upregulation is associated with a more favorable prognosis.[44] Through a loss-of-function screening of the phosphatome to identify genes suppressing tumor initiation in HCC, PTPRF emerged as one of the top-scoring tumor suppressor candidates. Functional studies underscore the tumor-suppressive properties of PTPRF. It suppresses cell proliferation and colony formation in Huh7 and SK-Hep1 cells and inhibits HepG2 xenograft tumor growth in nude mice. Importantly, the phosphatase activity of PTPRF is essential for its tumor suppressor function. Mechanistically, PTPRF exerts its effects by modulating phosphorylation events within the ERK1 and ERK2 pathways. PTPRF directly interacts with upstream factors of ERK, including v-src avian sarcoma viral oncogene homologue (SRC) and PP2A. It facilitates the removal of phosphate groups from SRC at Y416 and PP2AC (the catalytic subunit C of PP2A) at Y307. This activity results in the suppression of SRC activity and the activation of PP2A.[44] In our recent study, we conducted a rigorous analysis and successfully identified and quantified the phosphorylation of PTPRF at S1299. Our findings unveiled a significant increase in expression (log FC = 0.728; *P*-value = 4.42×10^{-15}) of these phosphorylation events in tumor tissues compared to their nontumor counterparts. In summary, PTPRF serves as a crucial tumor suppressor in HCC, exerting its effects through the modulation of key signaling pathways and phosphorylation events.

Dual-Specificity Phosphatase (CDC25C). Dual specificity phosphatase (CDC25C) is a cell division cycle protein that plays a critical role in cell cycle regulation. In the context of liver cancer, it has been studied for its involvement in the dysregulation of the cell cycle, which is a common characteristic of cancer cells. Furthermore, CDC25C is a phosphatase enzyme that helps regulate the cell cycle by promoting the progression from the G2 phase to the mitotic (M) phase. It does so by activating cyclin-dependent kinases (CDKs) through dephosphorylation, which is necessary for the cell to enter mitosis. In some studies, overexpression of CDC25C has been observed in liver cancer (HCC) tissues. This overexpression is believed to contribute to uncontrolled cell division and tumor growth. High levels of CDC25C expression in liver cancer have been associated with a poor prognosis and more aggressive tumor behavior. It is considered a potential biomarker for assessing the severity and prognosis of liver cancer. Because of its crucial role in cell cycle regulation and its potential as a prognostic marker, CDC25C has been explored as a therapeutic target in liver cancer. Several studies have provided evidence of the relationship between CDC25C and various human cancers. Ozen and Ittmann demonstrated a close association between CDC25C and the occurrence, development, and prognosis of prostate cancer.[45] Wang et al. further supported this by confirming that CDC25C overexpression plays a significant role in the development of squamous cell carcinoma of the female vulva.[46] Moreover, Xia et al. found that CDC25C serves as a predictor of poor prognosis in lung adenocarcinoma and may function in cell cycle regulation and FAS-mediated apoptosis.[47] Additionally, one study observed increased levels of CDC25B and CDC25C in both mouse and human skin cancers.[48] In previous reports, it has been documented that CHK2 phosphorylates CDC25C at Ser216 and Ser287 residues. This phosphorylation event creates a binding site for 14-3-3 proteins, leading to the translocation of CDC25C from the nucleus to the cytoplasm. This translocation effectively terminates the role of CDC25C in the cell cycle regulation.

In the current study, we successfully identified and quantified CDC25C phosphorylated at S216, revealing its overexpression (log FC = 1.88; *P*-value = 8.2×10^{-45}) in tumor patients when

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compared to nontumor patients. The phosphorylation of CDC25C at S216 is a critical component of the regulatory mechanism that activates CDC25C and plays a pivotal role in cancer progression. Abnormal regulation of CDC25C phosphorylation, including changes at S216, can lead to the dysregulation of the cell cycle, a common hallmark of cancer cells. Such alterations can result in uncontrolled cell division and contribute to tumor growth. This discovery underscores the potential significance of CDC25C and its phosphorylation sites, such as S216, as promising targets for therapeutic intervention. Modulating the phosphorylation status of CDC25C, particularly at S216, represents a potential strategy to regain control over the aberrant cell division observed in cancer cells. Elevated CDC25C expression was associated with significantly shorter DFS and OS in HCC patients, indicating its potential significance in HCC prognosis. To elucidate the likely pathogenic mechanism of CDC25C in HCC, we conducted PPI network analysis and GO enrichment analysis. This analysis revealed ten interacting proteins linked to CDC25C: CDK1, CCNB1, CHEK2, CHEK1, PLK1, CCNA2, YWHAZ, TP53, CCNB2, and WEE1. Additionally, GO enrichment analysis indicated that these genes are primarily involved in cell cycle regulation. Inhibition of CDC25C activity could potentially slow down or halt the uncontrolled cell division seen in cancer cells. It is important to note that while CDC25C is an interesting target in liver cancer research, treatments targeting this protein are still in the experimental stages, and clinical applications are evolving.

Dual-Specificity Phosphatase 9. Dual-specificity phosphatase 9 (DUSP9) is an enzyme that plays a critical role in the regulation of cellular signaling pathways by dephosphorylating specific tyrosine and threonine residues on proteins. DUSP9 has been of interest in cancer research because of its potential involvement in cancer development and progression. DUSP9 is known to act as a tumor suppressor in some contexts. Its enzymatic activity allows it to negatively regulate the activity of signaling molecules involved in cell proliferation and survival. By dephosphorylating specific targets, DUSP9 can inhibit signaling pathways that promote cancer cell growth. DUSPs, also known as MKPs, regulate MAPKs by dephosphorylating phosphotyrosine and phosphoserine/phosphothreonine residues on ERK, c-Jun N-terminal kinase, and p38.[49] This pathway is crucial for cell growth and differentiation. Dysregulation of MAP kinase signaling is common in cancer, including HCC. DUSP9's role in dephosphorylating and inactivating specific kinases can influence cell cycle progression. We aim to understand alterations in DUSP9 expression and activity in HCC patients that may have diagnostic and prognostic implications. High DUSP9 expression could potentially be associated with better outcomes in some cases because of its tumor-suppressing properties. More ongoing research aims to elucidate DUSP9 its specific functions, clinical relevance, and potential as a target for therapeutic intervention in HCC. In the current study, we have successfully identified and quantified DUSP9 phosphorylated at S16 and S189, revealing its overexpression (log FC = 1.78; *P*-value = 1.45×10^{-34}) and (log FC = 0.92; *P*-value = 2.52×10^{-16}) in tumor patients when compared to nontumor patients. The phosphorylation of these specific serine residues can have significant implications for the function of DUSP9 and its role in cellular processes in HCC. DUSP9 plays a crucial role in regulating cell proliferation and emerges as a potential predictor of recurrence following surgical intervention in HCC. These findings position DUSP9 as a promising candidate for both prognostic assessment and therapeutic targeting in HCC. Further investigations are essential to delve deeper into the precise mechanisms and regulatory pathways through which DUSP9 influences HCC.

Protein Phosphatase 1 Regulatory Subunit 12A. Protein phosphatase 1 regulatory subunit 12A (PPP1R12A), also known as myosin phosphatase target subunit 1, serves as a regulatory subunit of myosin phosphatase, playing a pivotal role in the regulation of smooth muscle contraction and cytoskeletal dynamics.[4] In the context of liver cancer, specifically HCC, our current study delves into the potential roles of PPP1R12A in the development and progression of this disease. Emerging evidence suggests that PPP1R12A may function as a tumor suppressor by inhibiting cell proliferation and promoting apoptosis. Moreover, Merlin, recognized as a tumor suppressor, becomes activated upon the dephosphorylation of serine 518 by the PPP1R12A−PP1cδ enzyme.[50] The loss of Merlin results in a coordinated upsurge in both Wnt/β-catenin signaling and the PI3K/AKT pathway, both of which contribute to chemoresistance in colorectal cancer.[51] However, as of now, no studies have delved into the relationship between PPP1R12A and clinical outcomes in HCC. Additionally, PPP1R12A is a key regulator of the actin cytoskeleton, a critical factor in cell migration and invasion. Dysregulation of PPP1R12A can profoundly influence these processes, potentially fostering tumor metastasis. Moreover, PPP1R12A's intricate interactions with various signaling pathways implicated in cancer, including the Rho/ROCK pathway, underscore its significance. Activation of these pathways can trigger alterations in the actin cytoskeleton, thereby enhancing cancer cell motility. Notably, PPP1R12A expression levels have been scrutinized for their prognostic value in liver cancer. In certain cases, elevated PPP1R12A expression has been correlated with poor prognosis and advanced disease stages.

Our ongoing research endeavors aim to uncover the multifaceted role of PPP1R12A in disease progression and explore its potential as a therapeutic target. As we delve deeper into its functions, our goal is to shed light on opportunities for therapeutic intervention, not only in liver cancer but also in other cancer types. In this study, we identified and quantified the overexpression of phosphorylated PPP1R12A at S422 (log FC = 1.17; *P*-value = 7.33×10^{-41}), at S896 (log FC = 0.79; *P*-value = 2.72×10^{-12}), at S871 (log FC = 0.84; *P*-value = 1.23×10^{-21}), and S862 (log FC = 0.78; *P*-value = 1.17×10^{-16}). This post-translational modification can profoundly impact the activity of PPP1R12A. Importantly, phosphorylation at S422 can influence the function of myosin phosphatase and, consequently, cellular processes related to cytoskeletal dynamics. Research has shown that phosphorylation events, including those occurring at S422, can significantly influence the cellular functions of PPP1R12A. These alterations can have direct implications for processes such as cell migration, invasion, and motility, all of which are critical in cancer progression, including liver cancer. Understanding the phosphorylation of PPP1R12A at S (422, 896, 871, and 862) and its functional consequences holds promising therapeutic implications. Depending on its specific role in a particular cancer context, the possibility of targeting PPP1R12A or the signaling pathways it regulates as a potential therapeutic strategy warrants exploration. Research focused on PPP1R12A-S422 in liver cancer aims to determine its clinical significance, including its potential value as a prognostic marker or therapeutic target. The phosphorylation status of PPP1R12A at S422 may exhibit variations among individual patients and could be associated

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with different disease outcomes. In summary, the phosphorylation of PPP1R12A represents a post-translational modification that can profoundly impact PPP1R12A's functions in liver cancer. Its specific role and clinical implications may depend on various factors, including the cancer's stage and subtype. Ongoing research endeavors aim to elucidate the significance of PPP1R12A in liver cancer and its potential as a target for therapeutic intervention.

Protein Phosphatase 1 Catalytic Subunit Beta. Protein phosphatase 1 catalytic subunit beta (PPP1CB) is a catalytic subunit of PP1, which is a crucial enzyme involved in the regulation of various cellular processes, including cell cycle progression, signal transduction, and gene expression. In liver cancer (HCC), the role of PPP1CB may be of interest because of its involvement in cellular signaling and its potential impact on cancer development and progression. PPP1CB is a serine/threonine phosphatase that plays a central role in dephosphorylating target proteins. It is involved in regulating various signaling pathways, including those related to cell growth, apoptosis, and DNA repair. Dysregulation of these pathways can contribute to cancer development. Research on PPP1CB in liver cancer may focus on its clinical significance, including its potential as a prognostic marker or therapeutic target. Understanding how PPP1CB expression and activity are altered in liver cancer patients may have diagnostic or therapeutic implications.

Depending on its specific role in liver cancer, PPP1CB or the signaling pathways it regulates could be explored as potential therapeutic targets. Modulating the activity of PPP1CB or its downstream targets may offer a strategy for cancer treatment. PPP1CB likely interacts with a network of proteins and signaling pathways. Studying these interactions may provide insights into its role in liver cancer and potential points of intervention. In summary, PPP1CB is a protein phosphatase with diverse roles in cellular signaling and regulation. Its involvement in liver cancer is an area of interest, and ongoing research aims to elucidate its specific functions, clinical relevance, and potential as a target for therapeutic intervention in HCC and other cancer types. PPP1CB-S311 is a serine/threonine phosphatase, and its phosphorylation can affect its enzymatic activity. In HCC, alterations in the phosphorylation status of PPP1CB-S311 might impact its ability to dephosphorylate target proteins involved in signaling pathways relevant to cancer. In the current study, we successfully identified and quantified PPP1CB phosphorylated at S311, revealing its overexpression (log FC = 1.07; *P*-value = 5.95×10^{-36}) in tumor patients compared to nontumor patients. Phosphorylation events on PPP1CB can contribute to dysregulations in signaling pathways and cellular processes, potentially promoting cancer cell growth, survival, or metastasis. It is worth noting that there has been limited research on PPP1CB in liver cancer. This study represents a significant contribution to the field, as it addresses this knowledge gap and sheds light on the role of PPP1CB-S311 phosphorylation in HCC for the first time.

Protein Phosphatase 1 Regulatory Subunit 9A. Protein phosphatase 1 regulatory subunit 9A (PPP1R9A), also known as neurabin-1, is a protein that plays a role in cytoskeletal organization and cellular signaling, which is crucial for cell shape, motility, and adhesion. Dysregulation of cytoskeletal dynamics can influence cancer cell migration and invasion, processes relevant to cancer progression. PPP1R9A interacts with signaling pathways that are implicated in cancer. For example, it can interact with and modulate the activity of proteins involved in the Wnt signaling pathway, which plays a role in cell proliferation and differentiation. Dysregulation of these pathways can contribute to cancer development. PPP1R9A is involved in regulating cell adhesion, which is essential for cancer cells to attach to and invade surrounding tissues. Altered cell adhesion is a hallmark of metastatic cancer cells. PPP1R9A is believed to be imprinted in a tissue-specific manner. A study examining human PPP1R9A expression in various embryonic tissues and the placenta reported monoallelic expression in skeletal muscle but not in other tissues.[52] As of now, there have been no publications regarding the imprinting status of PPP1R9A in HCC. PPP1R9A likely interacts with a network of proteins and signaling pathways. Studying these interactions may provide insights into its role in HCC and potential points of intervention. We identified and quantified PPP1R9A phosphorylated at T932, revealing its overexpression (log FC = 1.43; *P*-value = 3.29×10^{-19}) in tumor patients compared to nontumor patients. Phosphorylation at T932 on PPP1R9A is a post-translational modification that can modulate its function. Understanding how this phosphorylation event affects PPP1R9A's activity is crucial, as PPP1R9A is involved in cytoskeletal regulation, signaling pathways, and cell adhesion. PPP1R9A interacts with signaling pathways implicated in cancer, such as the Wnt signaling pathway. Phosphorylation events on PPP1R9A may impact these pathways, potentially contributing to cancer cell growth and metastasis. The phosphorylation of PPP1R9A-T932 is a critical post-translational modification that may impact its functions relevant to HCC. Understanding the specific role and clinical implications of PPP1R9A-T932 phosphorylation in HCC is a subject of ongoing research with the potential to improve our understanding of HCC progression and its treatment.

Protein Phosphatase 1 Catalytic Subunit Alpha. Protein phosphatase 1 catalytic subunit alpha (PPP1CA), one of the three isoforms of PPP1C, has been implicated in cancer progression. Its association with aggressive metastasis and poor prognosis has been observed in various tumor types. PPP1CA plays a role in promoting tumor cell proliferation and metastasis by activating the MAPK signaling pathway.[53] This could explain the high rate of lymph node metastasis seen in patients with elevated PPP1CA expression, as indicated by the bc-GenExMiner database. Additionally, PPP1CA can interact with cyclin D1 to phosphorylate RB or dephosphorylate breast cancer susceptibility protein-1 (BRCA1), leading to cell cycle deregulation and increased tumor cell proliferation. Therefore, inhibiting PPP1CA holds promise as a strategy to curb breast cancer proliferation and metastasis. Inhibiting PPP1CA presents a potential therapeutic avenue, with heightened significance in the context of triple-negative breast cancer patients with elevated BRCA1 mutation rates.[54] This approach may hold considerable promise for achieving therapeutic effects in this specific subset of breast cancer and other cancer cases. Currently, limited research exists on the mechanisms through which PPP1CA is involved in HCC development. In this study, we report for the first time that PPP1CA was found to be upregulated in HCC samples, with log FC value of 0.873 and corresponding *P*-value of 4.64×10^{-23}. This result identified the p-site at T320. Ongoing research efforts are dedicated to elucidating the precise functions of PPP1CA in HCC and assessing its clinical relevance, paving the way for potential advancements in HCC diagnosis and treatment.

Protein Phosphatase 1 Regulatory Subunit 13L. Protein phosphatase 1 regulatory subunit 13L (PPP1R13L),

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also known as the inhibitor of apoptosis-stimulating protein of p53, is a protein that plays a crucial role in regulating cell survival and apoptosis. PPP1R13L is known to inhibit apoptosis by interacting with and inhibiting the activity of the tumor suppressor protein p53. Dysregulation of apoptosis is a hallmark of cancer, including HCC, as it allows cancer cells to evade programmed cell death. PPP1R13L has been implicated in promoting cell proliferation and may influence cancer cell invasion and metastasis. Metastasis is a critical factor in the aggressiveness of cancer, and proteins that regulate this process are of interest in cancer research. PPP1R13L likely interacts with a network of proteins and signaling pathways. Studying these interactions may provide insights into its role in HCC and potential points of intervention. In the current study, we successfully identified and quantified PPP1R13L phosphorylated at Y132, revealing its overexpression (log FC = 1.22; *P*-value = 3.19×10^{-18}) in tumor patients when compared to nontumor patients. The phosphorylation of this specific tyrosine residue can have implications for the function of PPP1R13L and its role in cellular processes in HCC.

Protein Phosphatase 1 Regulatory Subunit 3E. Protein phosphatase 1 regulatory subunit 3E (PPP1R3E) is a vital regulatory component of PP1, a serine/threonine phosphatase known for its significant role in governing various cellular processes. These processes encompass critical aspects such as cell cycle progression, signal transduction, and gene expression, which are central in cancer development and progression, including HCC. PPP1R3E's role as a regulatory subunit of PP1 offers it the capacity to influence these essential signaling pathways and cellular functions. Abnormality of these pathways is a defining feature of cancer, including HCC. PPP1R3E may potentially modulate PP1 activity and its downstream targets, affecting pivotal cellular functions relevant to cancer initiation and growth. Investigating how PPP1R3E expression and activity are altered in HCC patients holds promise for diagnostic and prognostic applications. Depending on its precise role in HCC, PPP1R3E or the signaling pathways under its influence could be explored as prospective therapeutic targets. Manipulating PPP1R3E activity or targeting its downstream effectors may present a strategy for cancer intervention. The phosphorylation of PPP1R3E at S33 and S16 bears substantial implications for its role in the development and progression of HCC. These phosphorylation events exert profound influence over protein activity and play a pivotal role in modulating critical signaling pathways associated with cancer.

In our recent study, we conducted a rigorous analysis and successfully identified and quantified the phosphorylation of PPP1R3E at S33 and S16. Our findings unveiled a pronounced less expression (log FC = −1.10; *P*-value = 3.37×10^{-23}) of these phosphorylation events in tumor tissues as compared to their nontumor counterparts. The perturbation of phosphorylation events is a hallmark feature of cancer, inclusive of HCC. Phosphorylation events, as observed in proteins such as PPP1R3E, can significantly contribute to the disruption of these pathways, thereby potentially fostering cancer cell proliferation, bolstering cell survival, or promoting metastatic behavior.

Comprehending the specific functional role of PPP1R3E-S33 and S16 phosphorylation in the context of HCC, along with its clinical implications, assumes paramount importance. It may pave the way for the development of innovative diagnostic or prognostic markers. Depending on the precise mechanistic insights we gain regarding its role within HCC, the exploration of therapeutic strategies targeting PPP1R3E or the associated signaling pathways may emerge as a promising avenue for therapeutic intervention. Dedicated and ongoing research endeavors are diligently working toward unraveling the full spectrum of significance pertaining to PPP1R3E-S33 and S16 phosphorylation in HCC, along with its potential as a bona fide therapeutic target.

Protein Phosphatase 1 Regulatory Subunit 12B. Protein phosphatase 1 regulatory subunit 12B (PPP1R12B), also known as myosin phosphatase target subunit 2, is a regulatory subunit intricately involved in the complex regulatory mechanisms of cellular processes. It particularly influences cell motility and contractility through its association with the myosin phosphatase complex. PPP1R12B has garnered significant interest because of its potential implications in the initiation and progression of this aggressive form of liver cancer. PPP1R12B is a vital component of the myosin phosphatase complex, capable of engaging with various signaling pathways pertinent to cancer progression. Its role in orchestrating crosstalk between these pathways can substantially influence the development of HCC. The ongoing and meticulous research endeavors in this realm aim to uncover the precise functional dimensions of PPP1R12B in HCC, discern its clinical significance, and explore its therapeutic promise. A comprehensive comprehension of PPP1R12B's role within the HCC milieu holds the potential to revolutionize diagnostics, prognostics, and treatment modalities for this formidable liver cancer. In this study, we report for the first time that PPP1R12B was found to be downregulated in HCC samples, with log FC values of −1.068 and −1.0679 and corresponding *P*-values of 1.13×10^{-19} and 1.13×10^{-19}, respectively. These results identified the p-sites at S900 and S43. However, it is essential to recognize the role and importance of PPP1R12B in liver cancer. Research in this field is dynamic and continually evolving as is our understanding of the molecular mechanisms underpinning cancer progression, especially in the context of liver cancer. Consequently, ongoing investigations in this area hold the promise of improved treatment strategies for liver cancer patients.

Protein Phosphatase 4 Regulatory Subunit 2. Protein phosphatase 4 regulatory subunit 2 (PPP4R2) is a regulatory subunit of protein phosphatase 4 (PP4). PP4 is a serine/threonine protein phosphatase that plays a crucial role in regulating various cellular processes, including cell cycle progression, DNA repair, and gene expression. The regulatory subunits of PP4 exhibit a high degree of conservation across mammals, yeast, and plants. This conservation extends to the heterotrimeric complex, PP4C-PP4R2-PP4R3 (also known as PPH3-YBL1046W-PSY2), which shares significant homology between humans and *Saccharomyces cerevisiae*. In both organisms, this complex plays a critical role in the DNA damage response.[55] While PP4 is known to be overexpressed in various human cancers, including breast and lung tumors, stage II pancreatic ductal adenocarcinoma, colorectal carcinoma, and glioma, it also significantly increases the activity of c-Jun N-terminal kinase 1 in prostate cancer cell lines PC-3 and LNCaP. This increased activity is crucial for cell proliferation and drug resistance,[55] suggesting an oncogenic potential for PP4. However, conflicting studies have reported that the PP4R2 gene is deleted in patients with acute myeloid leukemia. This deletion is essential for proper DNA repair and leads to the protein functioning as a tumor suppressor.[55] Furthermore, PP4 activity is inhibited through the phosphorylation of its regulatory

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subunits, PP4R2 and PP4R3α. This phosphorylation results in the blockage of microtubule nucleation by γ-tubulin. The dysregulation of signaling pathways and cellular processes is a hallmark of cancer, including HCC. PPP4R2, as a regulatory subunit of PP4, could potentially influence these pathways. Modulating the activity of PPP4R2 or its downstream targets may offer a strategy for cancer treatment. Overall, PPP4R2 is a regulatory subunit of PP4 with diverse roles in cellular signaling and regulation.

In our present study, we have adeptly identified and quantified the phosphorylation of PPP4R2 at S218, revealing its overexpression (log FC = 1.37; *P*-value = 7.92×10^{-37}) in tumor patients when compared to nontumor patients. These results suggest that the phosphorylation of PPP4R2 at S218 may have important implications in the HCC. Dysregulation of signaling pathways and cellular processes is a hallmark of cancer, including HCC. Phosphorylation events on proteins like PPP4 can contribute to the dysregulation of signaling pathways, potentially promoting cancer cell growth, survival, or metastasis. It is crucial to understand the specific role of PPP4R2-S218 phosphorylation in HCC, and its clinical implications may help in developing diagnostic or prognostic markers. Targeting PPP4R2-S218 or the signaling pathways it regulates may be explored as a potential therapeutic strategy in HCC.

Protein Phosphatase 6 Regulatory Subunit 2 and 3. Protein phosphatase 6 regulatory subunit 2 and 3 (PPP6R2 and PPP6R3) are a regulatory subunit of protein phosphatase 6 (PP6), a serine/threonine phosphatase that plays a role in regulating various cellular processes, including cell cycle progression, signal transduction, and gene expression. In mammalian cells, PP6 functions as a holoenzyme by interacting with its regulatory subunits, PPP6R1, PPP6R2, and PPP6R3. The involvement of PPP6R2 and PPP6R3 in HCC research is of interest because of its potential influence on cellular signaling and its impact on cancer development and progression. Dysregulation of signaling pathways and cellular processes is a common feature of cancer, including HCC. PPP6R2 and PPP6R3, as regulatory subunits of PP6, may be involved in modulating these pathways. PP6 has been reported to participate in the regulation of the cell cycle, DNA damage repair, and tumorigenesis. In particular, PP6 has been identified as a component of the NF-κB interacting network in the TNFα signaling pathway and may be involved as a negative regulator of the NF-κB pathway.[56] Furthermore, investigating the specific role of PPP6R2 and PPP6R3 in HCC and its interactions with other cellular components and signaling pathways can provide valuable insights into the molecular mechanisms underlying this type of cancer. While the precise role of PPP6R2 and PPP6R3 in HCC is an area of ongoing research, its potential as a target for therapeutic intervention may also be explored in the future.

The phosphorylation of PPP6R2 at S796 holds significant implications for its role in cancer development and progression. Phosphorylation events can profoundly impact protein activity and influence critical signaling pathways associated with cancer. In our recent study, we conducted a comprehensive analysis and successfully identified and quantified the phosphorylation of PPP6R2 at S796. Our findings revealed a notable overexpression (log FC = 1.47; *P*-value = 1.22×10^{-11}) of this phosphorylation in tumor tissues compared to nontumor tissues. Furthermore, our findings regarding PPP6R3 and its phosphorylation sites (S509, T541, and S471) have shown significant overexpression with log fold changes of (log FC = 0.86; *P*-value = 5.96×10^{-31}), (log FC = 0.84; *P*-value = 9.56×10^{-19}), and (log FC = 0.86; *P*-value = 6.66×10^{-18}), respectively. The dysregulation of signaling pathways and cellular processes is a defining characteristic of cancer, including HCC.

Comprehending the specific role of PPP6R2-S796 phosphorylation in HCC and its clinical implications is pivotal, as it may pave the way for the development of novel diagnostic or prognostic markers. Depending on its precise function within the context of HCC, exploring the possibility of targeting PPP6R2/PPP6R3 or the associated signaling pathways could emerge as a promising avenue for therapeutic intervention. Ongoing research endeavors are dedicated to unraveling the full significance of PPP6R2/PPP6R2 in HCC and assessing its potential as a therapeutic target.

Phosphatase and Tensin Homologue. PTEN is a well-known tumor suppressor gene that is frequently inactivated in human cancers and plays a critical role in regulating cell growth and preventing the development of cancer. PTEN has garnered significant attention because of its potential involvement in cancer development and progression. PTEN functions primarily as a phosphatase, counteracting the activity of protein kinases. It acts on a critical cellular signaling pathway known as the PI3K/AKT pathway, which regulates various cellular processes, including cell proliferation, survival, and growth. PTEN's role is to dephosphorylate specific molecules within this pathway, thereby inhibiting its activity.[57] Dysregulation or loss of PTEN function can lead to the overactivation of the PI3K/AKT pathway, contributing to uncontrolled cell growth. In HCC, alterations in PTEN expression or mutations in the PTEN gene have been reported. These changes can disrupt the normal regulation of the PI3K/AKT pathway, promoting cancer cell survival and proliferation. As a result, PTEN is considered a crucial player in the development and progression of HCC. PTEN deficiency leads to Akt activation, while SHP2 deficiency causes JNK activation, resulting in increased expression and activation of c-Jun, a promoter of HCC development.[58] It is important to understand that the specific alterations in PTEN in HCC patients may have significant diagnostic, prognostic, and therapeutic implications. Additionally, reduced PTEN levels have been linked to increased expression of cancer stem cell markers (e.g., CD133, epithelial cell adhesion molecule, and CK19), poorer HCC prognosis, and higher rates of HCC recurrence.[59] These findings underscore the pivotal role of the PTEN/Akt/mTOR pathway in regulating malignant hepatic tumorigenesis and influencing HCC recurrence and OS by modulating cancer stem cells. PTEN also plays a critical role in regulating macrophage polarization and function within the tumor microenvironment. N-myc downstream-regulated gene 2 (NDRG2) recruits PP2A to regulate PTEN activity by dephosphorylating Ser380, Thr382, and Thr383 in the C-tail of PTEN.[60] Additionally, PTEN is speculated to be involved in JAK/STAT and NF-κB signaling pathways. Loss of NDRG2 in bone marrow-derived macrophages enhances phosphorylation of IκB kinases α/β (IKKα/β), p65, and IκBα following Akt activation, leading to a shift in macrophage polarization from an M2 phenotype toward an M1-like phenotype.[61] Altogether, PTEN dephosphorylation and activation via the NDRG2−PP2A complex contribute to cancer progression by increasing the number of M2 tumor-associated macrophages through inhibition of NF-κB and IκB phosphorylation. High PTEN expression or restoration of PTEN function could potentially be associated with improved outcomes in HCC patients, making it an attractive target for therapeutic intervention.

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The phosphorylation of PTEN at S294 assumes a pivotal role in its involvement in cancer development and progression. Phosphorylation events wield significant influence over protein functionality and have the capacity to modulate critical signaling pathways closely associated with cancer pathogenesis. Our recent study meticulously examined and successfully quantified the phosphorylation status of PTEN at S294. The findings underscored a remarkable overexpression pattern (log FC = 1.2; P-value = 1.06×10^{-16}) of this phosphorylation marker in tumor tissues in contrast to nontumor tissues. It is imperative to acknowledge that cancer, including HCC, is distinguished by the aberrant dysregulation of signaling cascades and cellular processes. Phosphorylation events affecting proteins can significantly contribute to the perturbation of these pathways, potentially fostering the uncontrolled proliferation, survival, or metastasis of cancer cells. A comprehensive comprehension of the specific role played by PTEN-S294 phosphorylation in HCC and its clinical implications assumes paramount importance. This understanding holds the promise of ushering in novel diagnostic or prognostic markers, with potential far-reaching implications. There lies an avenue of exploration concerning the viability of targeting PTEN or the pertinent signaling pathways as a promising approach to therapeutic intervention. Our ongoing research endeavors are wholeheartedly committed to unraveling the complete significance of PTEN-S294 in the realm of HCC and to assessing its potential as a robust therapeutic target.

Protein Phosphatase 2 Regulatory Subunit 5D. Protein phosphatase 2 regulatory subunit 5D (PPP2R5D), a serine/threonine dual-specific protein phosphatase, serves as a tumor suppressor with a pivotal role in diverse intracellular signaling pathways and processes in mammalian cells. Its functions encompass apoptosis, cell cycle regulation, cell proliferation, cell migration, cell transformation, and transcription.[4] This induction results in increased apoptosis and reduced proliferation in HCC cell lines. Furthermore, the activation of PP2A inhibits the rise in proliferating cell nuclear antigen-positive hepatocytes following DEN administration in vivo.[62] Moreover, PP2A plays a crucial role in modulating the sensitivity and activity of chemotherapy against HCC. Notably, the transcription of PP2A-B55δ, a PP2A subunit, is reduced in HCC cell lines, but administration of cisplatin (cDDP), a chemotherapy drug, upregulates PP2A-B55δ expression. PP2A-B55δ enhances the tumor-inhibitory effects of cDDP on cell migration, colony formation, proliferation, cell cycle progression, and apoptosis, thereby enhancing its therapeutic efficacy.[63] PP2A also potentiates the anticancer effects of erlotinib and bortezomib. Inhibition of CIP2A mediates the apoptotic effect of TD52, an erlotinib derivative, by downregulating p-Akt via PP2A in HCC cell lines.[64] It was reported that PPP2R5D is necessary for HCV infection in cultured hepatoma cells, and its function may involve binding to HCV NS5B. In this study, we identified and quantified the overexpression of phosphorylated PPP2R5D at S573 (log FC = 0.65; P-value = 1.4×10^{-15}). However, the clinical implications of this finding remain unclear, and the consequences of PP2A inhibition on the effectiveness of targeted therapies have not been fully elucidated.

Protein Phosphatase, Mg^{2+}/Mn^{2+}-Dependent 1E. Protein phosphatase, Mg^{2+}/Mn^{2+}-dependent 1E (PPM1E), also known as PP2CE, is a member of the protein phosphatase 2C (PP2C) family of phosphatases that bind manganese/magnesium ions (Mn^{2+}/Mg^{2+}) in their active center and function as single-subunit enzymes. PP2C phosphatases are known for their role in dephosphorylating and regulating various signaling molecules in cellular pathways. PPM1E is involved in the dephosphorylation of specific substrates, and its functions are associated with the regulation of cellular processes. PPM1E expression was elevated in human gastric cancer tissues compared to normal tissues, which was correlated with AMPK (p-AMPKα, Thr-172) dephosphorylation and mTOR complex 1 (mTORC1) activation. PPM1E upregulation, AMPK inhibition, and mTORC1 activation were also observed in human gastric cancer cell lines (AGS, HGC-27, and SNU601).[65] Currently, there is limited research on the mechanisms through which PPM1E is involved in HCC tumor development. It is important to study the role of PPM1E in HCC, which could contribute to our knowledge of liver cancer biology and potentially lead to new therapeutic strategies in the future. In our study, we observed that PPM1E was overexpressed in tumor patients compared to nontumor patients, and its overexpression was associated with a poor prognosis. PPM1E is phosphorylated at S535 and S548, and this phosphorylation was significantly upregulated (log FC = 0.77; P-value = 1.13×10^{-12} & log FC = 0.95; P-value = 6.15×10^{-10}). Additionally, it has not been reported that PPM1E is one of the underlying prognostic biomarkers and treatment targets for HCC. However, its functions and mechanisms of HCC progression are unknown. This study results for the first time show that PPM1E was overexpressed in HCC tumor patients compared to nontumor patients. To gain further insights into the functions and mechanisms of PPM1E in HCC progression, additional research is warranted. This research could involve experiments to elucidate the specific substrates that PPM1E dephosphorylates in HCC, its impact on signaling pathways relevant to cancer, and how its dysregulation contributes to tumor development and poor prognosis. Researchers in the field may find this information useful for further exploration and experimentation.

Slingshot Homologue 2. Slingshot homologue 2 (SSH2) is a protein phosphatase that plays a role in regulating actin cytoskeleton dynamics. The Slingshot family comprises SSH1, SSH2, and SSH3 proteins. SSH1 and SSH2 exhibit phosphatase activity in vitro, capable of dephosphorylating specific substrates, while SSH3 does not display this activity. Furthermore, existing literature suggests that SSH1 and SSH2 can colocalize with microfilaggrin, whereas the relationship between SSH3 and microfilaggrin localization remains unclear.[66] Currently, there is limited research on the mechanisms through which SSH3 is involved in tumor development, with only one study reporting that SSH3 may promote metastasis in colorectal tumor cells by affecting the LIMK1/Rac1 signaling pathway.[67] While SSH2 itself may not be a widely and extensively studied protein in the context of HCC, it is possible that it could indirectly impact HCC progression or metastasis through its involvement in actin cytoskeleton regulation, which can affect cell migration and invasion. Consequently, this study aims to elucidate the impact of SSH1, SSH2, and SSH3 on HCC, thereby providing further insights into the molecular pathogenic mechanisms involving SSH1, SSH2, and SSH3 in the development of HCC. In the current study, we quantified SSH1, SSH2, and SSH3 from the database. Interestingly, SSH2 phosphorylated at S811 was significantly upregulated (log FC = 0.74; P-value = 6.14×10^{-20}), while SSH3 phosphorylated at S9 and T79 was insignificant (log FC = 0.51; P-value = 1.08×10^{-12}) in tumor patients when compared to nontumor patients. Indeed, understanding the functions and roles of SSH2 and its post-translational modifications in HCC is crucial for developing

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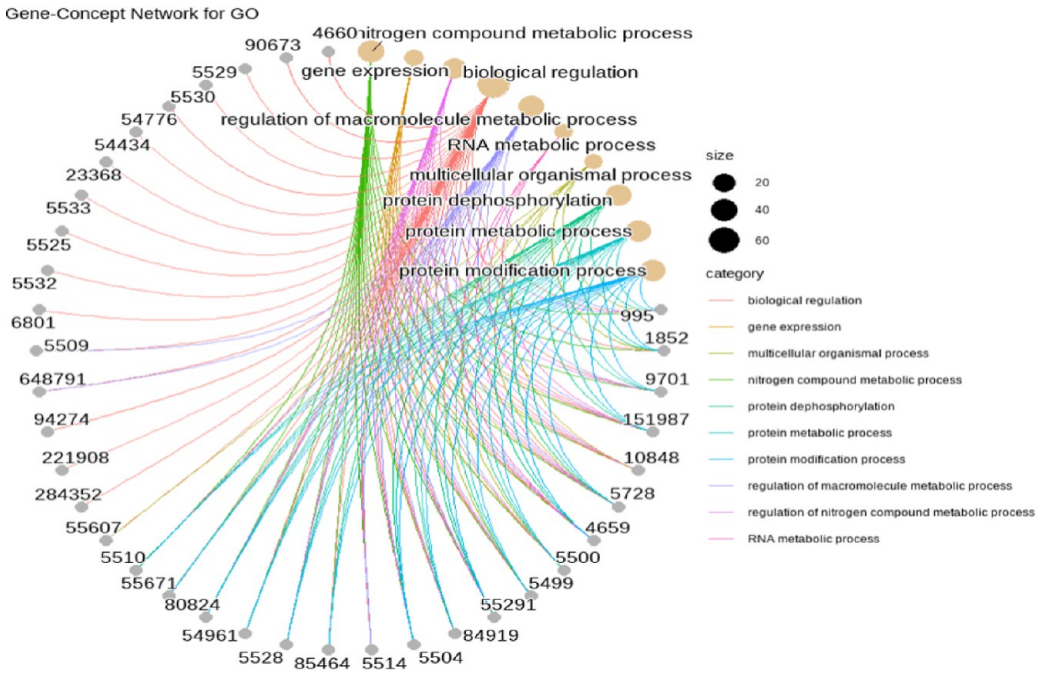

Figure 14. GO using GSEA of 31 significant genes' correlation with genes in significant BP.

targeted therapies and gaining insights into the molecular mechanisms of cancer progression. Further research into SSH2, particularly in HCC, may provide valuable information about its potential as a therapeutic target or diagnostic marker. Researchers may investigate how SSH2 and its phosphorylation at S811 contribute to the development and progression of HCC and whether targeting SSH2 could be a viable strategy for HCC treatment.

RNA Polymerase II Subunit A C-Terminal Domain Phosphatase. SSH2 is an essential protein involved in the regulation of RNA polymerase II transcription. While it may have roles in gene expression and RNA processing, there is limited information available regarding its direct involvement in HCC. Further research is required to understand if and how carboxy terminal domain phosphatase subunit 1 (CTDP1) may play a role in HCC development or progression. CTDP1, also known as Fcp1, is a phosphatase that associates with TFIIF. Its encoding gene is located in the 14th exon of 18q23. It is ubiquitously expressed in tissues[68] and is found in the nucleus and cytoskeleton during cell mitosis.[69] CTDP1 is known to dephosphorylate the C-terminal domain (CTD) of RNA polymerase II subunit A, promoting the gene expression cycle. RNA polymerase II plays a key role in mRNA production, during which CTD undergoes a cycle of phosphorylation and dephosphorylation. Before the formation of the preinitiation complex and transcription, CTD is dephosphorylated, and during transcription elongation, it becomes phosphorylated.[70] CTDP1 is a classic phosphatase for the heptapeptide repeat of CTD[71] and is necessary for initiating another cycle of mRNA synthesis. Additionally, CTDP1 regulates other substrates. It inactivates crucial mitotic substrates (e.g., USP44, CDC20, and WEE1) to dephosphorylate M-phase-promoting factor/CDK1, promoting exit from mitosis.[68] Furthermore, CTDP1 regulates transcription elongation and enhances the rate of elongation by RNAP II.[72] Altogether, anti-CTDP1 autoantibodies may neutralize the role of CTDP1 in dephosphorylation, including RNAP resumption, mitosis exit, and transcription elongation, inhibiting mRNA production and daughter cell formation. This may affect tissues with high metabolism rates, such as skin and mucosa, partially explaining the recurrent ulcers seen in Behcet's disease.

Partial deficiency of CTDP1 has been reported in congenital cataract facial dysmorphism neuropathy syndrome, an autosomal recessive developmental disease.[73] As the name suggests, a key feature is nervous system involvement, manifested by motor neuropathy leading to disability. Moderate, nonprogressive cognitive deficits and pyramidal signs are also associated neurological features. Nerve biopsy shows a demyelinating pathogenesis.[74] The role of CTDP1 in cancer, especially in HCC, has not been extensively studied and should be further investigated. Such research could prove to be highly valuable for the clinical diagnosis of HCC in the future. In our study, we successfully quantified CTDP1 and its phosphorylation sites, and we observed significant overexpression in tumor patients compared to nontumor patients. This marks the first quantification of CTDP1 in HCC. Interestingly, our study identified five phosphorylation sites on CTDP1 (S740, S831, S833, S474, and S869) that were significantly overexpressed, with log FC values of 0.81, 1.03, 1.02, 0.99, and 0.60, respectively and P. value $< 9.8 \times 10^{-21}$. This wealth of information suggests that CTDP1 could play a substantial role in the development and progression of HCC. It may provide valuable insights into potential therapeutic targets or avenues for further research, ultimately enhancing our understanding of the molecular mechanisms underlying HCC.

The proper balance between phosphorylation and dephosphorylation processes, regulated by protein kinases and phosphatases, is crucial for maintaining cellular functions and signaling pathways. Abnormality of these processes can contribute to various diseases and underscores the importance of studying and understanding the roles of phosphatases in health and disease. The pursuit of novel biomarkers for early HCC detection and diagnosis stands as a pivotal objective in cancer research. However, the task presents significant

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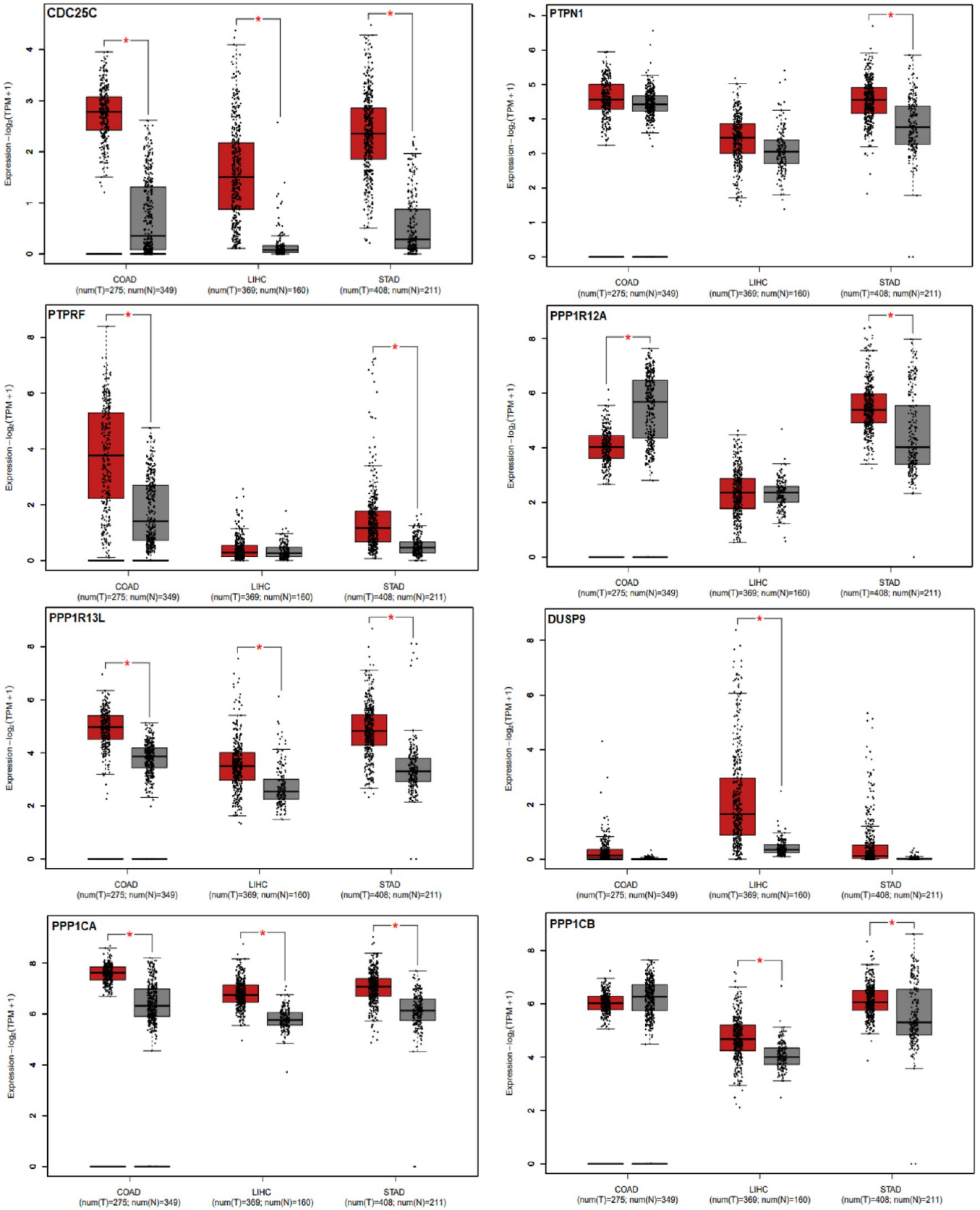

Figure 15. continued

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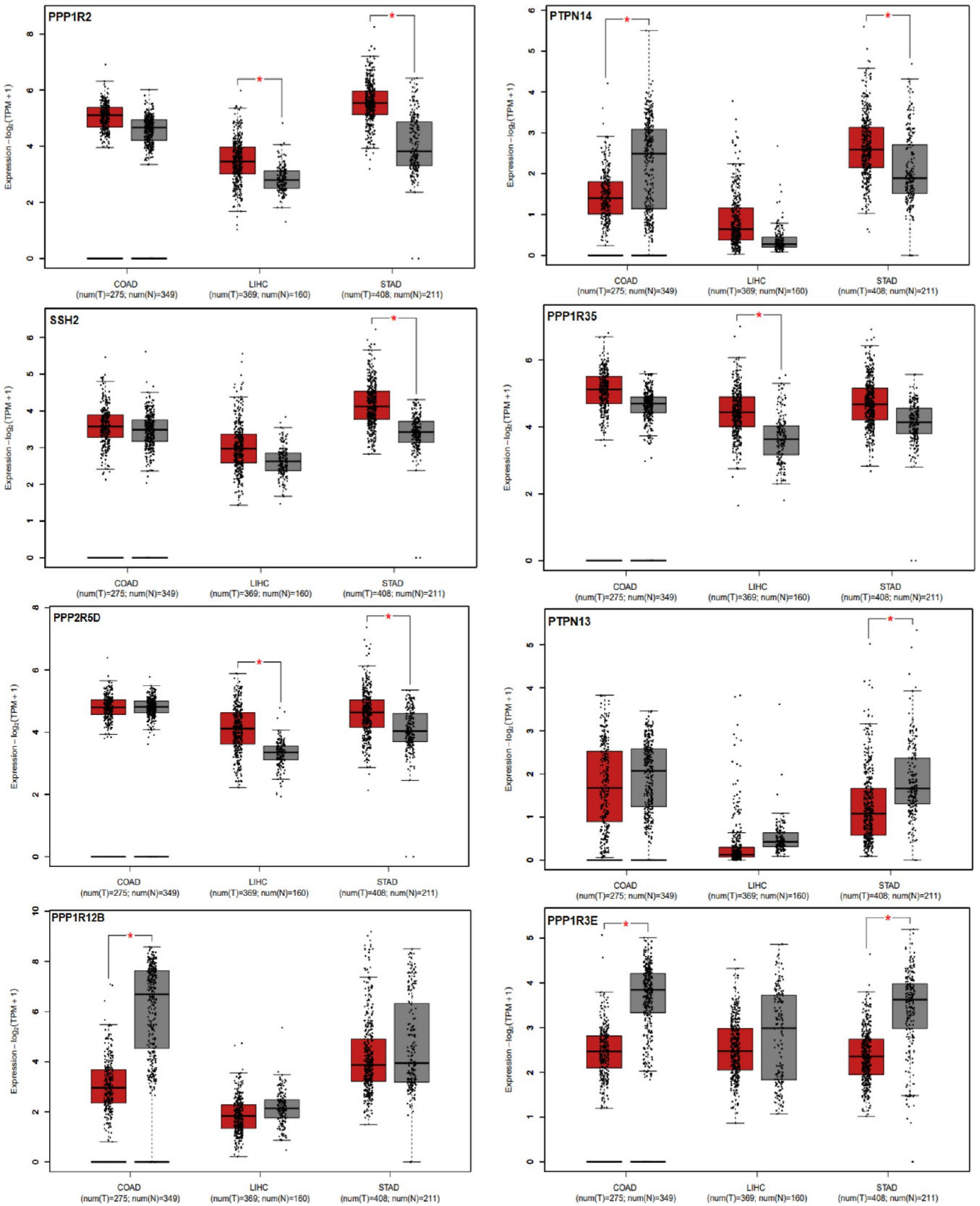

Figure 15. Expression of the protein phosphatase family in HCC for the 16 genes compared between normal liver tissues (denoted by a black box) and HCC (represented by a red box), derived from the GEPIA online tool (*$p < 0.01$; tumor vs normal).

challenges in isolating changes directly tied to cancer transformation, amidst the backdrop of alterations caused by diverse factors such as stress, tissue regeneration, inflammation, or the body's systemic response to the tumor. This intricate landscape underscores the imperative of conducting thorough and meticulous studies aimed at discerning the precise molecular

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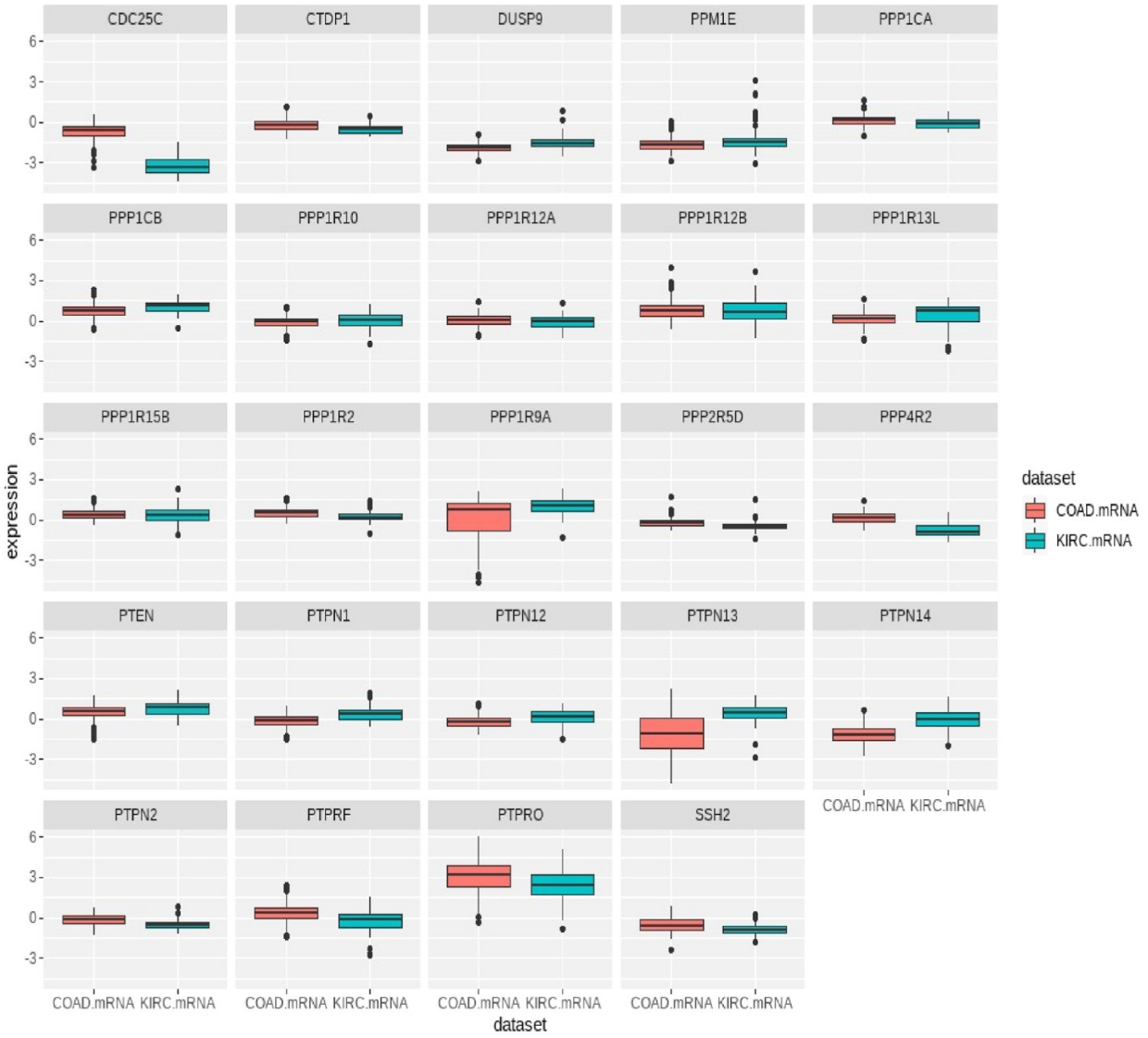

Figure 16. MRNA expression of the protein phosphatase family in HCC for the 20 genes compared between COAD mRNA and KIRC mRNA, derived from the RTCGA package in the R program.

modifications most pertinent to the development and progression of cancer. In this pursuit, advanced techniques in proteomics and genomics emerge as invaluable instruments. They empower researchers to analyze expansive sets of molecular data, unveiling potential biomarkers that could prove instrumental in the early detection and diagnosis of HCC. The current study has revealed that a range of phosphatases, including PP1, PTEN, PP2A, PTPN2, PTPN3, PTPN13, PTPN13, PTPN14, PTPRO, PTPRF, CDC25C, PPP4, PPP6, DUSP9, SSH2, and CTDP1, contribute to altering the characteristics of the liver microenvironment and driving HCC development. They achieve this by regulating the activities of various cellular components, including parenchymal cells, stromal cells, and immune cells that constitute the liver microenvironment. These actions underscore how protein phosphatases can impact HCC progression and influence the liver microenvironment's features through intracellular signaling pathway regulation in liver cells. These results accentuate the significance of protein phosphatases in modulating the constituents of the liver microenvironment and their potential as therapeutic targets. Ongoing and rigorous research endeavors aim to unravel the intricate functional dimensions of these proteins and the phosphorylation sites in HCC. The ultimate goal is to fully decipher their clinical relevance and therapeutic prospects and may revolutionize the management of HCC by paving the way for innovative diagnostic tools, prognostic markers, and targeted therapies.

To gain a deeper understanding of the pathways regulated by these phosphorylated proteins, we conducted an enrichment analysis of KEGG pathways. Among the pathways analyzed, the most significantly enriched ones included insulin resistance, insulin signaling, adherens junction, and oocyte meiosis. Our GO analysis revealed marked enrichment in BP, CC and MF. In addition, we performed an extensive GO analysis of the 31 significant genes, revealing their involvement in important BP including protein dephosphorylation, regulation of dephosphorylation, peptidyl tyrosine dephosphorylation, nitrogen compound metabolic process, RNA metabolic process, and regulation of phosphatase activity, as illustrated in Figure 14.

We constructed a PPI network for these DEGs using the String network. After screening, we identified 31 hub genes, with 15 key genes standing out. These key genes, including PTP1B,

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Table 5. OS Analysis for HCC Patient Groups at 1, 3, and 5 Years[a]

#	time (day)	*n*.risk	*n*.event	*n*.censor	surv	cumhaz	gender	lower	upper
1	0	31	0	0	1	0	female	1	1
2	100	31	0	0	1	0	female	1	1
3	200	29	0	2	1	0	female	1	1
4	300	28	0	1	1	0	female	1	1
5	400	27	0	1	1	0	female	1	1
6	500	25	0	2	1	0	female	1	1
7	600	23	0	2	1	0	female	1	1
8	700	23	0	0	1	0	female	1	1
9	800	23	0	0	1	0	female	1	1
10	900	20	1	2	0.952381	0.047619	female	0.865518	1
11	1000	17	3	0	0.809524	0.205806	female	0.657853	0.996163
12	1100	10	6	1	0.52381	0.619071	female	0.348394	0.787547
13	1200	9	1	0	0.471429	0.719071	female	0.298457	0.744647
14	1300	6	3	0	0.314286	1.098039	female	0.164085	0.601978
15	1400	3	2	1	0.188571	1.548039	female	0.071718	0.495823
16	1500	1	2	0	0.062857	2.381373	female	0.009691	0.40769
17	1600	1	0	0	0.062857	2.381373	female	0.009691	0.40769
18	1700	1	0	0	0.062857	2.381373	female	0.009691	0.40769
19	1800	1	0	0	0.062857	2.381373	female	0.009691	0.40769
20	0	120	0	0	1	0	male	1	1
21	100	119	0	1	1	0	male	1	1
22	200	116	0	3	1	0	male	1	1
23	300	111	0	6	1	0	male	1	1
24	400	104	0	6	1	0	male	1	1
25	500	98	0	6	1	0	male	1	1
26	600	93	0	5	1	0	male	1	1
27	700	91	0	2	1	0	male	1	1
28	800	86	0	5	1	0	male	1	1
29	900	80	5	2	0.941176	0.060108	male	0.892462	0.99255
30	1000	67	11	1	0.810127	0.209029	male	0.730548	0.898373
31	1100	56	11	0	0.677121	0.384579	male	0.583874	0.78526
32	1200	52	4	0	0.628755	0.458005	male	0.53311	0.74156
33	1300	38	13	1	0.469251	0.745661	male	0.372962	0.590398
34	1400	17	20	1	0.219925	1.480438	male	0.145739	0.331873
35	1500	2	15	0	0.025873	3.38977	male	0.006606	0.101345
36	1600	2	0	0	0.025873	3.38977	male	0.006606	0.101345
37	1700	2	0	0	0.025873	3.38977	male	0.006606	0.101345
38	1800	2	0	0	0.025873	3.38977	male	0.006606	0.101345

[a]Cumhaz: Cumulative hazard transformation. Time: the time points at which the curve has a step. *n*.risk: the number of subjects at risk at *t*. *n*.event: the number of events that occur at time *t*. Lower: lower confidence limit for the survival curve. Upper: upper confidence limit for the survival curve. *n*.censor: the number of subjects who exit the risk set, without an event, at time *t*. (For right censored data, this number can be computed from the successive values of the number at risk). Surv: the estimate of survival at time $t + 0$. The latter occurs when a set of survival curves is created from a single Cox model.

CDC25C, PTPRF, PTPRO, PPP1R12A, PPP1R13L, PPP2R5D, PPP1R37, PPP1R3E, and PPP1CA, held significant relevance. Furthermore, elevated expression levels of these key genes were strongly associated with poorer survival in HCC patients. Our observations, including survival analysis, gene-level, and protein-level expression analysis, confirmed that the overexpression of these genes at diagnosis could be considered unfavorable prognostic indicators, reducing OS in HCC patients. These unfavorable genes are primarily involved in pathways such as oocyte meiosis, mRNA surveillance, insulin resistance, focal adhesion, cell cycle, and proteoglycans in cancer pathways. To validate our findings, we utilized the TCGA database to compare the expression differences of these key genes between tumor and normal tissues. Our research benefits from the integration of data from three databases CPTAC, TCGA, and RTCGA, enhancing the credibility of our results. Finally, we conducted immunohistochemical analysis and screened potential small analytical drugs for HCC, further extending the scope of our study.

To fully comprehend the roles of these genes in HCC, we conducted an expression analysis using TCGA data, focusing on the top 16 significantly implicated genes. Our objective was to compare their expression levels across various cancer types. Specifically, we selected stomach adenocarcinoma (STAD), colon adenocarcinoma (COAD), and liver hepatocellular carcinoma (LIHC) for comparison because of their propensity for liver metastasis. Investigating these particular genes will significantly contribute to differentiating primary liver cancer (HCC) from metastatic lesions in the liver. Through exploring distinct gene expression patterns in these cancers, we aim to uncover potential diagnostic markers for accurately distinguishing between primary and metastatic liver malignancies. The

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results are depicted in Figure 15, and the analysis was carried out using GEPIA 2 software. We assessed the expression levels of individual members of the protein phosphatase family in three cancer types: COAD, LIHC, and STAD, using data from the TCGA database. Notably, we observed higher expression levels in tumor samples compared to nontumor samples in our analysis. We employed a Student's *t*-test to calculate the *p*-values for expression differences of protein phosphatase family genes between normal controls and cancer samples, setting the threshold parameters for the *p*-value and fold change at 0.05 and 1.5, respectively. Our analysis revealed consistent trends: in STAD, the expression levels of CDC25C, PTPN1, PTPRF, PPP1R12A, PPP1R13L, PPP1CA, PPP1CB, PPP1R2, PPP2R5D, SSH2, PPP1R18, and PTPN14 were significantly higher in tumor tissues compared to normal tissues. In COAD, CDC25C, PTPRF, PPP1R12A, PPP1R13L, PPP1CA, PTPN14, and PPP1R18 showed substantial increases in expression compared to normal samples. In LIHC; CDC25C, PPP1R13L, DUSP9, PPP1CA, PPP1CB, PPP1R2, PPP1R35, and PPP2R5D were notably overexpressed relative to normal samples, while PTPN13, PPP1R12B, and PPP1R3E exhibited lower expression levels than normal tissues. In summary, our findings indicate that the expression levels of CDC25C, PPP1R13L, and PPP1CA are significantly upregulated in STAD, COAD, and LIHC, suggesting their potential utility as diagnostic biomarkers. Additionally, PPP1R12B and PPP1R3E are significantly downregulated in both COAD and STAD, while PTP13 is markedly downregulated in STAD.

TCGA is a comprehensive cancer genomics database (https://cancergenome.nih.gov/) containing genomic information from over 2000 primary neoplasms and matched normal samples. For our study, we obtained case information related to mRNA expression profiles and clinical features from the TCGA database, specifically the RTCGA mRNA. We utilized the RTCGA package in the R programming language to download the data. In our analysis, we specifically focused on the COAD mRNA and kidney renal clear cell carcinoma (KIRC) mRNA data sets. All data analysis was conducted using the R programming, and the results are presented in Figure 16 and in Table S5 (The mRNA Expression). It is essential to recognize the role and importance of the protein phosphatase family in liver cancer. As our understanding of the molecular mechanisms underpinning cancer progression, especially in the context of liver cancer, advances, it may pave the way for the development of targeted therapies that can specifically address the dysregulation of protein phosphatases family and its phosphorylation sites.

There was no statistically significant difference observed in OS between the groups (*P*-value = 0.56). The OS probabilities for HCC patients at 1, 3, and 5 years are presented in Table 5. We performed a multivariable Cox regression analysis to identify predictors of recurrence after liver resection in our cohort. Well-established predictors of poor clinical outcomes, including tumor size, tumor number, and vascular invasion, were validated in our cohort of patients. In addition to these, a fold increase in expression also emerged as a significant predictor of recurrence, with an HR > 1.0 and $P < 0.05$. The results revealed that most of the higher expression genes were significantly correlated with worse survival in HCC (*P*-value <0.05). A higher expression resulted in a significant shorter DFS and OS in HCC patients, suggesting that CDC25C, PTPN1, PPP2R5D, PPP1R37, and PPP1R13L may play an important role in the prognosis of HCC. As a result, ongoing investigations in this field offer the promise of improved treatment strategies for liver cancer patients. Interestingly, inhibitors targeting CDC25C, PPP1R13L, PTPN1, PPP2R5D, and PPP1CA have not been studied either individually or in combination with other anticancer agents, such as chemotherapy drugs or targeted therapies, to enhance the effectiveness of cancer treatment or mitigate HCC.

CONCLUSIONS

The field of liver cancer research is continually evolving, and one area of investigation is the role of protein phosphatases in better understanding and treating this complex disease. In this groundbreaking study, we explored the phosphoproteome and proteomic profiles of protein phosphatases within the context of HCC. This investigation is pioneering, as it establishes a novel protein signature. The observed dysregulation of protein phosphatases highlights distinct structural variations and potential migratory behaviors between tumor and paired nontumor tissues. Moreover, the significant alterations in phosphorylation sites may have biological relevance to HCC malignancy. These findings provide a valuable resource for the broader scientific community involved in HCC research and hold potential for the development of new drugs. Our comprehensive survival analysis, considering multiple intersecting markers, sheds new light on the complex interactions among markers in predicting cancer patient outcomes. The results reveal that protein phosphatases are highly expressed in HCC and are associated with a poor prognosis. Additionally, KEGG and GO pathway analyses suggest that protein phosphatases may exert their effects on liver cancer through various targets and pathways, ultimately promoting HCC. This information implies that protein phosphatases could play a significant role in the development and progression of HCC. Notably, in our study, we found significantly upregulated levels of CDC25C, PPP1R13L, and PPP1CA, which emerge as promising avenues. This significant expression could serve as potent diagnostic and prognostic markers to enhance the effectiveness of HCC cancer treatment, offering efficiency and accuracy in patient assessment. In essence, our research provides novel insights into the phosphoproteomic landscape of protein phosphatases in HCC, contributes valuable information to the HCC research field, and highlights a potential biomarker combination for enhanced diagnostic and prognostic capabilities.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c07787.

Detailed information on demographic and clinical characteristics of patients with HCC in the study; identification of 105 protein phosphatases with significant changes (*p*-values <0.05) between samples; comprehensive overview of GO analysis using GSEA; highlights of significant MF, CC, and BP associated with studied molecular pathways; comparison of OS between two groups based on median risk scores; exploration of mRNA expression in COAD and KIRC data sets; and insights into differential mRNA expression patterns associated with these cancer types (XLSX)

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Author Contributions

The manuscript was collectively written by all authors. All authors have provided their consent and approval for the final version of the manuscript. A.M. contributed to the methodology, formal analysis, and data curation. A.M. was responsible for drafting the original manuscript. A.M. and N.A. contributed to manuscript review and editing. A.M. supervised the project, administered the research, and acquired funding.

Notes

The authors declare no competing financial interest.

Ethics Statement: The data used in our study were exclusively sourced from the CPTAC and TCGA data sets and were not derived from our own clinical samples. It is important to emphasize that our current study received ethical approval and consent from the ethics committee.

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ABBREVIATIONS

HCC	hepatocellular carcinoma
AFP	alpha-fetoprotein
CPTAC	clinical proteomic tumor analysis consortium
TCPA	The Cancer Proteome Atlas
TCGA	The Cancer Genome Atlas
PP1	protein phosphatase 1
KIRC	kidney renal clear cell carcinoma
LIHC	liver hepatocellular carcinoma
STAD	stomach adenocarcinoma
COAD	colon adenocarcinoma
NES	normalized enrichment scores

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