Original Paper

Comprehensive and Integrative Analysis Reveals the Diagnostic, Clinicopathological and Prognostic Significance of Polo-Like Kinase 1 in Hepatocellular Carcinoma

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Polo-like kinase 1 • Hepatocellular carcinoma • Data mining • Cell cycle pathway

Abstract

Background/Aims: Liver cancer has the second highest cancer-related death rate globally and has relatively few targeted therapeutics. Polo-like kinase 1 (PLK1) is a fascinating trigger of the cell cycle; however, the still-rudimentary understanding of PLK1 at present is a significant barrier to its clinical applications. Here, we comprehensively clarified the clinicopathological value and potential functions of PLK1 in hepatocellular carcinoma (HCC). **Methods:** HCC-related microarrays, RNA-sequencing datasets and published studies were deeply mined and integrated from The Cancer Genome Atlas, Gene Expression Omnibus, ArrayExpress, Oncomine, literature databases, and immunohistochemistry experiments. Meanwhile, the associations between PLK1 expression and its clinicopathological implications and prognostic value in HCC patients were assessed. The standardized mean difference, summary receiver operating characteristic curve and the corresponding area under the curve, hazard ratios, odds ratios (ORs), and their 95% confidence intervals (CIs) were examined by STATA 12.0. Additionally, several bioinformatics methods were used to identify the potential function of PLK1 in HCC. **Results:** Comprehensive analyses revealed that PLK1 was significantly increased in HCC (standardized mean difference = 1.34, 95% CI: 1.03–1.65, $P < 0.001$). The results of diagnostic tests specified that in the summary receiver operating characteristic curve, the area under the curve was 0.88 (95% CI: 0.85–0.90). Furthermore, an elevated PLK1 level significantly predicted unfavorable overall survival (hazard ratio = 1.78, 95% CI: 1.10–2.88, $P = 0.019$) and was correlated with female gender (OR = 0.73, 95% CI: 0.56–0.95, $P = 0.017$), tumor thrombus (OR = 3.97, 95% CI: 1.46–10.78, $P < 0.001$), metastasis (OR = 3.46, 95% CI: 1.33–9.01, $P = 0.011$), pathologic stage (OR = 1.56, 95% CI: 1.17–2.07, $P = 0.002$), Barcelona Clinic Liver Cancer stage (OR = 5.76, 95% CI: 2.17–15.28, $P < 0.001$) and histologic grade (OR = 2.33, 95% CI: 1.12–4.87, $P = 0.024$). Through bioinformatics methods, we determined that enhancing the proliferative effect of PLK1 in HCC was associated with a series of hub genes and the activation P. Lin, D.-y. Wen contributed equally to this work.

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of the cell cycle pathway. **Conclusions:** These findings substantiated that PLK1 may be an independent prognostic biomarker in HCC and may facilitate the development of targeted precision oncology.

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Introduction

Liver cancer, of which hepatocellular carcinoma (HCC) is the predominant form, is the second leading cause of death related to cancer globally, being outranked by only lung cancer [1, 2]. In 2017, the numbers of newly diagnosed cases of and deaths from HCC in the United States are assumed to reach 40,710 and 28,920, respectively [3]. Despite the rapid pace of technological advances, the five-year survival rate of patients with HCC after initial diagnosis remains at approximately 14% [4]. Hepatocarcinogenesis is considered a complex and multistep process, both etiologically and biologically, but the specific perturbed pathways involved in the initiation of HCC remain only partially understood [5, 6]. Furthermore, pivotal biomarkers verified as specific driver molecules or mutations in HCC are still relatively few in number, which leads to a paucity of potentially therapeutic interventions [7, 8]. In light of these considerations, identifying biomarkers that can reliably estimate the carcinogenesis and deterioration of HCC has tremendous value in addressing these present challenges.

Polo-like kinase 1 (PLK1), a fascinating trigger of the cell cycle, is mainly involved in mitotic entry, the G2/M checkpoint, spindle assembly, chromosome segregation, cytokinesis, and DNA replication [9-12]. The irreplaceable role of PLK1 in the cell cycle is apparent, as the perturbation of PLK1 leads to a susceptibility to various diseases, especially cancers. Therefore, dysregulated PLK1 is frequently perceived as a tumor marker that is closely correlated with tumor prognosis [13-15]. Although it has long been speculated that PLK1 overexpression vigorously promotes the carcinogenesis and progression of HCC, this phenomenon remains obscure. Interestingly, recent studies have proposed several novel nonmitotic functions of PLK1. For example, Diab et al. identified that PLK1 could be a proviral host factor for hepatitis B virus and that it positively regulated hepatitis B virus replication [16]. This raised the suggestion that PLK1-targeted therapy could be effective in strategies combining antiviral and anti-tumor treatments. Additionally, some researchers have recently provided new insights into the molecular function of PLK1 in regulating autophagy. However, the results are not conclusive [17-19]. These studies will prompt a major change in how knowledge of PLK1 is conceptualized.

While the role of PLK1 in HCC is interesting and many aspects still need to be explored, we believe that clarifying its clinical value and identifying its underlying molecular mechanism could offer exciting prospects. Accordingly, we have performed the first multi-platform analysis of PLK1, which includes a description of the overexpression of PLK1 and the relationship of PLK1 with the clinicopathological features in The Cancer Genome Atlas (TCGA). The above findings were verified using the Gene Expression Omnibus (GEO), ArrayExpress and Oncomine databases. The HCC specimens obtained from in-house patients were evidently supplemental samples. Using bioinformatics methods, the co-operative genes and regulators of PLK1 in HCC were fully mined and visualized. Through the integration of rich and varied clinical data sources with *in silico* analysis, we have facilitated a better interpretation of the oncogenic role of PLK1 in the onset and progression of HCC and a deeper understanding of its biological roles.

Materials and Methods

TCGA dataset

To investigate the expression pattern and clinical role of PLK1 in HCC, we first downloaded genomic RNA-seq raw count data and clinical data from TCGA, which provided 371 primary HCC patients with gene expression data and clinical follow-up information [20]. Among these patients, a total of 50 had PLK1

expression data in both HCC and paired adjacent non-tumor tissues. The related data were processed using a log2 transformation. Based on the median level of PLK1 expression, the HCC cases were categorized into two groups to evaluate the correlation between PLK1 and the clinicopathological parameters and clinical outcomes. To make the results more reliable, we also computed the expression pattern and clinical outcomes of PLK1 in HCC via the Gene Expression Profiling Interactive Analysis (GEPIA) online database [21]. This is a newly available resource for analyzing the differential expression of genes and linking TCGA survival data to the expression of genes in units of transcripts per million, which is considered to be the most suitable unit for comparing RNA-seq data.

Data mining

The expression of PLK1 in 38 cancer cell lines was obtained from the Cancer Cell Line Encyclopedia [22]. We also searched for and downloaded PLK1-related microarray and RNA-seq datasets from GEO, ArrayExpress, and Oncomine. The following keywords were used for the searches for HCC: (malignant* OR neoplas* OR cancer OR tumour OR tumor OR carcinoma) AND (hepatocellular OR hepatic OR liver OR HCC). We extracted all of the expression data for PLK1 and the relevant clinical parameters from the datasets being searched out. Additionally, published studies containing data reporting the expression of PLK1 in HCC were also searched for and identified with the following databases: Web of Science, PubMed, Cochrane Library, EMBASE, Medline, CNKI and Wan Fang. The following search strategy was used: (PLK1 OR STPK13 OR "polo-like kinase 1") plus the part used for HCC aforementioned.

Immunohistochemistry

Three tissue microarrays containing 336 HCC and 133 normal tissues were used for the analysis. Immunohistochemical staining (IHC) was carried out using a rabbit polyclonal primary antibody to PLK1 (1:1500 dilution; Abcam, ab21738). Two pathologists (GC and YWD) scored the intensity of PLK1 expression via the immunoreactivity scoring system (IRS) independently. Under light microscopy, a four-tiered grading system that included "no staining" as 0, "weak staining" as 1, "intermediate staining" as 2 and "strong staining" as 3 was used to evaluate staining intensity. Meanwhile, the positive cell rates were scored from 0 to 4 under the following system: 0, no stained cells; 1, <10% of the cells were stained; 2, 11–50% of the cells were stained; 3, 51–80% of the cells were stained; and 4, more than 80% of the cells were stained. Depending on the multiplication of the staining intensity and positive cell rates of PLK1, an IRS ranging from 0 – 12 was generated [23]. HCCs were categorized into positive-PLK1 group with the IRS ≥ 6 , or negative-PLK1 group with the IRS < 6.

Statistical processing

SPSS 24.0 (Chicago, IL, USA) and STATA software, Version 12.0 (StataCorp, College Station, TX, USA) were used to statistical analyses. Relevant figures were produced using R and GraphPad Prism 5 (San Diego, CA, USA). The relationships between PLK1 expression level and the clinicopathological characteristics of HCC patients were calculated using the chi-squared test. The public microarray or RNA-seq datasets used for comparing PLK1 expression were log2-transformed. Data are presented in the format of the mean $\pm$ standard deviation in each of the datasets. A receiver operating characteristic (ROC) curve and the corresponding area under the ROC curve (AUC) for each dataset were performed. Kaplan-Meier (K-M) method was used to draw the survival curve, and differences in survival were test for significance using the log-rank test.

To draw a comprehensive conclusion, we integrated data from multiple sources by meta-analysis. For the PLK1 expression pattern in HCC, the total standardized mean difference > 0 and its 95% confidence interval (CI) also greater than 0 suggested that PLK1 is remarkably overexpressed in tumors compared with that in adjacent non-tumor tissues. Then, we further studied the comprehensive performance of the PLK1 expression pattern in distinguishing HCC tissues from non-tumor tissues, for which we drew summary ROC curves and calculated the AUC values, calculated the 95% CIs, and determined the sensitivity and specificity. For the correlations between PLK1 expression and the clinicopathological features, the odds ratio (OR) with the 95% CI were utilized to explore the significance of the integrated results. Only those clinical parameters that were reported by no fewer than 3 studies were included in the meta-analysis. We also obtained hazard ratios (HRs) with their 95% CIs, either directly or extracted from the K-M survival curves, using Engauge Digitizer software. A combined HR and its 95% CI were computed by STATA to reveal the prognostic significance of PLK1 in HCC. A pooled HR > 1 and its 95% CI not crossing 1 is implicative of an inferior outcome for HCC patients with PLK1 overexpression.

Cochran's Q test and I^2 statistics were employed to evaluate heterogeneity across the included studies. A P-value < 0.1 or an I^2 > 50% indicated heterogeneity. A random-effects model or fixed-effects model was selected to carry out the meta-analysis according to the heterogeneity. The level of statistical significance was $P < 0.05$.

Interactions network

To show the interactions between PLK1 and other molecules, we generated a causal interaction network between PLK1 and other biological entities via the SIGnaling Network Open Resource (SIGNOR) database, which is designed to facilitate molecular insights into causal interactions [24]. The interactions score threshold was set to 0.4.

Gene set enrichment analysis

Since only a few studies have revealed the exact pathways that PLK1 regulates in HCC, gene set enrichment analysis (GSEA) was performed between the high and low PLK1 expression tumors to identify the related Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways regulated by PLK1 using data downloaded from TCGA. GSEA is a gene enrichment method used to identify significantly enriched or depleted groups of genes in a full list of genes, which are associated with phenotypes [25]. GSEA software (v3.0; Broad Institute, Cambridge, MA, USA) was downloaded from the Broad Institute website. The false discovery rate value and normalized enrichment score were calculated and used to rank the pathways enriched in different phenotypes. The gene set permutations were conducted 1000 times. The top 10 enriched genes of interest of the KEGG pathway were visualized using R. Genes with a ranked score greater than 0.9 were identified as the core co-operative genes with which PLK1 interferes during HCC tumorigenesis.

Identification of the co-operative gene set

To further study the clinical value of the hub genes, the expression patterns of these hub genes were displayed using matrix plots. Scatterplots of the relationships between PLK1 expression and the hub genes were obtained from GEPIA. Furthermore, SurvExpress was used to perform multivariate survival analysis and risk categorization of the gene set. SurvExpress is a database for the confirmation of multi-markers for gene expression in cancers [26]. Using this online tool, we input and analyzed the prognostic significance of the 18-gene signature in HCC. Then, the algorithm calculated the prognostic index (PI), which was used to analyze the prognostic value of the panel of genes.

Potential regulators of PLK1

The identification of potential regulators of PLK1 were identified from the intersection of 12 in silico databases, including the miRWalk, miRanda, MicroT4, miRMap, miRDB, miRNAmap, RNA22, PITA, RNAhybrid and Targetscan databases. The microRNAs (miRNAs) that were simultaneously predicted in no less than 4 of the databases were selected for subsequent study. Furthermore, the publications were assessed for validated mRNA-regulator interactions, which were also collected in the miRWalk program. Then, the relationships between PLK1 and the miRNAs were visualized by a plot that was produced using Cytoscape software.

Determination of PLK1 alterations

The underlying mechanism relevant to genetic alterations of PLK1 remains unknown; therefore, we analyzed the methylation and copy number variation (CNV) status of PLK1. By using the cBioPortal database, we obtained the relationships between the expression profiles and gene alterations (including methylation, amplifications and copy number) of PLK1 in HCC.

Results

An overview of the procedure used to analyze the data is shown in Fig. 1. This study mainly included the mining of TCGA, a verification by other public databases, and an aggregation of the IHC experimental results. Furthermore, bioinformatics analyses were performed to understand the molecular events relevant to PLK1 in HCC.

Fig. 1. Flow chart of the present study.

Overall browsing for PLK1 expression

First, we noticed the overall expression of PLK1 in 33 types of cancer based on GEPIA, which computed the TCGA data in the form of transcripts per million. The expression of PLK1 was significantly upregulated in 20 tumors, including HCC (Fig. 2). Furthermore, the PLK1 expression patterns of 1854 cell lines representing 38 distinct tumor types are shown in Fig. 3. The mean expression of PLK1 in the liver tumor cell lines ranks 15th among the 38 cancer cell lines.

Clinical implications of PLK1 in HCC based on TCGA

We analyzed the data downloaded from the TCGA portal and obtained the same result as that from GEPIA, in which PLK1 expression was dramatically elevated in HCC tissue (8.439 ± 1.760 vs. 4.790 ± 1.041 , $P < 0.001$; Fig. 4A). The area under the ROC curve for PLK1 expression was 0.960 ($P < 0.001$), which suggests that the capacity of PLK1 to discriminate HCC tissues from non-tumor tissues is moderate (Fig. 4B). As presented in Table 1, the HCC patients were categorized into PLK1-positive and negative groups according to different grouping criteria, and differences between both groups were explored by the chi-squared

Fig. 2. The expression patterns of PLK1 across 33 cancer types and paired non-tumor samples. Each dot represented a distinct tumor or normal sample. Data was obtained through the Gene Expression Profiling Interactive Analysis (GEPIA).

Fig. 3. The PLK1 expression patterns of 1854 cell lines representing 38 distinct tumor types. Data was obtained through the Cancer Cell Line Encyclopedia (CCLE).

test or Fisher's exact test. Clinicopathological relevance analysis of the HCC patients demonstrated that PLK1 expression was closely related to age, gender, pathologic stage, pathologic T stage, histologic grade, tumor status, alpha-fetoprotein (AFP) and vascular tumor cell type (Table 1). Interestingly, PLK1 expression increased progressively during the multistep transformation of low-grade malignancies into high-grade malignancies (Fig. 4C, D). Nonetheless, there was not a statistically significant correction between the expression of PLK1 and other clinicopathological features. More importantly, survival curves were drawn, which were based on the median level of the PLK1 expression, by K-M analysis and the log-rank test. As shown in Fig. 4E and 4F, HCC patients with upregulated PLK1 expression had

Fig. 4. Relative PLK1 expression in hepatocellular carcinoma (HCC) tissues and its clinical significance based on TCGA. (A) Analysis of PLK1 mRNA expression in TCGA cohort. (B) Receiver operating characteristic (ROC) curve of PLK1 expression for the differentiation of HCC from non-tumor tissues. (C) The expression level of PLK1 in different pathological stage. (D) The expression level of PLK1 in different histological grade. (E) Kaplan-Meier (K-M) curve for overall survival (OS) stratified according to low or high expression of PLK1 in HCC. (F) K-M curve for disease free survival (DFS) stratified according to low or high expression of PLK1 in HCC. (G) OS analysis based on PLK1 expression by Gene Expression Profiling Interactive Analysis (GEPIA) [20]. (H) DFS analysis based on PLK1 expression by GEPIA.

an inferior overall survival (OS) and disease-free survival compared to those with downregulated PLK1 expression. These results were also validated by another data source, which was obtained from the GEPIA (Fig. 4G, H). Further univariate and multivariate analyses confirmed that PLK1 is an independent predictor of poor prognosis in patients with HCC (HR = 2.208, 95% CI: 1.329–3.667, $P = 0.002$, Table 2).

Verification of the PLK1 expression pattern via other public databases

A total of 36 microarray or RNA-seq datasets were collected from the GEO, Oncomine, and Array-Express datasets and were used to evaluate the expression pattern of PLK1[27-61]. In addition, two studies reported the mRNA expression

Table 1. The relationships between PLK1 expression and clinicopathological features in HCC based on TCGA data

Clinicopathological features	n	PLK1 expression level PLK1 Low Expression (< Median)	PLK1 High Expression (≥ Median)	Chi-squared value	p-value
Age				3.932	0.047
<60	169	75	94		
≥60	201	110	91		
Gender				4.277	0.039
Male	250	134	116		
Female	121	51	70		
Race				1.160	0.282
Asian	168	78	90		
White	184	96	88		
Pathologic stage				10.345	0.001
I-II	257	142	115		
III-IV	90	32	58		
T				9.720	0.002
T1-T2	275	149	126		
T3-T4	93	33	60		
N				-	0.623
N0	252	123	129		
N1-N3	4	1	3		
M				-	0.369
M0	266	131	135		
M1	4	3	1		
Histologic grade				27.793	<0.001
G1-G2	232	138	94		
G3-G4	134	45	89		
Tumor status				10.943	0.001
Tumor free	201	117	84		
With tumor	151	61	90		
AFP				22.544	<0.001
<400	225	134	91		
≥400	65	17	48		
Vascular tumor cell type				2.368	0.124
None	206	117	89		
Micro+Macro	109	52	57		

Table 3. The relationships between PLK1 expression and clinicopathological features in HCC in-house

Clinicopathological features	n	PLK1 expression level		Chi-squared value	P-value
		Negative	Positive		
Age				1.004	0.316
<60	260	102	158		
≥60	76	25	51		
Gender				1.191	0.275
Male	272	99	173		
Female	64	28	36		
Pathologic stage				0.910	0.340
I-II	236	92	144		
III-IV	93	31	62		
T				4.203	0.04
T1-T2	204	85	119		
T3-T4	125	38	87		
N				0.043	0.836
N0	303	112	191		
N1-N3	23	9	14		
Histologic grade				5.205	0.023
G1-G2	231	95	136		
G3-G4	91	25	66		
AFP				4.978	0.026
<400	37	10	27		
≥400	43	18	25		
Diameter				1.192	0.275
>5cm	57	21	36		
≤5cm	28	7	21		

nine samples (62.2%) were in the PLK1-positive group and 127 cases (37.8%) in the PLK1-negative group. Moreover, 21 non-tumor tissues (16.4%) exhibited positive PLK1 expression, and 107 (83.6%) were negative (Fig. 8A, B). The IRS values demonstrated that PLK1 protein expression was markedly up-regulated in HCC samples (5.595 ± 3.066) than in non-cancer tissues (1.680 ± 2.223 , $P < 0.001$, Fig. 8E).

The correlations between PLK1 expression in HCC and the clinicopathological features of HCC are listed in Table 3. We found close associations between PLK1 expression and the pathologic T stage ($P = 0.004$), histologic grade ($P = 0.023$) and AFP ($P = 0.026$), indicating that PLK1 might be functionally implicated in the development of HCC. However, no positive correlations between PLK1 expression and age, gender, pathologic N stage or tumor diameter were observed.

K-M survival analysis of PLK1 expression with OS between different subgroups was also performed. As presented in Fig. 8F, patients in the PLK1-positive group had a worse OS (log-rank, $P = 0.028$) than those in the PLK1-negative group. Then, we further conducted univariate and multivariate analyses to confirm whether PLK1 expression is an independent predictor for the prognosis of HCC. The results from the univariate analysis showed that PLK1 expression was strongly correlated with the prognosis of HCC (HR = 2.109, 95% CI: 1.061–4.194, $P = 0.033$). We included parameters that were profoundly related to the prognosis of HCC patients in the multivariate analysis and confirmed that PLK1 expression was still a predictor for the outcomes of HCC patients (HR = 2.082, 95% CI: 1.046–4.143, $P = 0.037$) (Table 4).

Meta-analysis of all-inclusive data

To obtain results for a comprehensive analysis of PLK1 expression, we combined data from the TCGA, GEO, Oncomine, and ArrayExpress databases with the IHC staining results

Fig. 6. Receiver Operating Characteristic (ROC) curves of PLK1 expression for the differentiation of HCC from non-tumor tissues based on public databases.

Fig. 7. Receiver Operating Characteristic (ROC) curves of PLK1 expression for the differentiation of HCC from non-tumor tissues based on another several public databases.

and that the sensitivity and specificity were 0.81 (95% CI: 0.75–0.87) and 0.8 (95% CI: 0.73–0.86), respectively (Fig. 10).

Associations between PLK1 expression and several clinicopathological features of HCC patients were also computed. PLK1 overexpression was identified as being significantly associated with female gender (OR = 0.73, 95% CI: 0.56–0.95, $P = 0.017$; $I^2 = 0.00\%$, $P = 0.782$; Fig. 11A), metastasis (OR = 3.46, 95% CI: 1.33–9.01, $P = 0.011$; $I^2 = 50.6\%$, $P = 0.108$; Fig. 11B), Barcelona Clinic Liver Cancer (BCLC) stage (OR = 5.76, 95% CI: 2.17–15.28, $P < 0.001$; $I^2 = 0.00\%$, $P = 0.409$; Fig. 11C), tumor thrombus (OR = 3.97, 95% CI: 1.46–10.78, $P < 0.001$; $I^2 = 55.0\%$, $P = 0.064$; Fig. 12A), pathologic stage (OR = 1.56, 95% CI: 1.17–2.07, $P = 0.002$; $I^2 = 46.1\%$, $P = 0.084$; Fig. 12B), and histologic grade (OR = 2.33, 95% CI: 1.12–4.87, $P =$

Fig. 8. The Immunohistochemical analysis of PLK1 in HCC. (A, B) A normal liver tissue showing low PLK1 expression. (C, D) A HCC tissue showing high PLK1 expression. (A, C: Magnification $\times 100$; B, D: Magnification $\times 400$). (E) immunoreactivity scoring system (IRS) results indicated that PLK1 staining scores were higher in HCC tissues than non-tumor tissues. (F) Kaplan-Meier (K-M) curve indicated HCC patients in PLK1 positive group had poorer overall survival.

for a meta-analysis. A total of 39 studies reported a difference between the mRNA expression levels of PLK1 in HCC and non-cancer tissues. These studies showed that PLK1 expression was higher in the tumor group (standardized mean difference = 1.34, 95% CI: 1.03–1.65, $P < 0.001$; Fig. 9) when using a random-effects model. Meta-analysis results for the diagnostic tests specified that the AUC of the summary ROC was 0.88 (95% CI: 0.85–0.90)

0.024; $I^2 = 74.1\%$, $P = 0.002$; Fig. 12C). PLK1 overexpression did not exhibit an association with age (OR = 0.82, 95% CI: 0.64–1.05, $P = 0.123$; $I^2 = 30.1\%$, $P = 0.168$; see online suppl. material, Fig. S1A), hepatitis B (OR = 0.79, 95% CI: 0.42–1.49, $P = 0.471$; $I^2 = 10.8\%$, $P = 0.339$; Fig. S1B), tumor number (OR = 1.11, 95% CI: 0.29–2.25, $P = 0.878$; $I^2 = 71.1\%$, $P = 0.016$; see online suppl. material Fig. S1C), tumor diameter (OR = 1.49, 95% CI: 0.93–2.39, $P = 0.102$; $I^2 = 0.00\%$, $P = 0.455$; see online suppl. material Fig. S1D) or AFP (OR = 1.38, 95% CI: 0.46–4.15, $P = 0.571$; $I^2 = 85.0\%$, $P < 0.001$; see online suppl. material, Fig. S1E), (see online suppl. material, Fig. S1).

Five datasets contained information about PLK1 expression and the OS of HCC patients. Pooled data analysis revealed a significant relationship between up-regulated PLK1 expression and poor OS, and the HR estimate of this analysis was 1.78 (95% CI: 1.10–2.88, $P = 0.019$; Fig. 13). This analysis also revealed minor heterogeneity in the data ($I^2 = 56.5\%$, $P = 0.056$).

Interactions between PLK1 and other molecules

Considering that proteins rarely function alone, a systems-level understanding of the function of PLK1 is necessary. The assembly of a causal interaction network for PLK1 could provide an informative and global view of its molecular interactions. On the basis of

Table 4. Univariate and multivariate analyses of OS in HCC patients of immunohistochemistry by Cox regression analysis

Variables	Univariate analysis		Multivariate analysis	
	Hazard ratio (95%CI)	P value	Hazard ratio (95%CI)	P value
Age (≥ 60 / <60)	1.719 (0.969–3.051)	0.064		
Gender (male/female)	1.374 (0.672–2.808)	0.384		
Pathologic stage (III–IV/I–II)	1.720 (1.007–2.941)	0.047	0.514 (0.203–1.299)	0.160
Tumor (T3–T4/T1–T2)	2.297 (1.346–3.920)	0.002	3.745 (1.491–9.406)	0.005
Lymph node metastasis (yes/no)	1.308 (0.521–3.284)	0.568		
Histologic grade (G3–G4/ G1–G2)	1.442 (0.784–2.653)	0.239		
AFP (≥ 400 / <400)	1.387 (0.795–2.420)	0.249		
Diameter (>5 cm/ ≤ 5 cm)	1.412 (0.698–2.858)	0.338		
PLK1 (Positive/Negative)	2.109 (1.061–4.194)	0.033	2.082 (1.046–4.143)	0.037

Fig. 9. Forest plot of standard mean differences (SMD) for evaluating PLK1 expression between hepatocellular carcinoma (HCC) and non-tumor tissues.

Fig. 10. Summary receiver operating characteristic (SROC) curve for distinguishing hepatocellular carcinoma (HCC) from non-tumor tissues using PLK1.

Fig. 11. Meta-analysis of the association between PLK1 and (A) female gender, (B) metastasis and (C) Barcelona Clinic Liver Cancer (BCLC) stage. Results are presented as individual and pooled odds ratio (OR), and 95% confidence interval (CI).

the SIGNOR database, the causal interaction network of PLK1 contained 42 nodes that were selected with a combined score of > 0.4 (Fig. 14).

Pathways significantly regulated by PLK1 in HCC

According to the GSEA results, high PLK1 expression was significantly related to the cell cycle, base excision repair and oocyte meiosis (Fig. 15). We noted that among these pathways, the cell cycle had the most significant variation (Fig. 16). These findings suggested that PLK1 is involved in the development and progression of HCC through several progression-associated pathways. Additionally, 18 genes were determined as being the core interaction genes in the PLK1-regulated tumorigenesis of HCC: CCNF, PLK1, TPX2, RFC4, POLD1, LMNB1, BUB1, KIFC1, CCNB2, SKA3, SPC25, CCNB1, CKAP2L, SGO1, EZH2, PKMYT1, TROAP, and GINS1.

Analysis of the co-operative gene set

An expression matrix plot of co-operative genes was plotted (Fig. 17), in which the density of the color in each block represents the median expression value of a gene. Furthermore, the correlations between the expression of PLK1 and the hub genes were generated based on the TCGA dataset via GEPIA (Fig. 18). We analyzed the prognostic value of the expression of the core interaction genes in TCGA using SurvExpress. The HCC patients from TCGA were divided into two groups, the low-risk and high-risk groups, according to the median point of the PI score (Fig. 19). We also assessed the expression levels of these genes in different risk groups. PLK1, TPX2, RFC4, POLD1, LMNB1 and GINS1 were noted as having remarkably higher expression in the patients in the high-risk group (Fig. 20A). Furthermore, K-M curves suggested that a higher PI was evidently related to a poor prognosis and shorter

Fig. 12. Meta-analysis of the association between PLK1 and (A) tumor thrombus, (B) pathologic stage and (C) histologic grade in HCC. Results are presented as individual and pooled odds ratio (OR), and 95% confidence interval (CI).

OS time (HR = 2.20, 95% CI: 1.56–3.11, $P < 0.001$; Fig. 20B), which confirmed that the PI score of the core interaction genes could function as an indicator with moderate predictive power for the survival of HCC patients.

Regulatory network

A miR-mRNA regulatory network is a complicated biological system. miRNAs reduce the expression of downstream targets by binding to the 3'-untranslated regions of mRNAs. Thus, exploring which miRNAs bind to PLK1 could provide a deeper understanding of the biological mechanisms in which PLK1 is involved. Using the 12 online predictive tools mentioned above, a total of 119 miRNAs appeared on 4 or more platforms. These miRNAs were screened, and 17 validated genes were obtained from miRWalk. Finally, a total of 136 potential or identified miRNAs that could regulate and control the expression of PLK1 were identified (Fig. 21).

PLK1 genetic alteration status

As shown in Fig. 22A, of 440 cases with HCC, a genetic alteration of PLK1 was detected in 27 cases as provided by the online database cBioportal. Four types of CNV (shallow deletion, diploid, gain and amplification) of PLK1 were detected in HCC based on TCGA data (Fig. 22B). PLK1 diploid was the main type of CNV, and a relatively rare PLK1 amplification was observed. We also examined the relationships between DNA methylation of the PLK1 promoter and mRNA expression level. Interestingly, when assessing the relationship between PLK1 mRNA expression and DNA methylation data, we found that PLK1 mRNA levels were negatively correlated with the methylation level of a CpG locus in HCC patients (Fig.

Fig. 13. Forest plot of hazard ratio for the prediction of overall survival using PLK1.

Fig. 14. A causal interaction network displayed an informative and global view of molecules interacted with PLK1. Data was obtained through the SIGnaling Network Open Resource (<http://signor.uniroma2.it/>) [23].

Fig. 15. Bar plot displayed Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways computed by Gene Set Enrichment Analysis (GSEA) [24]. Blue-to-red color corresponded to normalized enrichment score (NES). The length of the bar represented the number of genes enriched in the pathway.

22C). These results provided us with a clue that epigenetic regulation of PLK1 mRNA expression might be a key factor related to its clinical significance in HCC. Furthermore, K-M analysis revealed that cases with PLK1 alterations had a shorter median OS (45.07 vs. 58.84 months; Fig. 23A) and median disease-free survival (13.63 vs. 20.99 months; Fig. 23B); however, there was no statistically significant difference, probably due to the limited number of cases involved.

Discussion

Here, we deeply mined and integrated large-scale datasets to enhance our understanding of the mechanism by which the upregulated expression of PLK1 significantly promotes the tumorigenesis of HCC and deterioration of HCC patients. Additionally, high PLK1 expression could be a robust predictor of an inferior prognosis in patients with HCC. Using bioinformatics analysis, we identified major determinants of and co-operative molecules involved in the mechanism of regulating PLK1 expression in HCC. Additionally, we provided an insight into how PLK1 functions as the core component of the cell cycle during its participation in the tumorigenesis of HCC. The utilization of various distinct data sources and bioinformatics tools in the current study has facilitated integrated solutions that are not possible in studies with a single platform. Our results proposed that PLK1 functions as an oncogenic player by interacting with several molecules and modifying the cell cycle in HCC, and this could provide a superior prognostic predictive biomarker and guide anti-PLK1 targeted treatment for HCC.

Throughout the course of the great advances in high-throughput sequencing over the past decades, an ever increasing amount of sequencing data have provided the means for testing gene signatures [72]. We, for the first time, collected microarray, RNA-seq, RT-PCR and IHC data to corroborate that there is a sharp upregulation of PLK1 expression in HCC. This evidence indicated that PLK1 could be an indispensable molecular biomarker that is involved in the onset of HCC. The fact that PLK1 was overexpressed in multiple types of cancer suggests that it could be a more general oncogene (Fig. 2). Another distinctive feature of the current study is that PLK1 functioned as a major driving force of tumor progression toward the stages of metastasis and differentiation. These findings strongly suggested that PLK1 is a major factor in the onset of HCC and in the deterioration of HCC patients. Another highlight of this study is that the prognostic value of PLK1 expression in HCC was evidently confirmed in the form of a meta-analysis. Thus, PLK1 expression could be an independent prognostic factor in patients with HCC.

Fig. 16. The expression profiling changes of genes in cell cycle pathway between the different expression of PLK1. Each lattice represented the expression of a gene in a patient, blue indicated that the gene was down-regulated, and red indicated that the gene was up-regulated.

Fig. 17. Expression matrix plots based on co-operative genes of PLK1. The density of color in each block represented the median expression value of a gene in tumor or non-tumor.

Therefore, depicting the precise biological processes that are controlled by PLK1 in HCC seems most appropriate. However, the still-rudimentary understanding of PLK1 at present is a significant barrier to its clinical application. PLK1 is a mitotic serine/threonine protein kinase, and oncogenic PLK1 has recently been considered as a subtype-related biomarker of HCC [73]. Wang et al. reported that the progression of HCC could be inhibited by PLK1-targeting chitosan nanoparticles, which apparently reflected the fact that PLK1 exerted functions that are relevant to the inappropriate proliferation of HCC cells. Furthermore, the overexpression of SLAMF3 exhibits tumor suppressor potential via inhibiting the expression and phosphorylation of PLK1 [74]. Several drugs that target PLK1 are currently being tested in preclinical trials, and these may develop into novel therapeutics for the management of cancer [75-77]. Nevertheless, the results of preclinical trials still do not translate to the clinical effectiveness of PLK1 in monotherapy. Hence, combination treatments may be functionally implicated for improving efficacy. Currently, volasertib is arguably one of the most clinically advanced PLK1 inhibitors *in vitro* and *in vivo*, and it has also been tested in combination with other inhibitors [78, 79]. A second but still-unresolved issue troubling the clinical application of PLK1 in HCC is that relatively little is known about its clinical value and corresponding mechanisms. Thus, we conducted this study and its findings are expected to offer a new means to treat HCC.

We also used several bioinformatics methods to explore the underlying mechanism of PLK1 in relation to its clinical significance. Tumorigenicity has been increasingly recognized as a complex mechanism, and no genes have been identified that can induce carcinogenesis on their own. Thus, GSEA was established from the view of gene set level analysis. GSEA can make up for the deficiency of traditional strategies that focus on the expression of

Fig. 18. Correlations between the expression of PLK1 and the hub genes were generated based on the TCGA dataset via online database GEPIA [20].

Fig. 19. A risk score of HCC patients using 18 hub genes. (A) The expression pattern of these genes in low and high score groups. (B) The survival status of HCC patients. (C) Heatmap of these genes expression in HCC. The color from blue to red showed a trend from low expression to high expression.

Fig. 20. Survival analysis with SurvExpress [25]. (A) Different expression of the hub genes between high risk group and low risk group. (B) Kaplan-Meier curves for the five lncRNAs signature in TCGA HCC patients. Green and red indicated low-risk and high-risk groups, respectively.

individual genes. According to the GSEA report, PLK1 overexpression significantly perturbs the expression of genes relevant to the cell cycle in HCC. Normal liver tissues carefully control growth-related pathways and regularly release instructions to ensure cellular homeostasis [80-82]. The PLK family comprises a type of factor that has highly conserved domains from yeast to humans, which have important roles in the development of multiple tissues. Interestingly, PLK1 has distinguishing functions compared to those of PLK2-4 [83, 84]. As mentioned previously, PLK1 negatively regulates the level of p53

Fig. 21. The regulator network of PLK1. The red diamond was a miRNA that has been demonstrated in studies to regulate PLK1. The blue circle was a miRNA that was predicted to regulate PLK1.

Fig. 22. Genetic alterations of PLK1 in HCC(A) Genetic alterations status of PLK1 in HCC. Genetic alterations were found in 27 of 440 HCC patients (27%).(B) A plot showing the relationship between PLK1 mRNA expression and copy number alteration (CNA).(C) A plot showing the relationship between PLK1 mRNA expression and PLK1 promoter DNA methylation.

directly or indirectly [85, 86]. However, whether the upregulation of PLK1 downregulates p53 expression or vice versa is still under question. Since PLK1 plays multifunctional roles in the cell cycle, maintaining genomic stability, communicating between DNA damage and recovery competence, it is not surprising that the deregulation of PLK1 expression is associated with carcinogenesis and the progression of cancer.

From performing GSEA analysis, 18 genes (CCNF, PLK1, TPX2, RFC4, POLD1, LMNB1, BUB1, KIFC1, CCNB2, SKA3, SPC25, CCNB1, CKAP2L, SGO1, EZH2, PKMYT1, TROAP, and GINS1) in the front rank of PLK1-regulated genetic changes were identified as comprising the co-operative gene set of genomic instability caused by PLK1. Several genes have also been reported to be involved in the onset and progression of HCC [87-89]. Further survival analysis revealed that the gene set was strongly correlated with a shorter OS. This result has provided a useful conceptual frame-

Fig. 23. The prognostic value of PLK1 alterations in HCC. The K-M analysis shows the overall survival (OS, A) and the disease-free survival (DSF, B) of HCC with or without PLK1 alterations.

work for understanding the complex biology of cancer. Increasing amounts of evidence have revealed that miRNA-mediated regulatory networks are drivers acting as rheostats that fine-tune gene expression and maintain the functional balance of various gene networks [90, 91]. Therefore, we prospectively collected and validated miRNAs to find the switch for PLK1. Similarly, the insight that hundreds of miRNAs could distinctly regulate PLK1 has already led to profound changes in our understanding of the regulatory mechanism of PLK1. We also assessed genomic alterations of PLK1 in HCC using cBioPortal to explain its clinical significance. Interestingly, PLK1 expression was negatively related to the methylation level of a CpG locus in its promoter. Taken together with the results of the copy number status of PLK1, we speculate that PLK1 expression might be regulated by both CNV and promoter methylation. However, there are very few studies on the epigenetic regulation of PLK1 in HCC. More in-depth studies in the future could provide the possibility of using the PLK1 methylation profile as a biomarker in the clinical setting, and identify how PLK1 exactly influences the progression of HCC.

Collectively, integrated analytic approaches have been applied to large sets of data to provide a better understanding of the vital functions of PLK1 in hepatocarcinogenesis, and it can serve as a prognostic biomarker and effective therapeutic target for HCC. We anticipate that the intercommunication pathways of PLK1 in HCC will gain greater clarity and will be important to the development of novel target therapies designed to treat HCC.

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Disclosure Statement

The authors declare that there is no conflict of interests.

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