

# Expression of FOXM1 and PLK1 predicts prognosis of patients with hepatocellular carcinoma

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**Abstract.** Hepatocellular carcinoma (HCC) is one of the most frequently encountered malignant tumor types and to improve its treatment, effective prognostic biomarkers are urgently required. Cell cycle dysregulation is a significant feature of cancer progression. The aim of the present study was to estimate the expression levels of forkhead box protein M1 (FOXM1) and polo-like kinase 1 (PLK1), both of which have essential roles in cell cycle regulation, and determine their prognostic value in HCC. To this end, FOXM1 and PLK1 expression levels were assessed in The Cancer Genome Atlas and International Cancer Genome Consortium Japan HCC cohorts, and the associations between their co-expression were determined via Pearson's correlation analysis. Furthermore, the overall survival and disease-free survival in these cohorts for different FOXM1 and PLK1 expression statuses were analyzed. *In vitro* knockdown experiments were also performed using Huh7 cells. The results obtained indicated overexpression of FOXM1 and PLK1 in HCC tumor tissues as well as a positive correlation between FOXM1 and PLK1 expression. The results also suggested that both FOXM1 and PLK1 are required for HCC cell proliferation. In addition, upregulation of FOXM1 and PLK1 was indicated to be associated with poor prognosis of patients with HCC. However, only

their coordinated overexpression was identified as an independent prognostic factor for HCC.

## Introduction

In 2020, 905,677 patients were diagnosed with liver cancer and this malignancy was accountable for 830,180 cancer-associated mortalities worldwide (1). Hepatocellular carcinoma (HCC), which is a multigenic disease with heterogeneous pathological mechanisms and clinical manifestation, accounts for 75-85% of primary liver cancer cases (1) and is a major health problem worldwide. It has been observed that the use of ultrasound monitoring every 6 months (with or without  $\alpha$ -fetoprotein for its treatment) is associated with improved early detection as well as improved overall survival (OS); however, in clinical practice, implementation-related limitations frequently result in a high proportion of HCC cases only detected at late stages (2). Furthermore, despite the improvements associated with the use of antiangiogenic drugs and immunotherapy over the past decade, HCC prognosis is limited (3). The major unmet challenges related to HCC treatments include advancements in the treatment at earlier stages of the disease, applying the treatment to patients with liver dysfunction, the discovery and validation of predictive biomarkers and the development of more effective combinatorial or sequential treatment approaches (3,4). Therefore, the mechanisms of HCC require to be explored and the identification of valuable biomarkers is urgently required.

Genome-wide expression profiling has enabled the analysis of patient heterogeneity within a short period. It has been proposed that the expression of numerous genes, including forkhead box protein M1 (FOXM1) (5,6) and polo-like kinase 1 (PLK1) (7,8), may serve as a putative prognostic biomarker for HCC. Although several studies have indicated that FOXM1 and PLK1 overexpression are associated with poor cancer prognosis (9-11), the underlying mechanisms have remained to be fully elucidated. Furthermore, to the best of our knowledge, the effect of the association between FOXM1 and PLK1 on the development and prognosis of HCC has not been reported in any previous study.

FOXM1 belongs to a large family of Fox transcription factors, all of which have a conserved domain attached to DNA (winged helix) (12). Furthermore, it has an important role in regulating cell cycle progression via the stimulation

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**Abbreviations:** HCC, hepatocellular carcinoma; OS, overall survival; PLK1, polo-like kinase 1; FOXM1, forkhead box protein M1; TCGA, The Cancer Genome Atlas; ICGC, International Cancer Genome Consortium; FBS, fetal bovine serum; cDNA, complementary DNA; siRNA, small interfering RNA; CCK-8, Cell Counting Kit 8; EdU, 5-ethynyl-2'-deoxyuridine; DFS, disease-free survival; HR, hazard ratio; TGF, transforming growth factor

**Key words:** forkhead box protein M1, polo-like kinase 1, hepatocellular carcinoma, cell cycle

of the genes that are critical for G1-S and G2-M transition, including S-phase kinase-associated protein 2, PLK1, centromeric protein A and survivin (13,14), and is itself regulated during the cell cycle process. Transcriptional activation of FOXM1 depends on cyclin-dependent kinase and PLK1 kinase mediates its phosphorylation (15-17). Furthermore, FOXM1 is frequently expressed at a higher level than normal in a variety of human cancers (18-20). Furthermore, several studies have demonstrated that FOXM1 is a key transcription factor that is associated with HCC (21,22). It has also been reported that elevated FOXM1 expression is associated with a poor prognosis of the disease (23,24).

Cell cycle disorders are essential for tumor development and protein kinases, which have important roles in regulating the cell cycle, are valuable targets for cancer therapy. Specifically, PLK1, a member of the serine/threonine kinase family, promotes cell mitosis in mammalian cells (25,26). It has also been identified as an essential mitotic kinase that controls mitotic entry, spindle assembly, centrosome maturation and cytokinesis (27,28). PLK1 overexpression was reported to cause cell cycle overrides in tumor cells, resulting in the survival, enhanced proliferation and immune evasion of cancer cells (29-31). It has also been observed that its expression is increased in numerous cancer types, including lung, bladder, breast and liver cancers (32-34). In addition, several studies have indicated that PLK1 overexpression may serve as an important prognostic factor for HCC (35,36); however, the underlying associated mechanisms have remained elusive. Selective inhibitors of PLK1 potently cause mitotic arrest and induce tumor cell apoptosis (37-39), indicating that PLK1 is a potential target for antitumor treatment.

PLK1, a target of FOXM1, is required for FOXM1 transcription activation and the formation of a feedback loop (14,16). In addition, PLK1 and FOXM1 are essential for the cell cycle process and are related to HCC prognosis. However, the association between this feedback loop and HCC has remained to be investigated. Therefore, in the present study, the prognostic values of PLK1 and FOXM1 overexpression in HCC were estimated using data from The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium Japan (ICGC JP) HCC cohorts. Furthermore, molecular analyses and cell proliferation assays were performed to examine the role of PLK1 and FOXM1 in Huh7 cells and the results suggested that the feedback loop is required for the proliferation of Huh7 cells.

## Materials and methods

**Clinical cohorts.** Sequencing and clinical data of patients with HCC were obtained from two public cohorts, namely TCGA (<http://xena.ucsc.edu>) and ICGC (<https://dcc.icgc.org>). A total of 373 and 243 patients from the TCGA and ICGC JP cohorts, respectively, were included in the analysis. Patients with incomplete OS or disease-free survival (DFS) information were excluded. OS was defined as the time from the date of initial pathological diagnosis to the time of death or last follow-up, while DFS was defined as the time from first treatment to the time of tumor recurrence or death. The TCGA cohort was used as the exploration cohort (clinicopathological information is provided in Table I), while the ICGC JP cohort was used for validation.

**Cell lines and cell culture.** The human Huh7 cell line (American Type Culture Collection) was cultured in a growth medium consisting of Dulbecco's Modified Eagle's Medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 4.5 g/l glucose, 10% fetal bovine serum (FBS; Shanghai ExCell Biology, Inc.) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using the ReverTra Ace qPCR RT Master Mix with a gDNA Remover kit (Toyobo Life Science) according to the manufacturer's instructions. qPCR was then performed in a 25- $\mu$ l volume reaction mixture containing 12.5  $\mu$ l AceQ Universal SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd.), 2  $\mu$ l template cDNA (100 ng/ $\mu$ l) and 1  $\mu$ M of primers in a LightCycler 96 (Roche Diagnostics Co., Ltd.). The thermocycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 94°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. GAPDH was used as a normalization control. Each experiment was performed independently at least three times and the fold change in the expression of each gene was calculated using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method (40). Primers for qPCR were obtained from BioSune Biotechnology Co., Ltd. All primers were designed to cross an intron-exon junction sequence to minimize genomic DNA contamination. The primer sequences were as follows: qPCR-PLK1-forward (F), 5'-AAGAGATCCCGGAGGTCC TA-3' and qPCR-PLK1-reverse (R), 5'-GCTGCGGTGAAT GGATATTT-3'; qPCR-FOXM1-F, 5'-CGTGGATTGAGG ACCACTTT-3' and qPCR-FOXM1-R, 5'-TCTGCTGTGATT CCAAGTGC-3'; qPCR-GAPDH-F, 5'-ACAACCTTTGGTA TCGTGGAAGG-3' and qPCR-GAPDH-R, 5'-GCCATCAC GCCACAGTTTC-3'.

**Immunoblotting.** Cells were lysed in radioimmunoprecipitation assay cell lysis buffer (Beyotime Institute of Biotechnology) containing protease inhibitors (cat. no. HY-K0010; MedChem Express) and phosphatase inhibitor cocktail (cat. no. 78427; Thermo Fisher Scientific, Inc.). Protein concentrations were determined via a bicinchoninic acid protein assay (Pierce; Thermo Fisher Scientific, Inc.). Proteins were separated using 10% SDS-PAGE [gels using 1X running buffer and transferred to polyvinylidene difluoride membranes (MilliporeSigma)]. Afterwards, the membranes were blocked by 5% skimmed milk (Anchor; Fonterra Co-operative Group) at room temperature for 1 h and incubated with primary antibodies against FOXM1 (rabbit; cat. no. A2493; dilution, 1:1,000; Abclonal), PLK1 (rabbit; cat. no. 208G4; dilution, 1:1,000; Cell Signaling Technology, Inc.) and GAPDH (mouse; cat. no. AC002; dilution, 1:10,000; Abclonal) at 4°C overnight. Subsequently, the membranes were cleaned twice with TBS with 0.1% Tween-20 and incubated with secondary goat anti-rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP (dilution, 1:5,000; cat. no. G21234; Thermo Fisher Scientific, Inc.) or goat anti-mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, HRP (dilution, 1:5,000; cat. no. G21040; Thermo Fisher Scientific, Inc.) at room temperature for 1 h. Proteins were detected via enhanced chemiluminescence (Vazyme

Biotech Co., Ltd.) with a digital luminescent image analyzer (Tanon-4200; Tanon Science and Technology Co., Ltd.). The intensities of protein bands were semi-quantified using ImageJ software (ImageJ bundled with 64-bit Java 1.8.0\_172; National Institutes of Health).

**RNA interference.** Human FOXM1 small interfering RNA (siRNA), human PLK1 siRNA and control siRNA were obtained from Shanghai GenePharma, Co., Ltd. The siRNA oligonucleotides were transfected into Huh7 cells (at 70% confluence) using Lipofectamine RNAiMAX (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. siRNAs were mixed with Opti-MEM reduced serum medium (Gibco; Thermo Fisher Scientific, Inc.) and Lipofectamine RNAiMAX and incubated for 20 min at room temperature. Subsequently, siRNA/Lipofectamine in Opti-MEM was diluted 1:5 (corresponding to a final concentration of 50 nM) in differentiation medium, and added to the cells. Thereafter, cells were incubated for 48 h at 37°C with 5% CO<sub>2</sub>. The following siRNAs were used for the experiments, which had the following sequences: siFOXM1-1, 5'-GCUGGG AUCAAGAUUAUUATT-3'; siFOXM1-2, 5'-GGCUGCACU AUCAACAAUATT-3'; siPLK1-1, 5'-CCCUCACAGUCCUCA AUAATT-3'; siPLK1-2, 5'-GGCAACCAAAGUCGAAUA UTT-3'; si negative control (NC), 5'-ACGUGACACGUUCGG AGAATT-3'.

**Cell counting kit 8 (CCK-8) assay.** A CCK-8 kit (Vazyme Biotech Co., Ltd.) was used to measure the proliferation of Huh7 cells. A total of 1,000 cells in a volume of 100 µl per well were cultured in six replicate wells in a 96-well plate in medium containing 10% FBS and 1% penicillin-streptomycin at 37°C with 5% CO<sub>2</sub> for 6 h. When cells had adhered, CCK-8 reagent (10 µl) was added to 90 µl DMEM to generate the working solution, of which 100 µl was added per well and incubated for 2 h. This assay was performed at 0, 24, 48, 72 and 96 h. The optical densities were measured at a spectral wavelength of 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.). Six replicates were analyzed for each time point.

**5-Ethynyl-2'-deoxyuridine (EdU)-DNA synthesis assay.** To measure the DNA replication activity of the Huh7 cells, a Cell-Light EdU Apollo488 *in Vitro* Kit (cat. no. C10310-3; Guangzhou RiboBio Co., Ltd.) was used. Huh7 cells were seeded in 96-well plates at a density of 8x10<sup>3</sup> cells per well. After 24 h, the cell culture medium was replaced with 50 µM EdU solution diluted with growth culture medium, followed by incubation for 2 h. The cells were then processed using the Cell-Light EdU Apollo488 *in Vitro* Kit according to the manufacturer's protocol. Images were captured with an Olympus fluorescence microscope (BX53; Olympus Corporation).

**Cell-cycle analysis.** A total of 1x10<sup>6</sup> cells were washed twice with PBS and fixed overnight with 1 ml pre-cooled 75% ethanol at 4°C. Thereafter, cells were collected by centrifugation (500 x g; 5 min; 4°C), washed twice in PBS and incubated with propidium iodide (5 µg/ml, Sigma) and RNase A (0.1 mg/ml; Thermo Fisher Scientific, Inc.) for 30 min at 4°C in the dark. A 40-µm screen filter was then used to filter

Table I. Baseline of characteristics of the patients (n=373).

Characteristic	N (%)
Age, years	
<50	70 (18.8)
50-59	99 (26.5)
60-69	121 (32.4)
≥70	83 (22.3)
Sex	
Male	252 (67.6)
Female	121 (32.4)
Stage	
I	173 (49.6)
II	86 (24.6)
III-IV	90 (25.8)
Virus status	
None	199 (56.2)
HBV	99 (28.0)
HCV	48 (13.8)
HBV+HCV	7 (2.0)

HBV, hepatitis B virus; HCV, hepatitis C virus.

the cell suspension and remove any adhesive cells. This was followed by flow cytometry to analyze the DNA content using the BD LSRFortessa system (BD Biosciences). FlowJo\_v10 software (Tree Star, Inc.) was used to estimate the proportion of cells in the G0/G1, S and G2/M phases.

**Pharmacological inhibitor of PLK1.** The pharmacological PLK1 inhibitor BI 2536 (HY-50698) was purchased from MedChemExpress. The drugs were reconstituted in DMSO and aliquots were stored at -20°C. An equivalent amount of DMSO was used for each experiment as a vehicle control. For cell proliferation and cell cycle assays, Huh7 cells were incubated with 10 nM BI 2536 for 24 h.

**Statistical analysis.** Statistical analysis was performed using R software (version 4.0.3) for survival analysis and Cox analysis, and GraphPad Prism (version 8.0; GraphPad Software, Inc.) for others. Kaplan-Meier curves were used to estimate OS and DFS. The log-rank test was used to compare patient survival times between high and low gene expression groups and hazard ratios (HRs) were calculated using the Cox proportional hazards model. The combined expression of FOXM1 and PLK1 was dependent on FOXM1 expression and PLK1 expression, thus they were analyzed respectively in Multivariable Cox analysis. Due to the crossing of survival curves, the 'TSHRC' package (v0.1-6; <https://CRAN.R-project.org/package=TSHRC>) of R software, which is a two-stage procedure for comparing HR functions, particularly suited for situations where HR functions cross, was used to perform a two-stage test rather than the log-rank test (41,42). Pearson's correlation coefficient was calculated to analyze the correlation between FOXM1 and PLK1 expression. Differences between

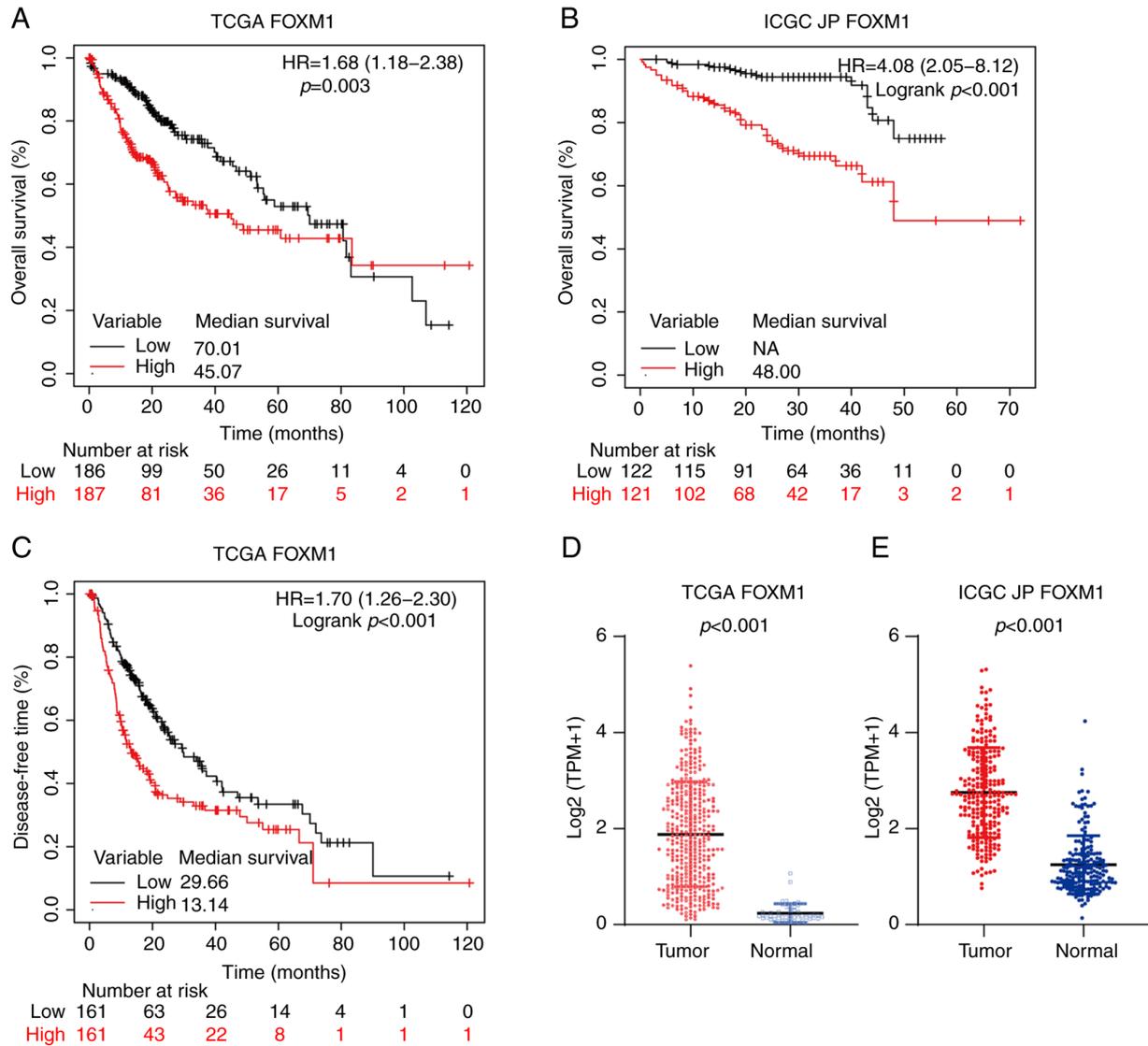


Figure 1. Overexpression of FOXM1 is associated with a poor prognosis in HCC. Kaplan-Meier survival curve estimates of overall survival in the (A) TCGA (n=373) and (B) ICGC JP (n=243) cohorts. The patients were divided into two subgroups of high and low FOXM1 expression. (C) Kaplan-Meier survival curve estimates of disease-free survival in the TCGA cohort (n=322). Expression of FOXM1 in HCC and non-tumor liver tissues determined by transcriptome sequencing from the (D) TCGA and (E) ICGC JP datasets. FOXM1, forkhead box protein M1; HR, hazard ratio; TCGA, The Cancer Genome Atlas; ICGC JP, International Cancer Genome Consortium Japan; HCC, hepatocellular carcinoma; NA, not available.

2 groups were analyzed using the unpaired Student's t-test with or without Welch's correction. One-way ANOVA followed by Tukey's post-hoc test was used for comparisons between multiple groups. Values are expressed as the mean  $\pm$  standard deviation.  $P<0.05$  was considered to indicate a statistically significant difference.

## Results

**Baseline characteristics.** The TCGA cohort was used as the exploration cohort and the ICGC JP cohort was used for validation. The baseline characteristics of the TCGA data set analyzed in the present study are summarized in Table I. The mean age of TCGA cohort was 59.5 years and the percentage of men was 67.6%. The most prevalent hepatitis virus was HBV. The ICGC JP cohort contained sequencing and clinical data of 243 patients with HCC from Japan. The mean age of

the ICGC cohort was 67.5 years and the percentage of men was 74.9%. Most patients presented with primary tumors (98.8%) and approximately half of the patients had stage II tumors (45.3%).

**Overexpression of FOXM1 and PLK1 is associated with poor prognosis for HCC.** The expression of FOXM1 and PLK1 in HCC and their effect on survival were first analyzed (Fig. 1). Compared with non-tumor liver tissues, the tumor tissues exhibited significantly higher FOXM1 expression in the TCGA ( $P<0.001$ ; Fig. 1D) and ICGC JP ( $P<0.001$ ; Fig. 1E) cohorts. To investigate the prognostic value of FOXM1 and PLK1 expression in HCC, the patients with HCC were divided into low and high expression groups at the 50th percentile. In addition, in the TCGA cohort, patients with high FOXM1 expression levels ( $F^H$ ) had shorter OS [HR, 1.68; 95% confidence interval (CI), 1.18–2.38;  $P=0.003$ ; Fig. 1A] and DFS (HR, 1.70; 95% CI,

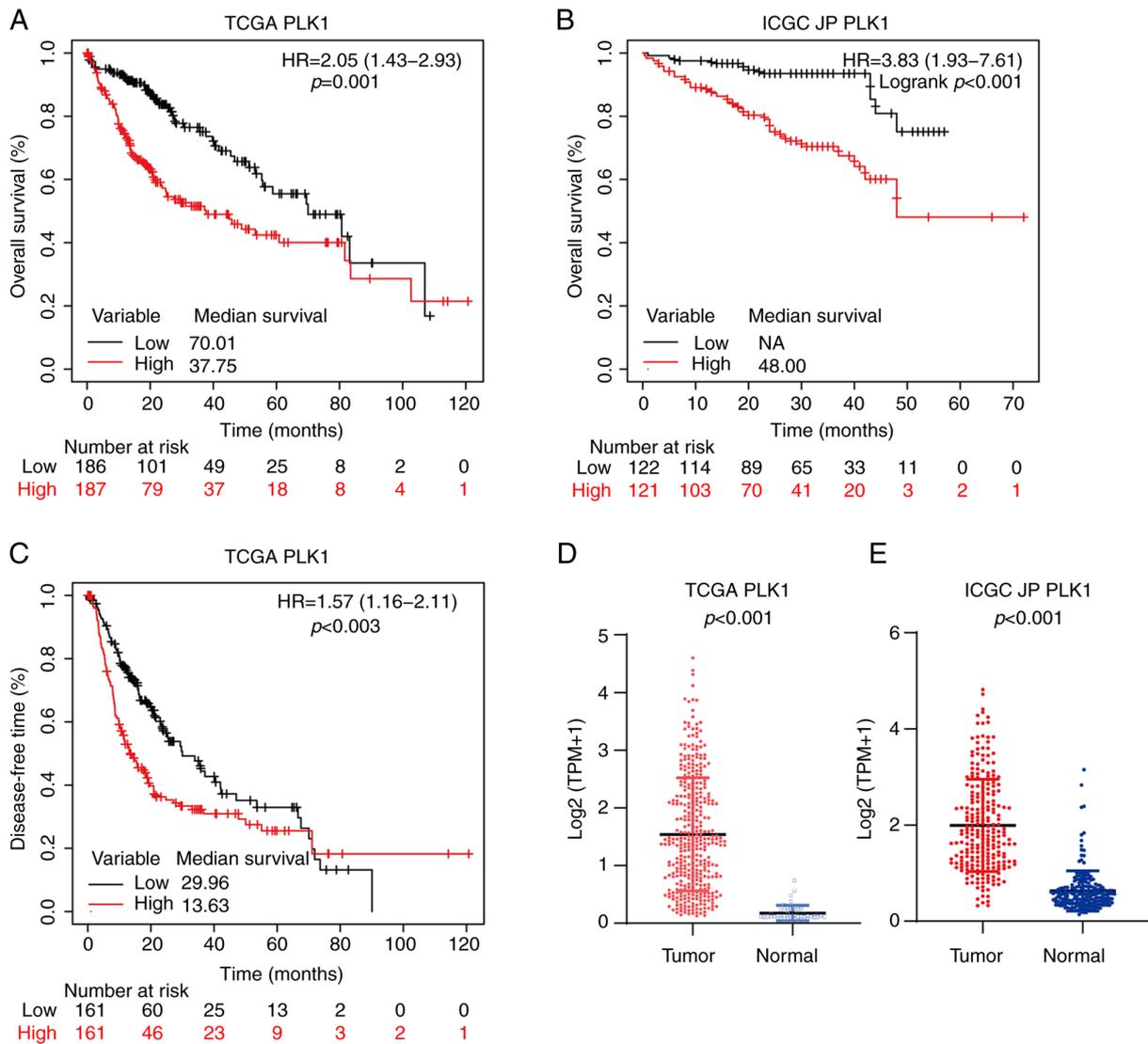


Figure 2. Overexpression of PLK1 is associated with poor prognosis in HCC. Kaplan-Meier survival curve estimates of overall survival in the (A) TCGA (n=373) and (B) ICGC JP (n=243) cohorts. The patients were divided into two subgroups of high and low PLK1 expression. (C) Kaplan-Meier survival curve estimates of DFS in TCGA (n=322) cohort. The expression of PLK1 in HCC and non-tumor liver tissues determined by transcriptome sequencing from the (D) TCGA and (E) ICGC JP datasets. PLK1, polo-like kinase 1; HR, hazard ratio; TCGA, The Cancer Genome Atlas; ICGC JP, International Cancer Genome Consortium Japan; HCC, hepatocellular carcinoma; NA, not available.

1.26-2.30;  $P<0.001$ ; Fig. 1C). Consistently,  $F^H$  was associated with poor prognosis in the ICGC JP cohort (HR, 4.08; 95% CI, 2.05-8.12;  $P<0.001$ ; Fig. 1B).

As presented in Fig. 2, similar to FOXM1, PLK1 was highly expressed in HCC tumor tissues in both the TCGA ( $P<0.001$ ; Fig. 2D) and ICGC JP ( $P<0.001$ ; Fig. 2E) cohorts. In addition, in the TCGA cohort, patients with high PLK1 expression level ( $P^H$ ) had shorter OS (HR, 2.05; 95% CI, 1.43-2.93;  $P<0.001$ ; Fig. 2A) and DFS (HR, 1.57; 95% CI, 1.16-2.11;  $P=0.003$ ; Fig. 2C). In the ICGC JP cohort, patients with  $P^H$  also had significantly poorer OS (HR, 3.83; 95% CI, 1.93-7.61;  $P<0.001$ ; Fig. 2B).

To assess the independent predictive value of  $F^H$  and  $P^H$ , logistic regression with a multivariate Cox proportional hazards model was utilized. After adjusting for age, sex, stage and virus status, stage and virus status were identified as independent prognostic factors for OS. However, neither  $F^H$  (HR, 1.14; 95% CI, 0.67-1.95;  $P=0.634$ ; Table II), nor  $P^H$  (HR, 1.73;

95% CI, 1.00-2.99;  $P=0.052$ ; Table II) were significantly and independently associated with a shorter OS.

*Combined overexpression of FOXM1 and PLK1 is associated with poor HCC prognosis.* FOXM1 and PLK1 are essential for cell cycle progression. PLK1 itself is a target of FOXM1 and phosphorylation of FOXM1 mediated by PLK1 is required for FOXM1 transcription activation (14,16). In both the TCGA ( $r^2=0.793$ ,  $P<0.001$ ; Fig. 3A) and ICGC JP ( $r^2=0.714$ ,  $P<0.001$ ; Fig. 3B) cohorts, a positive linear correlation between FOXM1 and PLK1 expression was observed, consistent with the presence of a feedback loop between them in HCC tissues. In the TCGA cohort, patients with combined high expression of FOXM1 and PLK1 ( $F^H$ - $P^H$ ) exhibited significantly shorter OS (HR, 2.04; 95% CI, 1.40-2.97;  $P<0.001$ ; Fig. 3C) and DFS (HR, 1.73; 95% CI, 1.26-2.38,  $P<0.001$ ; Fig. 3E). The median OS corresponding to the  $F^H$ - $P^H$  (33.02; Fig. 3C) group was shorter than that corresponding to

Table II. Cox regression proportional hazards model for the analysis of the prognostic values of FOXM1 expression and PLK1 expression for overall survival in patients with hepatocellular carcinoma.

Characteristic	Univariate		Multivariate	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (continuous)	1.01 (1.00-1.03)	0.089	1.01 (0.99-1.03)	0.292
Sex (female vs. male)	1.26 (0.88-1.80)	0.200	1.08 (0.72-1.63)	0.712
Stage				
II vs. I	1.42 (0.87-2.31)	0.164	1.11 (0.66-1.88)	0.691
III-IV vs. I	2.82 (1.86-4.28)	<0.001	2.00 (1.26-3.18)	0.003
Virus status (vs. none)				
HBV	0.34 (0.20-0.56)	<0.001	0.43 (0.24-0.78)	0.005
HCV	0.95 (0.56-1.61)	0.847	1.15 (0.64-2.05)	0.646
HBV+HCV	0.42 (0.10-1.74)	0.233	0.37 (0.08-1.61)	0.184
FOXM1 (high vs. low)	1.68 (1.18-2.38)	0.004	1.14 (0.67-1.95)	0.634
PLK1 (high vs. low)	2.05 (1.43-2.93)	<0.001	1.73 (1.00-2.99)	0.052

Patients with HCC were divided into the low expression group and the high expression group at the 50th percentile. HBV, hepatitis B virus; HCV, hepatitis C virus; FOXM1, forkhead box protein M1; PLK1, polo-like kinase 1; HR, hazard ratio.

Table III. Cox regression proportional hazards model for the analysis of the prognostic value of combined expression of FOXM1 and PLK1 for overall survival in patients with hepatocellular carcinoma.

Characteristic	Univariate		Multivariate	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (continuous)	1.01 (1.00-1.03)	0.089	1.01 (1.01-1.03)	0.150
Sex (female vs. male)	1.26 (0.88-1.80)	0.200	1.22 (0.79-1.89)	0.366
Stage				
II vs. I	1.42 (0.87-2.31)	0.164	1.03 (0.59-1.81)	0.905
III-IV vs. I	2.82 (1.86-4.28)	<0.001	1.97 (1.20-3.26)	0.008
Virus status (vs. none)				
HBV	0.34 (0.20-0.56)	<0.001	0.49 (0.25-0.96)	0.038
HCV	0.95 (0.56-1.61)	0.847	1.07 (0.58-1.98)	0.821
HBV+HCV	0.42 (0.10-1.74)	0.233	0.44 (0.10-2.01)	0.289
Combined FOXM1+PLK1 (high vs. low)	2.02 (1.39-2.95)	<0.001	1.94 (1.31-2.89)	0.001

Patients with HCC were divided into the low expression group and the high expression group at the 50th percentile. The combined expression of FOXM1 and PLK1 was defined as patients with high expression of FOXM1 and PLK1 or low expression of FOXM1 and PLK1. HR, hazard ratio; HBV, hepatitis B virus; HCV, hepatitis C virus; FOXM1, forkhead box protein M1; PLK1, polo-like kinase 1.

the F<sup>H</sup> (45.07; Fig. 1A) and P<sup>H</sup> (37.75; Fig. 2A) groups. These observations were validated in the ICGC JP cohort (Fig. 3B). Furthermore, the prognostic value of F<sup>H</sup>-P<sup>H</sup> expression in patients with HCC was confirmed using logistic regression with a multivariate Cox proportional hazards model. The results indicated that F<sup>H</sup>-P<sup>H</sup> expression was the most significant independent predictor of OS (HR, 1.94; 95% CI, 1.31-2.89; P=0.001; Table III).

Cancer immunotherapy has revolutionized cancer treatment. Antibodies against programmed death 1/ligand 1 and cytotoxic T-lymphocyte-associated protein 4 are effective

for the treatment of HCC (43-45). Given that the immune microenvironment has an important role in the response to immunotherapy (46,47), the impact of F<sup>H</sup>-P<sup>H</sup> expression on the immune microenvironment in patients with HCC was evaluated. Low activation of the transforming growth factor (TGF)- $\beta$  pathway is associated with better clinical outcomes for patients with cancer (32). Thus, the TGF- $\beta$  response score (48) were applied in patients with HCC and it was observed that the F<sup>H</sup>-P<sup>H</sup> group had a relatively higher score (P<0.001; Fig. 3F). Furthermore, CIBERSORT (49) has been used to analyze immune cell infiltration. Its

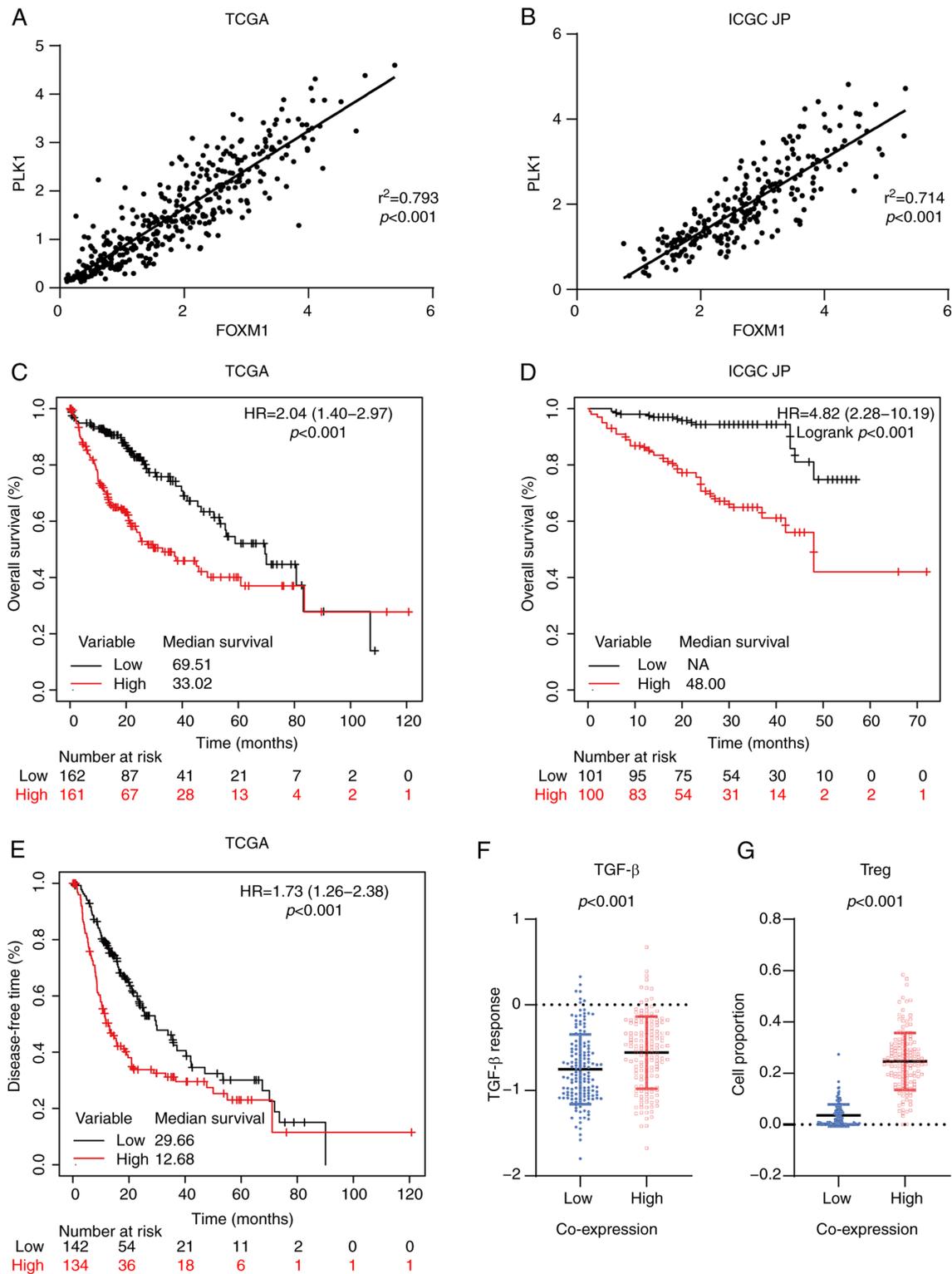


Figure 3. Coordinated overexpression of FOXM1 and PLK1 is associated with poor prognosis in HCC. Correlation between FOXM1 and PLK1 expression in HCC in the (A) TCGA and (B) ICGC JP cohorts based on Pearson's correlation analysis. Kaplan-Meier survival curve estimates of overall survival in the (C) TCGA (n=323) and (D) ICGC JP (n=201) cohorts. The patients were divided into two subgroups: High FOXM1+PLK1 and low FOXM1+PLK1. (E) Kaplan-Meier survival curve estimates of disease-free survival in the TCGA cohort (n=276). (F) Transforming growth factor-β response score of HCC in the TCGA cohort, stratified by the co-expression status of FOXM1 and PLK1. (G) Tumor-infiltrating Tregs in HCC in the TCGA cohort, stratified based on the co-expression status of FOXM1 and PLK1. FOXM1, forkhead box protein M1; PLK1, polo-like kinase 1; HR, hazard ratio; TCGA, The Cancer Genome Atlas; ICGC JP, International Cancer Genome Consortium Japan; HCC, hepatocellular carcinoma; NA, not available; Tregs, regulatory T cells.

application in the present study suggested that regulatory T (Treg) cells were significantly enriched in the F<sup>H</sup>-P<sup>H</sup> group (P<0.001; Fig. 3G). Of note, Treg cells are able to inhibit

T-cell proliferation and cytokine production and have a critical role in preventing tumor immune response (50,51). These results suggested that F<sup>H</sup>-P<sup>H</sup> expression is associated

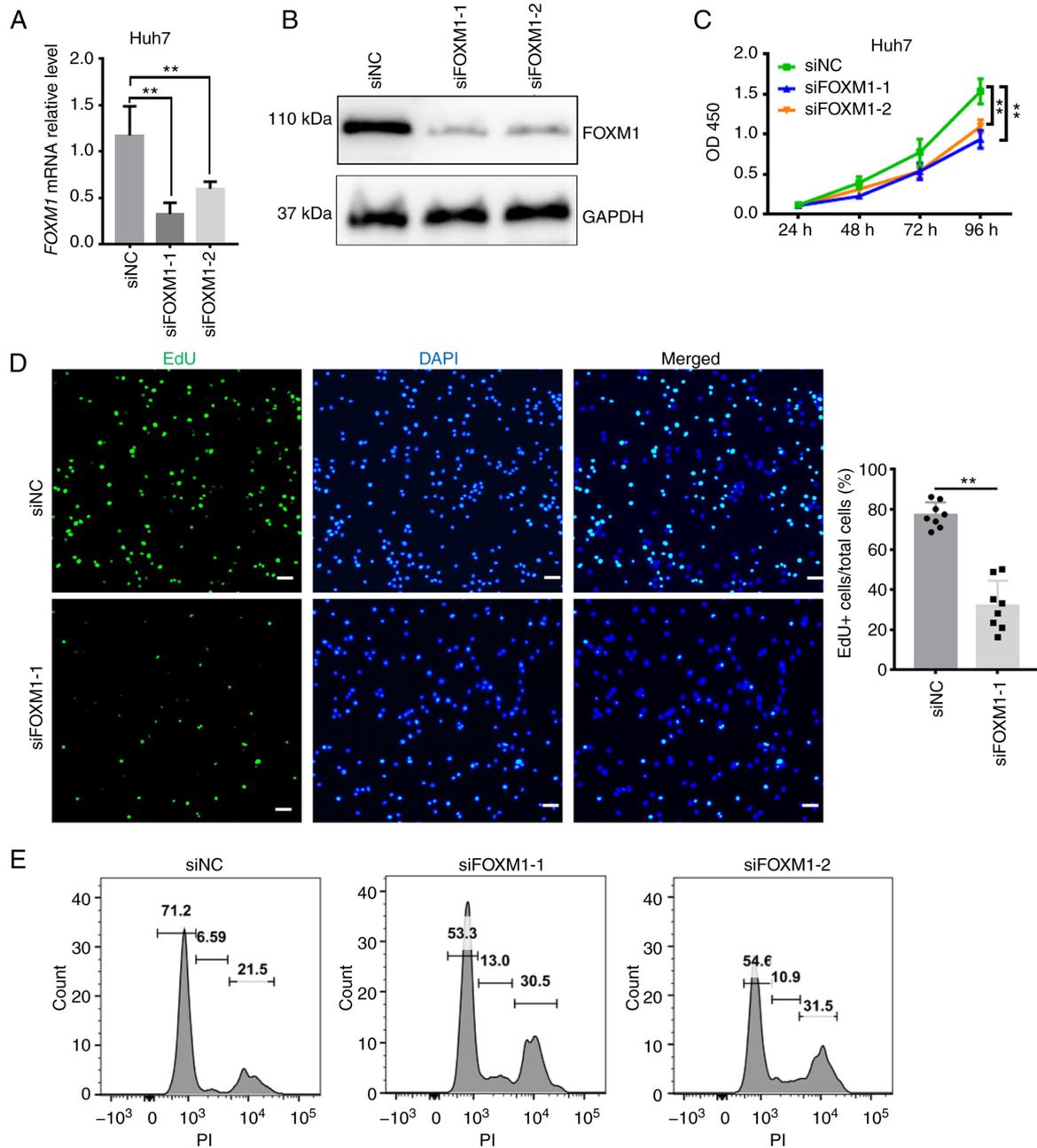


Figure 4. FOXM1 is required for Huh7 cell proliferation. Knockdown efficiency of FOXM1 confirmed using (A) reverse transcription-quantitative PCR and (B) western blot analysis. Decrease in the proliferation rate of Huh7 cells following FOXM1 knockdown as indicated by (C) a Cell Counting Kit-8 assay and (D) EdU staining (scale bars, 50  $\mu$ m). (E) DNA content analyzed using flow cytometry 24 h after transfection with siFOXM1 in Huh7 cells. Results are provided in histograms. Quantified values are expressed as the mean  $\pm$  standard deviation of three independent experiments. \*\* $P < 0.01$  vs. siNC. FOXM1, forkhead box protein M1; siRNA, small interfering RNA; siNC, negative control siRNA; siFOXM1, siRNA targeting FOXM1; PI, propidium iodide; EdU, 5-ethynyl-2'-deoxyuridine; OD450, optical density at 450 nm.

with a suppressive immune microenvironment and may hamper immunotherapy treatment in HCC.

#### *FOXM1 and PLK1 are required for HCC cell proliferation.*

Given that the overexpression of FOXM1 and PLK1 was associated with poor outcomes of HCC and based on their key regulatory roles in cell cycle progression, it was investigated whether FOXM1 and PLK1 influence HCC progression (Figs. 4 and 5). siRNA targeting FOXM1 and PLK1 was synthesized and the knockdown efficiency was measured in Huh7 cells (Figs. 4A and 5A and B). The cell proliferation

rate was estimated using CCK-8 and EdU assays. FOXM1 knockdown decreased Huh7 cell viability (Fig. 4C), as well as the percentage of EdU-positive Huh7 cells (Fig. 4D), indicating a lower proportion of cells entering the DNA replication phase of the cell cycle. PLK1 knockdown exerted a similar effect on Huh7 cells (Fig. 5C and E). To confirm the requirement of PLK1 for Huh7 cell proliferation, a pharmacological inhibitor of PLK1, BI 2536 (38), was applied during the CCK-8 assay. In the presence of the inhibitor, a significant decrease in the proliferation of Huh7 cells was observed (Fig. 5D). Furthermore, flow cytometry suggested that knockdown of FOXM1 (Fig. 4E) or

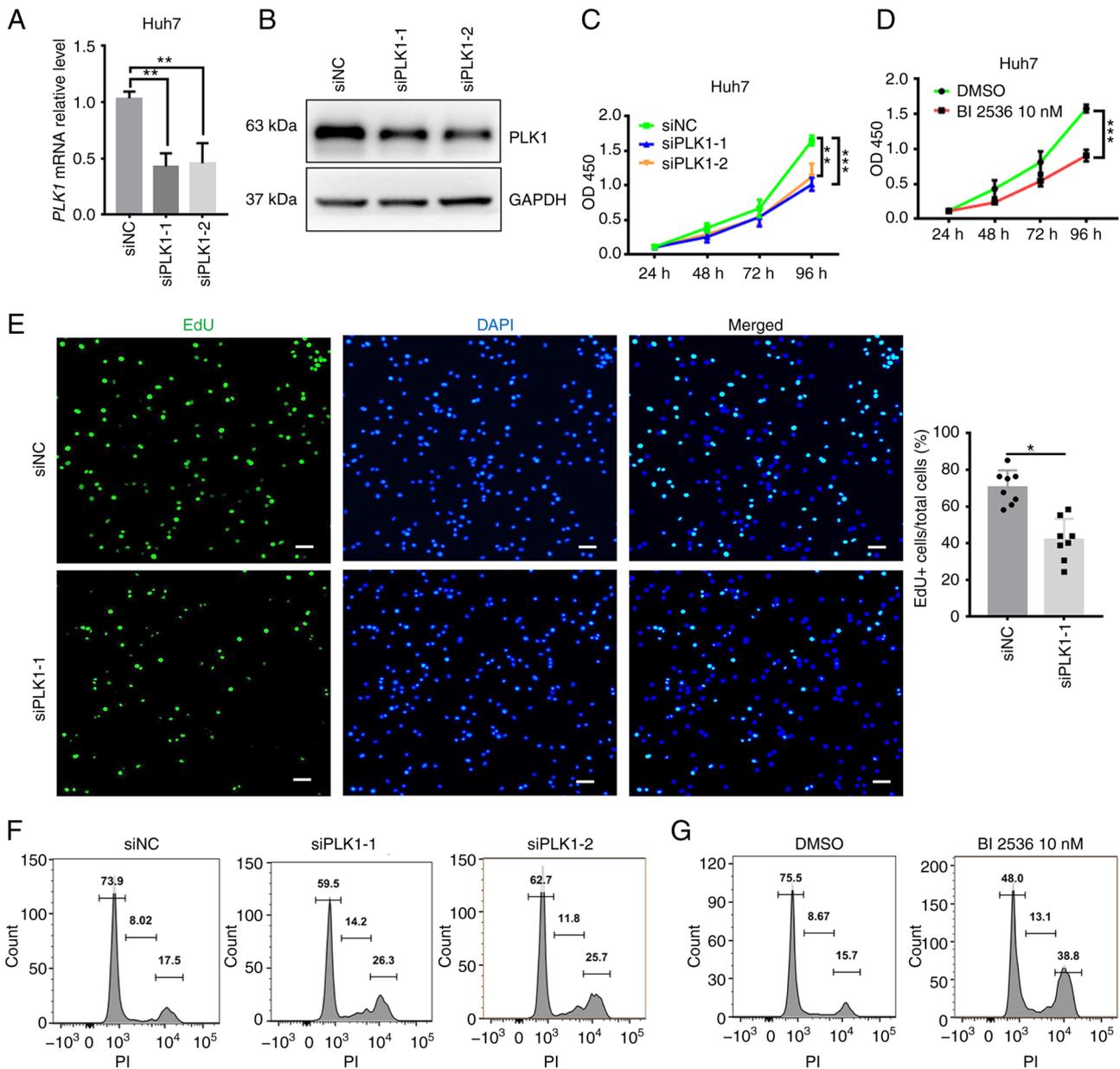


Figure 5. PLK1 is required for Huh7 cell proliferation. Knockdown efficiency of PLK1 confirmed using (A) reverse transcription-quantitative PCR and (B) western blot analysis. Cell proliferation rate analyzed using a Cell Counting Kit-8 assay after (C) transfection with siPLK1 or (D) treatment with 10 nM BI 2536 in Huh7 cells. (E) Decrease in the proliferation rate of Huh7 cells following PLK1 knockdown as indicated by EdU staining (scale bars, 50  $\mu$ m). DNA content of Huh7 cell analyzed via flow cytometry 24 h after (F) transfection with siPLK1 or (G) treatment with 10 nM BI 2536. Results are provided in histograms. Quantified values are expressed as the mean  $\pm$  standard deviation of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. siNC. PLK1, polo-like kinase 1; siRNA, small interfering RNA; siNC, negative control siRNA; siPLK1, siRNA targeting PLK1; PI, propidium iodide; EdU, 5-ethynyl-2'-deoxyuridine; OD450, optical density at 450 nm.

PLK1 (Fig. 5F) resulted in a marked increase in the proportion of cells in S and G2/M phase, as well as a decrease in the proportion of cells in G1 phase at 24 h after transfection. It appears that, as more cells progress away from G0/G1 phase, the cells are trying to proliferate but they exhibit cell cycle arrest in S and G2/M phase. Consistent with this observation, Huh7 cells treated with BI 2536 also exhibited a marked increase in the proportion of cells in S and G2/M phase (Fig. 5G). These results suggested that FOXM1 and PLK1 are required for HCC cell proliferation. Insufficient FOXM1 or PLK1 were thus indicated to hamper cell cycle progression as well as the proliferation of HCC cells, providing additional evidence that the coordinated

overexpression of FOXM1 and PLK1 is an independent prognostic factor for HCC.

## Discussion

HCC is the primary malignancy of the liver and complete surgical resection is the only curative approach. However, most patients with HCC are only diagnosed when the disease is already in the advanced stage, which is unsuitable for surgery. In addition, the prognosis associated with the systemic treatment of HCC is poor (3) and valuable prognostic biomarkers are urgently required.

Cell cycle dysfunction is a marked feature of tumor cells (52) and several regulators of the cell cycle have been proposed as putative prognostic biomarkers for cancer (53). In this regard, FOXM1 and PLK1, which are essential cell cycle regulators with prognostic value in HCC, have been extensively studied. Consistent with previous reports (23,35), the present results suggested that both FOXM1 and PLK1 were overexpressed in tumor tissues and associated with poor prognosis in patients with HCC. However, after adjusting for age, sex, stage and virus status, a multivariate Cox proportional hazards model indicated that neither FOXM1 nor PLK1 is able to independently serve as a prognostic factor for HCC.

PLK1 is a target of FOXM1 and is required for FOXM1 transcriptional activation. A positive linear correlation between FOXM1 and PLK1 expression was observed in HCC tissues. Insufficient FOXM1 or PLK1 may hamper the cell cycle and it may be speculated that the coordinated overexpression of FOXM1 and PLK1 may serve as an independent prognostic factor for HCC. The present results indicated that F<sup>H</sup>-P<sup>H</sup> expression was associated with significantly shorter OS and DFS in patients with HCC. In addition, after adjusting for age, sex, stage and virus status, an F<sup>H</sup>-P<sup>H</sup> expression status was indicated to be the most significant prognostic factor for patients with HCC. Furthermore, an F<sup>H</sup>-P<sup>H</sup> expression status was associated with lower TGF- $\beta$  response scores and a higher number of infiltrating Treg cells, suggesting that patients with HCC with F<sup>H</sup>-P<sup>H</sup> expression status harbor a suppressed immune microenvironment that leads to treatment failure.

In the present study, the requirement for FOXM1 and PLK1 expression in HCC cells was investigated via *in vitro* knockdown of FOXM1 and PLK1 in Huh7 cells. It was observed that either FOXM1 or PLK1 knockdown was able to hamper cell cycle progression as well as the proliferation of Huh7 cells. The antitumor activity of pharmacological inhibitors of PLK1 has been evaluated in clinical trials, but the efficacy was limited (54-56). The limited antitumor activity may be attributed to the low PLK1 or FOXM1 expression levels in these patients. For these patients, PLK1 was not the essential tumor-growth driving factor. Thus, the present results suggested that, to improve therapeutic efficacy in the future, it may be necessary to perform clinical trials involving patients with coordinated overexpression of FOXM1 and PLK1.

In conclusion, the present results indicated that FOXM1 and PLK1 were overexpressed in HCC tumor tissues and exhibited a positive linear correlation. FOXM1 and PLK1 are required for HCC cell proliferation. The present results also indicated that F<sup>H</sup> and P<sup>H</sup> expression were associated with poor prognosis for HCC; however, only the coordinated overexpression of FOXM1 and PLK1 was able to serve as an independent prognostic factor for HCC. Therefore, targeting FOXM1 or PLK1 is a potential treatment for improving HCC prognosis.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

BJ conceived and designed the study and also provided administrative support. WF and HM provided the study materials, performed the experiments and collected the public data. All the authors performed data analysis and interpretation, participated in writing the manuscript and all authors read and approved the final version of the manuscript. WF and HM confirm the authenticity of all the raw data. The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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