Apoptosis (2019) 24:135–144
https://doi.org/10.1007/s10495-018-1491-6

PPPDE1 promotes hepatocellular carcinoma development by negatively regulate p53 and apoptosis

Xingwang Xie[1,2] · Xueyan Wang[2] · Weijia Liao[3] · Ran Fei[2] · Nan Wu[2] · Xu Cong[2] · Qian Chen[3] · Lai Wei[2] · Yu Wang[1] · Hongsong Chen[2]

Published online: 13 November 2018

Abstract

We have previously identified that *PPPDE1* is a deubiquitinase (DUB) belonging to a cysteine isopeptidase family. Here we sought to explore the biological significance of *PPPDE1* in hepatocellular carcinoma and its underlying molecular mechanism. In the present study, we found that amplification and overexpression of *PPPDE1* were associated with poor prognosis in hepatocellular carcinoma (HCC). We also demonstrated that knocking down of *PPPDE1* could significantly block the clonal growth and tumorigenicity of human HCC cells, which revealed a critical role for *PPPDE1* in HCC development. Furthermore, we proved that *PPPDE1* is a key modulator of p53 protein level and its down stream apoptosis pathway. Taken together, these results suggested that *PPPDE1* is a putative HCC driver gene and extensive studies should be conducted in the future to investigate the role of *PPPDE1* in HCC and other tumors.

Keywords *PPPDE1* · Hepatocellular carcinoma · Driver gene · p53 · Apoptosis

Abbreviations

DUBs	Deubiquitinating enzymes
PPPDE	After Permuted Papain fold Peptidases of DsRNA viruses and Eukaryotes

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent human malignancies worldwide. It is the fifth most commonly diagnosed and the second most lethal neoplasm in men worldwide, and it was identified in an estimated 782,500 new cases and caused 745,500 deaths during 2012 [1]. However, fewer progresses have been made in the treatment of HCC over the last decade. Advanced-stage HCC still leads to a poor prognosis in most patients. Hence, it is of critical importance to explore new therapeutic molecular targets for HCC.

Cancer is a disease with both genetic and epigenetic causes that disrupt cellular pathways and results in uncontrolled cell growth [2]. The molecular pathogens of HCC is very complicated, comprising multiple genetic and epigenetic alterations, chromosomal aberrations, gene mutations, and altered molecular pathways [3]. Significant effort has been directed towards characterizing the genomic events that occur in HCC, with the prime goals of understanding the genetic basis of the disease and identifying new therapeutic targets.

Amplification of the chromosome 1q21-44 loci has been found in more than half of HCC patients, which suggests that this region may contain several vital oncogenes that could drive HCC carcinogenesis and development, such as

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10495-018-1491-6) contains supplementary material, which is available to authorized users.

✉ Yu Wang
wangyu@chinacdc.cn

✉ Hongsong Chen
chenhongsong2999@163.com

[1] Chinese Center for Disease Control and Prevention, Beijing 102206, China

[2] Peking University People's Hospital, Peking University Hepatology Institute, Beijing Key Laboratory of Hepatitis C and Immunotherapy for Liver Disease, Beijing 100044, China

[3] Laboratory of Hepatobiliary and Pancreatic Surgery, Affiliated Hospital of Guilin Medical University, Guilin 541001, China

CHD1L (1q21.1) [4, 5] and *BCL9* [6]. We have analyzed the cancer genomics data of HCC from TCGA (Liver hepatocellular carcinoma, LIHC) and found that a 1q44 coded gene, *PPPDE1* (*DESI2*), amplified in 72.4% (268/370) of analyzed HCC patients. We also found that the mRNA level of *PPPDE1* dramatically elevated in HCC tumor tissue, compared with adjacent non-tumor tissues, and significantly correlated with its DNA copy number.

We have previously identified that *PPPDE1* is a novel deubiquitinase (DUB) belonging to a cysteine isopeptidase family [7]. DUBs have been revealed to have vital roles in a series of biology events, such as cell cycle control [8], DNA damage [9], and tumors [10, 11]. Moreover, progress in understanding DUBs has made these proteases became drug targets for a large number of diseases, such as cancer [12–14] and infections [15]. Hence, we sought to explore the biological significance of *PPPDE1* in hepatocellular carcinoma and its underlying molecular mechanism.

Materials and methods

Copy number profiling and RNA sequencing data

Copy number variation (CNV) data and RNA Sequencing (RNA-seq) data of liver hepatocellular carcinoma (LIHC) in the Cancer Genome Atlas (TCGA) project (http://cancergenome.nih.gov) were obtained from the data hub of the UCSC Xena browser (https://xenabrowser.net/datapages/). The Copy number profile was measured experimentally using an Affymetrix Genome-Wide Human SNP Array 6.0. GISTIC2 method was applied using the TCGA FIREHOSE pipeline to produce gene-level copy number estimates. Subsequently, the estimated values were further thresholded to −2, −1, 0, 1, and 2, representing homozygous deletion, single copy deletion, diploid normal copy, low-level copy number amplification, or high-level copy number amplification. The RNA-seq was performed using an Illumina HiSeq 2000 RNA Sequencing platform and then the gene-level TPM (transcripts per million) estimates was calculated with RSEM methods and log2(x + 1) transformed. The CNV data of HCC cell lines were obtained from the Gene Expression Omnibus (GEO) database (GSE38207) [6]. The Copy numbers segmentation data of GSE38207 were kindly provided by the author and displayed using an Integrative genomics viewer (IGV2.3.52) [16].

Tissue samples and cell lines

All HCC cancer tissues and adjacent non-cancerous tissues were obtained from patients who underwent surgery at the affiliated hospital of Guilin Medical University (Guilin cohort). This study was approved by the Ethics Committee of Peking University People's Hospital and Guilin Medical University. All patients in this study provided written informed consent for sample collection and data analyses. The cell line HepG2 (wild-type *p53*) and NeHepLxHT (*p53* not determined) were purchased from the American Type Culture Collection (ATCC, USA). MHCC-97H (mutant *p53*, R249S) and MHCC-97 L (mutant *p53*, R249S) were kindly provided by the Academy of Military Medical Science (Beijing, China). All of the other cell lines used in this study were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), including Hep3B (*p53* homozygous deleted), PLC/PRF/5 (mutant *p53*, G199*/R249S), SK-HEP-1 (wild-type *p53*) and Huh7 (*p53* mutant, Y220C/del).

Plasmids and antibodies

Human *PPPDE1*, FLAG-tagged *PPPDE1* and *PPPDE1* mutant (FLAG-PPPDE1-C108S, C108S) open reading frame (ORF) sequence were cloned into a lentiviral expression vector pLenti-PGK V5-LUC Neo [17]. ShRNAs specifically targeting *PPPDE1* (DEK1: AGTATCTTGTATCGCTGTTTA, DEK4: CCACAGCAATAGAGCAAGTTA), *MDM2* (M2K1: AGGAATTTAGACAACCTGAAAT, M2K2: AGCTCATCCTTTACACCAACTC, M2K3: AGGAACTTGGTAGTAGTCAATC) and a non-silencing control shRNA (NS: CTGAGGTGATAAACAGTTACA) were cloned into a lentiviral shRNA expression vector (HIV-H1; GeneCopeia, USA) containing an H1-driving shRNA expression cassette and a GFP reporter gene. Rabbit polyclone antibodies anti-PPPDE1, anti-p53, anti-Bax, anti-MDM2 and mouse monoclone antibody anti-GAPDH were from Proteintech Inc. Mouse monoclonal antibodies against human PPPDE1 (2P14) was generated by using purified human PPPDE1 protein as antigen to immunize BALB/c mice. The immune spleen cells were fused with SP2/0 myeloma cell line and supernatants from hybridoma cultures were used for purification of antibodies.

Quantitative real-time RT-PCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, USA), and cDNA synthesis was performed using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA) according to the manufacturer's instructions. Quantitative real-time RT-PCR (qRT-PCR) amplification was performed on a LightCycler 480 instrument using LightCycler 480 SYBR Green I Master Mix (Roche Diagnostic Ltd., Mannheim, Germany). The sequences of the primers used were as follows: *PPPDE1*, 5′-GAATACAAAGGCAATGCTTATC-3′and 5′- GGAGGCAACTCTGTAGAAAGG-3′; and GAPDH, 5′-CCACATCGCTCAGACACCAT-3′ and 5′-GGCAACAATATCCACTTTACCAGA

GT-3′. Relative *PPPDE1* mRNA expression was calculated by normalization to the mRNA expression level of GAPDH.

Transfection and western blot analysis

Plasmids were transfected into Huh7 with ViaFect™ Transfection Reagent (Promega) or into MHCC-97H with TurboFect Transfection Reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. Total cell lysates were prepared and separated on the NuPAGE ® Novex Bis-Tris Gel. Separated proteins were transferred onto nitrocellulose membrane using iBlot™ Gel Transfer Stacks and iBlot gel transfer device (Thermo Fisher Scientific). Then desired proteins were detected with corresponding antibodies.

Immunohistochemistry (IHC)

Paraffin-embedded tissue slides were heated in EDTA buffer (pH 9.0) for antigen retrieval. PPPDE1 protein was detected with mouse anti-human PPPDE1 antibody (2P14) and followed by EnVision detection system (Dako, Denmark). Sections were then counterstained with hematoxylin.

Lentiviral production, infection and stable cell lines establishment

The recombinant lentiviral expression vector as well as assistant vectors: pVSVg (AddGene 8454) and psPAX2 (AddGene 12260) were co-transfected into HEK293T cells. Viral supernatants were collected 48 h later, centrifuged at 500 g for 5 min to remove debris, clarified by filtrated through 0.45 μm filter (Millipore), and then concentrated with Macrosep® Advance Centrifugal Device (100K, PALL Corporation). Lentivirus titers were determined with the QuickTiter lentivirus titer kit (Cell Biolabs, San Diego, CA). The concentrated virus was used to infect HCC cell lines at optimal MOI. Infected cells were then subjected to antibiotics selection for the establishment of stable cell lines.

Colony formation assay

The colony formation assay was performed with HCC stable cell lines after lentivirus infection. For each group, a single-cell suspension of 1000 cells was plated in 6-well plates in triplicates and cultured in DMEM supplemented with 10% FBS for 14 days. After most of the cell clones had expanded to > 50 cells, they were fixed with 4% paraformaldehyde for 10 min, and dyed with 0.05% crystal violet for 15 min at room temperature.

Cell proliferation assay

Cell proliferation was evaluated by MTS assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (Promega) according to the manufacturer's instructions. Briefly, 5000 cells/well were seeded in a 96-well plate after transduced with lentiviral shRNA for 12 wells for each shRNA-transduced group. At 1, 2, 4 and 7 day after seeding, 20 μl of CellTiter 96 Aqueous One solution was added to each well for triplets well each shRNA-transduced group, and the plate was incubated for 1–4 h. Plates were read at 490 nm and 650 nm (background) in a microplate reader (Molecular Devices). After subtraction of background, the relative cell number was calculated.

Xenograft tumor growth assay

2×10^6 Huh7 and MHCC-97H cell stably expressing non-silencing control (NS) and shRNA against *PPPDE1* (DEK1 and DEK4) were suspended in PBS and transplanted to the left sides of the back of BALB/c Nude mice (male, 4 week) under anesthesia. Tumors were measured with Vernier calipers every week and tumor volumes were calculated with the following formula: $\pi/6 \times$ large diameter $\times$ (small diameter) [2]. All experiments were performed under protocols approved by the Experimental Animal Center of Peking University People's Hospital.

Expression profiling and data analysis

Gene expression profiling was performed using Affymetrix Human Gene 2.0 ST Array according to the protocol described in the Affymetrix GeneChip Expression Analysis Technical Manual. Microarray data preprocessing and differentially expressed genes (DE genes) analysis was performed with Bioconductor packages. Gene ontology (GO) functional enrichment analysis of DE genes was performed using the PANTHER classification system (http://pantherdb.org/). Enriched KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways for DEGs were analyzed with mas3.0 molecule annotation system (http://mas.capitalbiotech.com/mas3/).

Flow cytometric analysis for apoptosis

Huh7 were subjected to annexin-V-FITC (eBioscience) and 7-amino-actinomycin D (7-AAD) labeling followed by fluorescence flow cytometry analysis. All annexin-V positive cells were considered apoptotic cells and included in calculation.

Results

Amplification and overexpression of *PPPDE1* in HCC

Firstly, we identified a 1q44 coded gene, *PPPDE1* (*DESI2*), amplified in 72.4% (268/370) of analyzed HCC patients in TCGA LIHC cohort using the thresholded GISTIC2 value calculated with the CNV profiling data (Fig. 1a). We found that the mRNA level of *PPPDE1* dramatically elevated in HCC tumor tissue compared with adjacent non-tumor tissues by analyzing the LIHC RNA-seq data (Fig. 1b). Remarkably, we also found that the mRNA expression level significantly correlated with DNA copy number of *PPPDE1* ($p < 0.001$), which suggested that the overexpression of *PPPDE1* in HCC was mainly attributed to copy number gain (Fig. 1c).

We then validated the mRNA overexpression of *PPPDE1* in an independent cohort of 120 HCC patients (Guilin cohort). qRT-PCR analysis showed that *PPPDE1* significantly overexpressed in the HCC tissues compared with the corresponding non-tumor tissue and 10 cases of normal liver tissue (Fig. 2a). To further analyze the clinical relevance of *PPPDE1* overexpression in hepatocellular carcinoma, we carried out a clinicopathological analysis of patients in Guilin cohort. 94 patients with overall survival data and 26 patients with recurrent-free survival data were subjected to the subsequent analyses. Among these 94 HCC patients, there is no significant correlation between the mRNA expression level of *PPPDE1* and all analyzed clinical factors, including gender, age, alcohol consumption, tumor size, AFP level, HBV infection, Cirrhosis, TNM stage, Multinodularity, lympho-invasion, venous-infiltration, metastasis and recurrence (Table S1). However, Kaplan–Meier survival analysis showed that the mRNA level of *PPPDE1* in primary HCC tumor tissue linked to bad prognosis of HCC patients in Guilin cohort. Patients with higher *PPPDE1* expression tent to have a shorter overall survival time and recurrent free survival time (Fig. 2b, c).

In order to further determine the prognostic value of *PPPDE1* mRNA level in HCC, we performed univariate and multivariate Cox regression analysis of prognosis factors, including *PPPDE1* expression, among patients in the Guilin cohort. A univariate analysis showed that *PPPDE1*, along with age, tumor size, TNM stage, venous-infiltration, and metastasis were associated with overall survival (Table S2). But, none of these factors were associated with

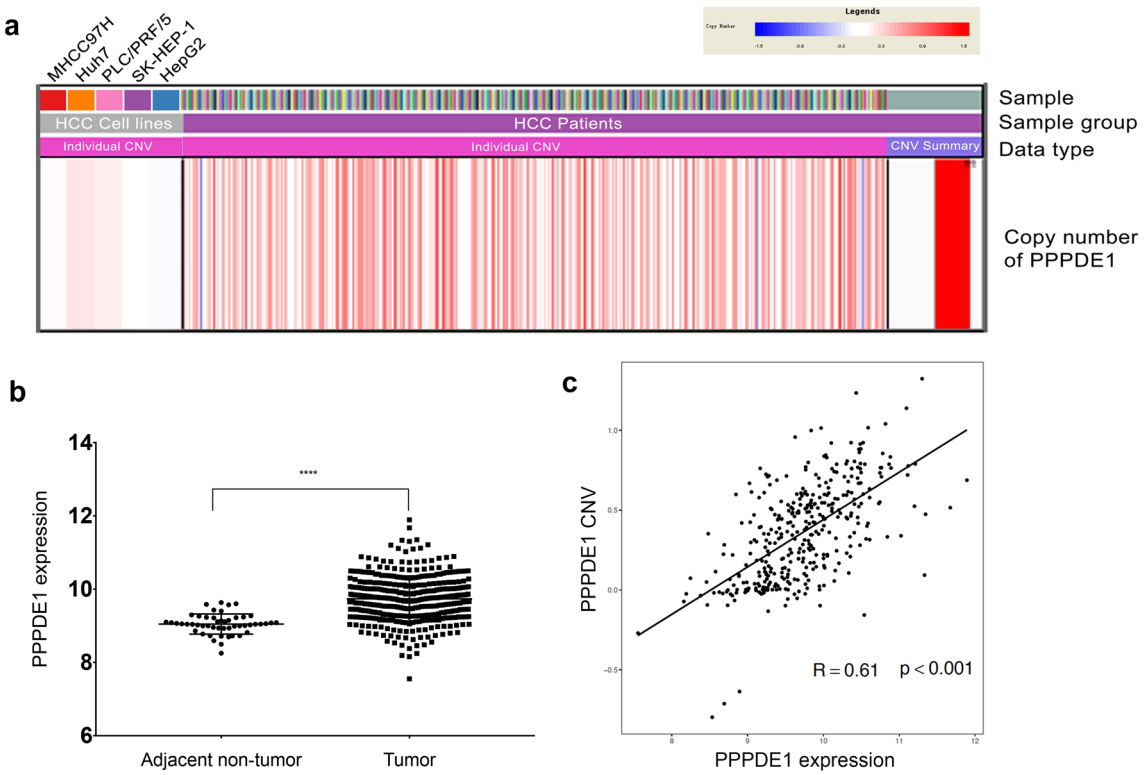


Fig. 1 Amplification and overexpression of *PPPDE1* in HCC. **a** The copy number of *PPPDE1* coding region in HCC tissues and HCC cell lines. **b** The mRNA expression (log2(x + 1) transform of TPM value) of *PPPDE1* in HCC tissues from TCGA LIHC dataset. **c** The correlation of copy number and mRNA expression of *PPPDE1* in HCC patients from TCGA LIHC dataset

recurrent-free survival. The limited numbers of the cases with available recurrent-free survival data may account for this negative result. In a multivariate Cox regression model, the mRNA level of *PPPDE1* was an independent prognostic factor for overall survival (hazard ratio for death, 1.737; 95% confidence interval [CI], 1.078–2.80; p = 0.023) (Table S3). Again,none of these factors were associated with recurrent-free survival.

Furthermore, we found that PPPDE1 protein also overexpressed in HCC tissue and cell lines by western blot analysis using a rabbit anti-PPPDE1 antibody (20517-1-AP, Proteintech group) (Fig. 2d, e). Although this antibody could stably detect PPPDE1 protein by western blot, and it also detected a 55kd non-specific targeted protein even in PPPDE1 negative normal liver or non-tumor tissue, which produced a strong non-specific signals in both HCC and normal liver tissues by IHC analysis (data not shown). In order to further determine the protein expression pattern and the location of PPPDE1 in HCC with IHC analysis, we used purified PPPDE1 protein to generate a mouse monoclonal antibody against human PPPDE1 (2P14), which only specifically detect PPPDE1 protein by western blot.

IHC staining of HCC and non-tumor tissue sections with 2P14 produced excellence signals that were consistent with the PPPDE1 protein expression status determined by western blot. By IHC analysis, we found that positive PPPDE1 staining presented in 41% (9/22) of the analyzed HCC patients. These positive staining located in the cytoplasm of HCC cell and showed a heterogeneously expression pattern in tumor (Fig. 2f).

PPPDE1 oncogene dependency in HCC cell lines

In order to determine the role of *PPPDE1* in the development of HCC, cell lines transfected with shRNAs against non-silencing control (NS) or *PPPDE1* (DEK1 and DEK4) were subjected to clonogenic assay. Remarkably, clone-forming ability was greatly reduced in these *PPPDE1*-amplified cell lines (MHCC-97H, Huh7 and PLC/PRF/5) by *PPPDE1* silencing, whereas cell lines without *PPPDE1* amplification (SK-HEP-1, HepG2) were not significantly affected (Figs. 1a, 3a–c). Silencing of *PPPDE1* dramatically upregulated the protein level of p53 in MHCC-97H but not in HepG2, which is consistent with the colony formation assay. These observations suggest that the regulation of p53 by *PPPDE1* is cell context dependent (Fig. 3c).

To exclude the possible off-target effects of shRNAs, we then performed RNAi rescue experiment. PPPDE1 ORF construct is insensitive to DEK1 mediated RNAi, because it lack the 3′ untranslated region (3′-UTR) that targeted by DEK1 shRNA. We found that delivery of PPPDE1 ORF expression cassettes to DEK1 transduced cell could restore high levels of PPPDE1 protein and completely rescued the growth defects induced by knocking down *PPPDE1* with shRNA (Fig. 3d–f). We detected a repeatable slightly increase in the clone formation potential of Huh7 after transduced a PPPDE1 expression lentivirus (Fig. 3d, e). We also performed cell proliferation assay to evaluate the impact of overexpressed PPPDE1 on the proliferation of MHCC-97H and Huh7 and found that the proliferation rate was raised in both cell lines (Fig. 3g).

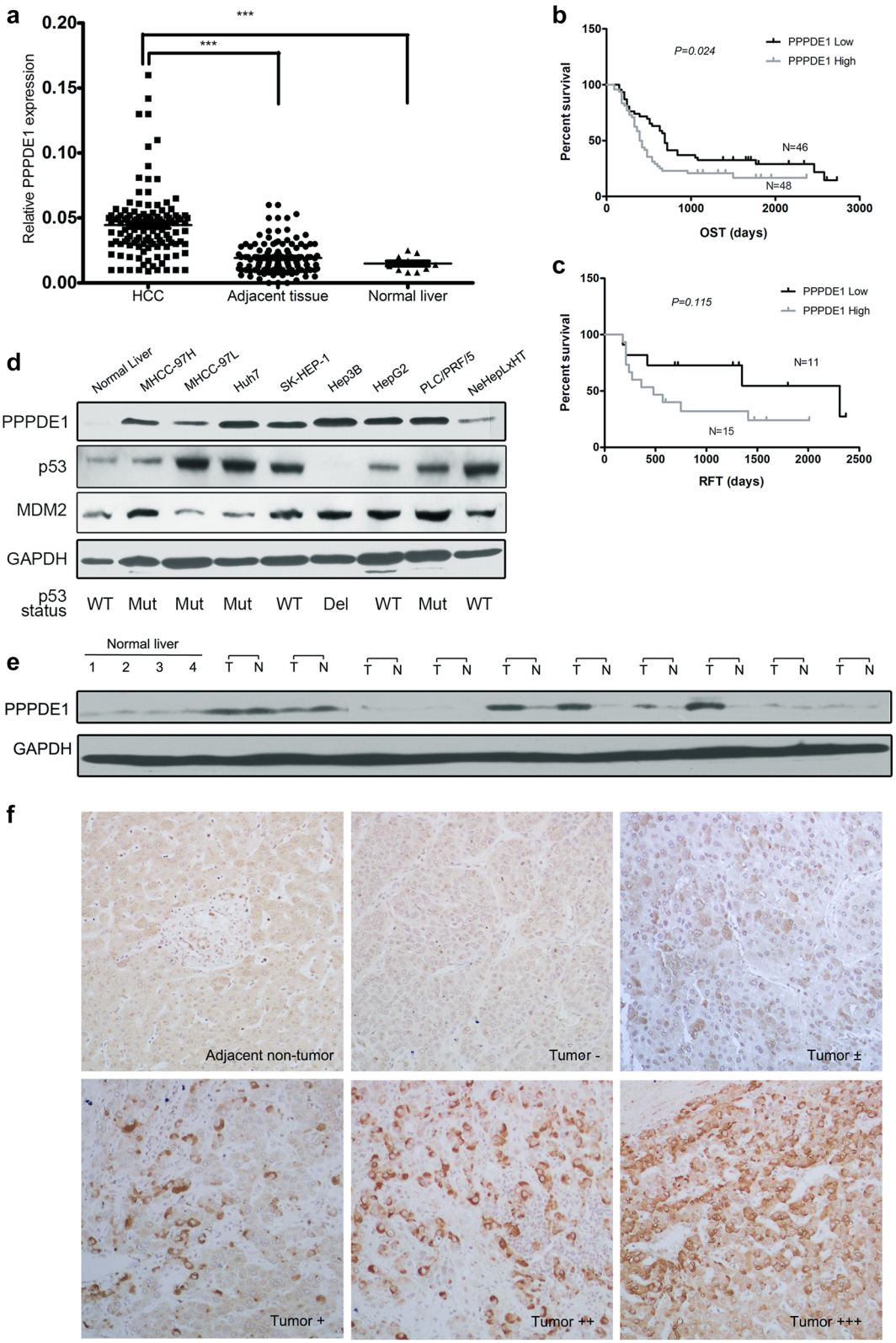
In order to further clarify the oncogene dependency on *PPPDE1* in human HCC cells, we evaluated the subcutaneous growth of established tumors from Huh-7 and MHCC-97H cells treated with lentivirus expressing shRNAs against non-silencing control and *PPPDE1*. Knocking down *PPPDE1* with two independent shRNAs dramatically suppressed the subcutaneous growth of established tumors from both Huh-7 and MHCC-97H cells (Fig. 3h–k).

We have previously shown that *PPPDE1* is a novel deubiquitinase that belongs to a cysteine isopeptidase family and the C108S mutant, in which 108 cysteine of PPPDE1 was substituted with serine, could destroy the deubiquitinating activity of PPPDE1. Strikingly, we showed that delivery of C108S mutant to DEK1 transduced cell failed to rescue the growth defects (Fig. 3d–f). These results established clear oncogene dependence of the expression and the deubiquitinating activity of PPPDE1 in human HCC cells.

PPPDE1 regulates p53 pathway and apoptosis

In order to get insight into the molecular mechanism of *PPPDE1* in HCC oncogene dependence, we measured the changes in gene expression profiles after knocking down *PPPDE1*. Huh7 cell cultured in vitro or in established subcutaneous tumors that transfected with shRNAs against non-silencing control (NS) or *PPPDE1* (DEK1 and DEK4) were subjected to expression profiling with Affymetrix Human Gene 2.0 ST Array. We identified a list of differentially expressed (DE) genes, including 78 down-regulated genes and 122 up-regulated genes, after knocking down *PPPDE1*. By Gene Ontology (GO) and pathway enrichment analysis, we found that p53 signaling pathway and cell cycle related pathways were dramatically affected by *PPPDE1* silencing (Fig. 4, Table S4).

We have previously identified that PPPDE1 could deubiquitinate and stabilize RPS7 proteins [7]. Ribosomal proteins (RPs) have been identified to modulate diverse biological process in addition to assembly a fully functional ribosome that is responsible for protein synthesis, especially in the ribosomal protein-MDM2-p53 signaling pathway [18, 19]. Several studies have revealed vital role of ribosomal protein S7 (RPS7) in modulating the MDM2-p53 pathway [20, 21], in cell proliferation and apoptosis, in tumorigenesis and metastasis [22–24].



Considering all these facts, we inferred that *PPPDE1* might modulate p53 pathway in HCC cells. We firstly investigated the protein level of p53 and its downstream Bax, an apoptosis effector protein. We found that overexpression of *PPPDE1* greatly reduced the protein level of p53 and Bax in a dose dependent manner in both Huh7

◂**Fig. 2** *PPPDE1* overexpressed in HCC and linked to bad prognosis. The mRNA expression level of *PPPDE1* in HCC tissues, paired non-tumor adjacent tissues (**a**) and its correlation with overall survival time (OST) (**b**) and recurrent-free survival time (RFT) (**c**) was determined by qRT-PCR analysis in patient from Guilin Cohort. ***p < 0.001. Total protein extraction from HCC cell lines were subject to immunoblotting with PPPDE1 (20517-1-AP, Proteintech), p53, MDM2 and GAPDH antibodies (**d**). Total protein from HCC tissues and paired non-tumor adjacent tissues were subject to immunoblotting with PPPDE1 and GAPDH (**e**). **f** The expression of PPPDE1 protein was determined by immunohistochemistry analysis using PPPDE1 antibody (2P14)

and MHCC-97H cell (Fig. 5a, b). We also showed that knocking down *PPPDE1* upregulated the protein level of p53 and Bax in Huh7 cells that cultured in vitro, while the protein level of MDM2 remained largely unchanged (Fig. 5c).

Huh7 only have a single copy of mutated *p53* coding DNA locus (Y220C/del), and MHCC-97H have a heterozygous *p53* coding DNA locus with one wild type copy and one mutant copy (R249S). As both MHCC-97H and Huh7 have mutant version of *p53* (Fig. 2d), we would wonder if *PPPDE1* is a regulator of both mutant and wild type *p53*. We further transfected FLAG-tagged wild type p53 into MHCC-97H together with *PPPDE1* or vector backbone, and determined the protein level of exogenous wild type p53 specifically by immunoblotting with anti-FLAG. We found that overexpression of *PPPDE1* could greatly down-regulated the protein level of exogenous p53 in MHCC-97H (Fig. 5d).

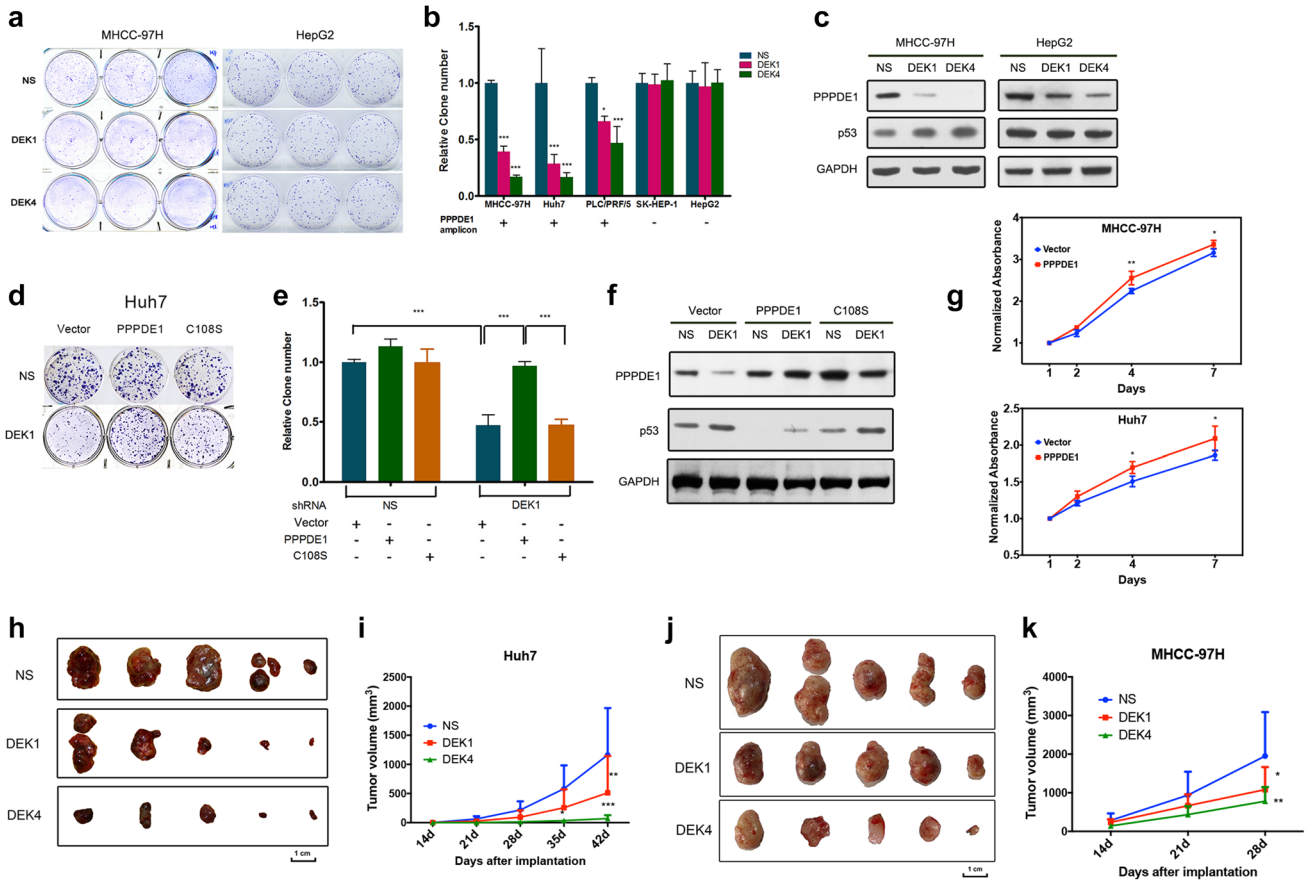


Fig. 3 *PPPDE1* Oncogene Dependency in Human HCC cell lines. **a, b** Clongenic assay of MHCC-97H and HepG2 infected with lentiviral shRNAs against non-silencing control and *PPPDE1* (DEK1 and DEK4) (**a**) and the quantification of clongenic assay of five HCC cell lines (three with *PPPDE1* amplification and three without) from three independent experiments (**b**). **c** The expression level of *PPPDE1*, p53 and GAPDH in MHCC-97H and HepG2 infected with lentiviral shRNAs (NS, DEK1 and DEK4) were determined by immunoblotting. **d, e** Clongenic assay of Huh7 cell infected with shRNAs against non-silencing control and *PPPDE1* (DEK1) and rescued with lentivirus expressing Luciferase, *PPPDE1* wild type protein or C108S mutant (**d**), and the quantification of clone numbers from three independent experiments (**e**). **f** The expression level of *PPPDE1*, p53 and GAPDH in Huh7 infected with lentiviral shRNAs and lentivirus expressing Luciferase, PPPDE1 wild type protein or C108S mutant were determined by immunoblotting. **g** Equivalent number of lentiviral vector or *PPPDE1* transduced cells was plated in 96-well plates, and cell proliferation assays were performed at the indicated time points with MTS assay. **h–k** HCC cells treated with lentivirus expressing shRNAs against non-silencing control and *PPPDE1* (DEK1 and DEK4) were implanted subcutaneously in BALB/c Nude for five different mice in each group. Tumors were harvest 42 days after Huh-7 implantation (**h, i**) and 28 days after MHCC-97H (**j, k**). Error bars denote ± SD. *p < 0.01, **p < 0.005, ***p < 0.001

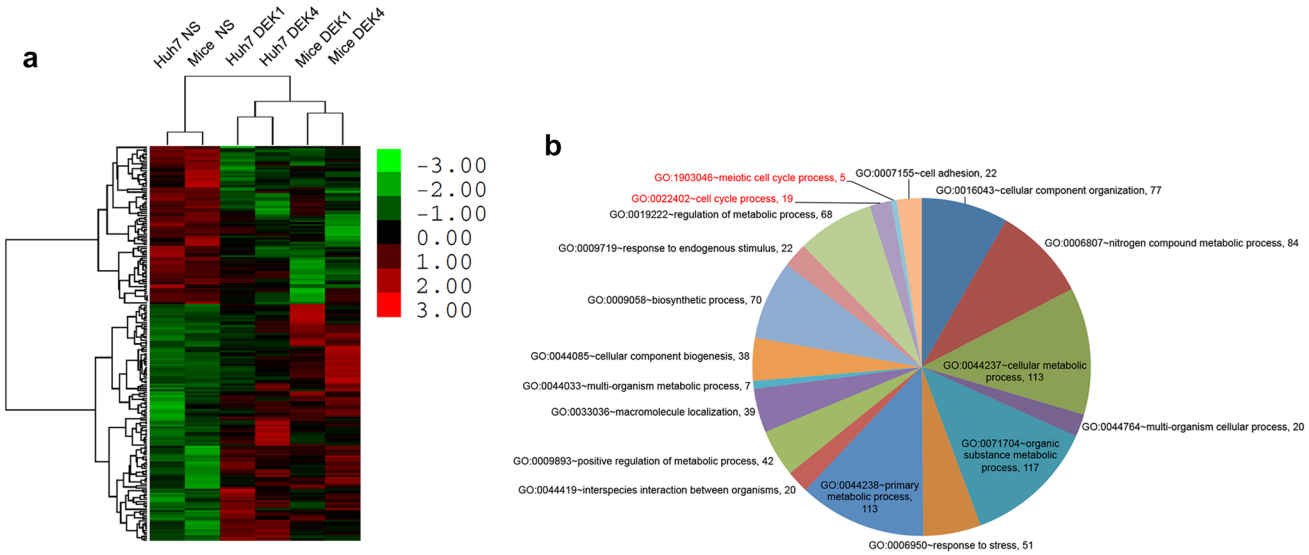


Fig. 4 Expression profiling of *PPPDE1* knock down cell and Xenograft tumor tissue. Huh7 cell or established subcutaneous tumors that transfected with shRNAs against non-silencing control (NS) or *PPPDE1* (DEK1 and DEK4) were subjected to expression profiling with microarray. Cluster analysis (**a**) and GO analysis (**b**) of 200 differential genes after *PPPDE1* silencing

MDM2 has been proved to be the key modulator of p53, so we sought to clarify if the regulation of p53 by *PPPDE1* involves MDM2. We firstly co-transfected MDM2 and *PPPDE1* into MHCC-97H cell and found that they could synergically down-regulate the protein level of p53 (Fig. 5d). To explore if the impact of *PPPDE1* on p53 is dependent on the activity of MDM2, we then knock *MDM2* down in MHCC-97H with lentiviral shRNA. We showed that the negative regulation effect of *PPPDE1* overexpression on p53 protein was largely limited without MDM2 (Fig. 5e).

Moreover, dramatically elevated p53 and BAX protein was also observed after knocking down *PPPDE1* in subcutaneous tumors established with Huh7 and MHCC-97H cells, which could explain why knocking down *PPPDE1* greatly suppressed subcutaneous tumor growth in xenograft tumor model (Fig. 5f, g).

Because one of the most important functions of p53 and BAX pathway is to promote apoptosis, we proposed that *PPPDE1* might regulate the apoptosis level of HCC cells which count for the oncogene dependence phenomenon in colony formation assay and subcutaneous tumorigenesis assay. We then monitored the apoptosis level after knocking down *PPPDE1* in Huh7 and found that silencing of *PPPDE1* greatly increased the apoptosis level of Huh7. Furthermore, the level of DNA damage induced apoptosis was also largely upregulated by knocking down *PPPDE1* (Fig. 5h, i).

Discussion

In this study we found that *PPPDE1* was amplified and overexpressed in HCC and it was linked to bad prognosis of HCC patients. In a multivariate Cox regression model, the mRNA level of *PPPDE1* was an independent prognostic factor for overall survival (hazard ratio for death, 1.737; 95% confidence interval [CI], 1.078–2.80; p = 0.023). We also demonstrated that, by knocking down of *PPPDE1* with lentiviral shRNA, the clonal growth and tumorigenicity of human HCC cells harboring the *PPPDE1* amplicon could be blocked. These results established a clear link between genotype (copy number status) and oncogene dependence of *PPPDE1* in HCC.

In this report we have also shown that it is possible to identify the underlying driver genes of human cancer through integrated analysis of CNV and RNA-seq data in the Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/) project. With the development of high-throughput sequencing technology, the cancer genomics data is accumulating at an unprecedented pace. For example, The TCGA project generates comprehensive genome-wide datasets that include data for CNV, somatic mutations, DNA methylation status, gene and exon expression, protein expression, pathway inference and phenotypes [17, 18]. These data provide a major opportunity for tumor biologist to identify key genes and pathways that are involved in carcinogenesis and the development of various types of tumors.

Furthermore, we proved that *PPPDE1* is a key modulator of p53 protein and its down stream apoptosis pathway.

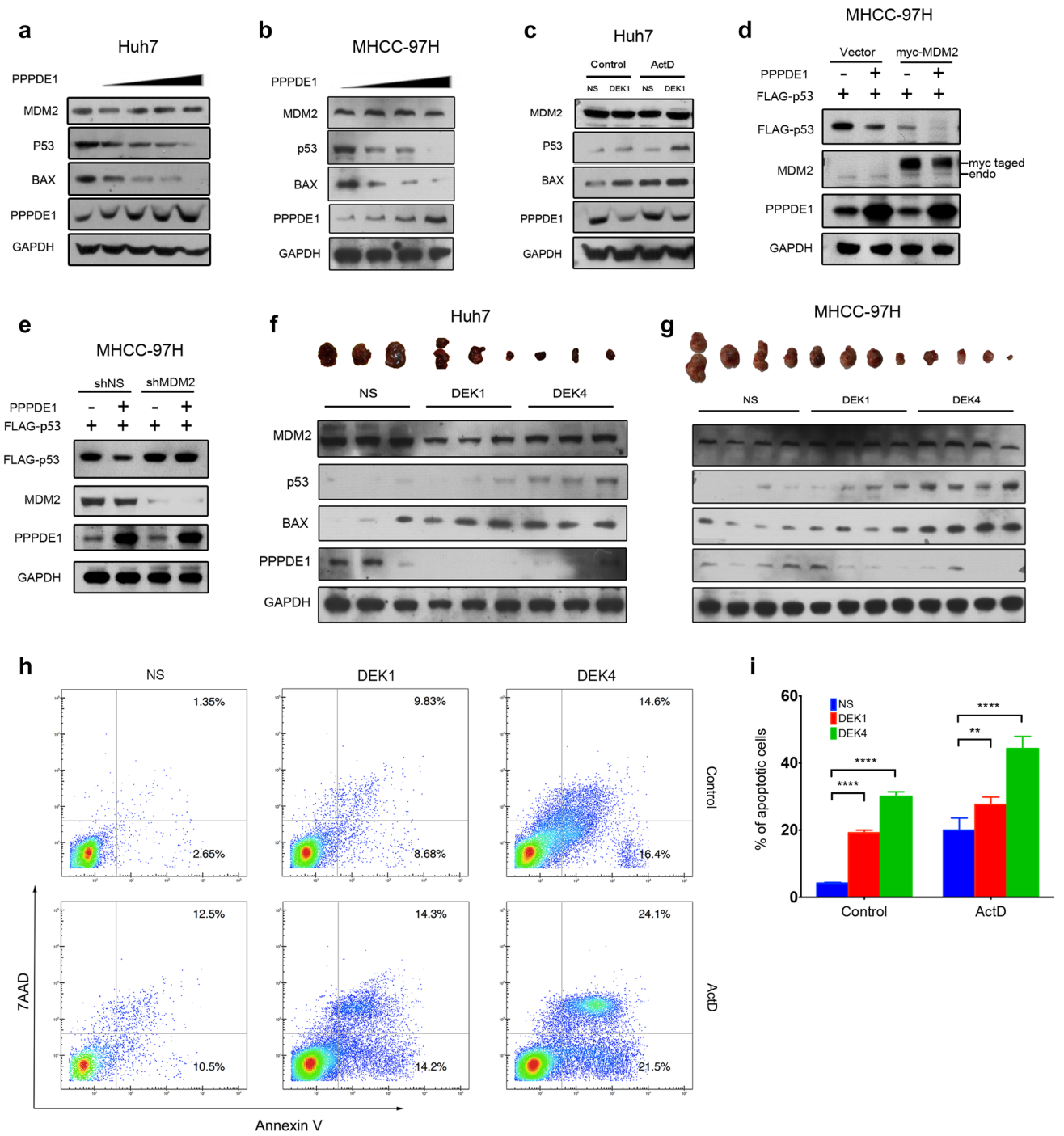


Fig. 5 *PPPDE1* regulates p53 pathway and apoptosis in HCC cell lines. **a, b** Total protein from HCC cell lines transfected with increasing amounts of *PPPDE1* expressing plasmid (0, 0.5, 1, 1.5 and 2 μg for Huh7 (**a**); 0, 0.5, 1 and 2 μg for MHCC-97H) was subjected to immunoblotting with MDM2, p53, Bax, PPPDE1 and GAPDH antibodies. **c** Huh7 transfected with shRNAs against non-silencing control (NS) or *PPPDE1* (DEK1) was subjected to immunoblotting with MDM2, p53, Bax, PPPDE1 and GAPDH antibodies. **d, e** Total proteins from MHCC-97H transfected with *PPPDE1* and FLAG-p53 together with myc-MDM2 (**d**) or shRNA against MDM2 (**e**) were subjected to immunoblotting with anti-FLAG, MDM2, PPPDE1 and GAPDH antibodies. **f, g** Total proteins from established subcutaneous tumors of Huh7 (**f**) or MHCC-97H (**g**) infected with lentivirus expressing shRNAs against non-silencing control (NS) and *PPPDE1* (DEK1 and DEK4) were subjected to immunoblotting with MDM2, p53, Bax, PPPDE1 and GAPDH antibodies. **h** After *PPPDE1* silencing, Huh7 was stained with Annexin-V or 7AAD and subjected to flow cytometry analysis to measure the apoptosis level. **i** The frequency of Annein-V positive cells was indicated. ***$p < 0.001$, ****$p < 0.0001$

By gene expression profiling analysis, we found that p53 signaling pathway were dramatically affected by *PPPDE1* silencing. We then found that the expression level of *PPPDE1* could dramatically modulate the protein level of p53 and Bax in a dose dependent manner in HCC cells. Strikingly, silencing of *PPPDE1* greatly increased the apoptosis level of HCC cells. TP53 is a transcription factor that plays a pivotal tumor suppression role by inducing apoptosis, cell cycle arrest and cellular senescence, and p53 deregulations represent the most prevalent molecular alterations in human cancers, including Hepatocellular Carcinoma [25, 26]. In this study, we uncovered a novel mechanism of suppressing the protein level of p53 in HCC.

We showed that *PPPDE1* could regulate the protein level of both mutated and wild type *p53* in HCC cell lines, which suggested *PPPDE1* probably play a universal role in p53 regulation in the development of HCC. We also showed that *PPPDE1* negatively regulate the protein level of p53 in a MDM2 dependent manner, which gave us a direction for further studies (Fig. 5d). Interestingly, we have previously showed that *PPPDE1* is a deubiquitinase and could deubiquitinate and stabilize RPS7 proteins [7]. In this study, we further revealed that its deubiquitinating activity is critical for oncogene dependence of *PPPDE1* in human HCC cells. Given that several studies have revealed vital role of ribosomal protein S7 (RPS7) in modulating the MDM2-p53 pathway [20, 21], in cell proliferation, in apoptosis, and in tumorigenesis and metastasis [22–24], we propose that *PPPDE1* might regulate p53 through the deubiquitinating of RPS7, which will be further clarified in our subsequent studies.

In summary, these results suggested that *PPPDE1* is a putative HCC driver gene and extensive studies should be conducted in the future to investigate the role of *PPPDE1* in HCC and other tumors.

Acknowledgements This work was supported by grants from the National Natural Science Foundation of China (No. 81201569, No. 81541151), the Beijing Natural Science Foundation (No. 7132186) and the National Key Sci-Tech Special Project of China (No. 2018ZX10302207, No. 2017ZX10203202).

Compliance with ethical standards

Conflict of interest There are no conflicts of interest to declare.

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A (2015) Global cancer statistics, 2012. CA 65:87–108
2. Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144:646–674
3. Farazi PA, DePinho RA (2006) Hepatocellular carcinoma pathogenesis: from genes to environment. Nat Rev Cancer 6:674–687
4. Cheng W, Su Y, Xu F (2013) CHD1L: a novel oncogene. Mol Cancer 12:170
5. Liu L, Dai Y, Chen J et al (2014) Maelstrom promotes hepatocellular carcinoma metastasis by inducing epithelial-mesenchymal transition by way of Akt/GSK-3beta/Snail signaling. Hepatology 59:531–543
6. Wang K, Lim HY, Shi S et al (2013) Genomic landscape of copy number aberrations enables the identification of oncogenic drivers in hepatocellular carcinoma. Hepatology 58:706–717
7. Xie X, Wang X, Jiang D et al (2017) PPPDE1 is a novel deubiquitinase belonging to a cysteine isopeptidase family. Biochem Biophys Res Commun 488:291–296
8. Stegmeier F, Sowa ME, Nalepa G, Gygi SP, Harper JW, Elledge SJ (2007) The tumor suppressor CYLD regulates entry into mitosis. Proc Natl Acad Sci USA 104:8869–8874
9. Nakada S, Tai I, Panier S et al (2010) Non-canonical inhibition of DNA damage-dependent ubiquitination by OTUB1. Nature 466:941–946
10. Schwickart M, Huang X, Lill JR et al (2010) Deubiquitinase USP9X stabilizes MCL1 and promotes tumour cell survival. Nature 463:103–107
11. Singh N, Singh AB (2016) Deubiquitinases and cancer: a snapshot. Crit Rev Oncol Hematol 103:22–26
12. Sippl W, Collura V, Colland F (2011) Ubiquitin-specific proteases as cancer drug targets. Future Oncol 7:619–632
13. Devine T, Dai MS (2013) Targeting the ubiquitin-mediated proteasome degradation of p53 for cancer therapy. Curr Pharm Des 19:3248–3262
14. Lim KH, Baek KH (2013) Deubiquitinating enzymes as therapeutic targets in cancer. Curr Pharm Des 19:4039–4052
15. Nanduri B, Suvarnapunya AE, Venkatesan M, Edelmann MJ (2013) Deubiquitinating enzymes as promising drug targets for infectious diseases. Curr Pharm Des 19:3234–3247
16. Robinson JT, Thorvaldsdottir H, Winckler W et al (2011) Integrative genomics viewer. Nat Biotechnol 29:24–26
17. Campeau E, Ruhl VE, Rodier F et al (2009) A versatile viral system for expression and depletion of proteins in mammalian cells. PLoS ONE 4:e6529
18. Warner JR, McIntosh KB (2009) How common are extraribosomal functions of ribosomal proteins? Mol Cell 34:3–11
19. Zhang Y, Lu H (2009) Signaling to p53: ribosomal proteins find their way. Cancer Cell 16:369–377
20. Chen D, Zhang Z, Li M et al (2007) Ribosomal protein S7 as a novel modulator of p53-MDM2 interaction: binding to MDM2, stabilization of p53 protein, and activation of p53 function. Oncogene 26:5029–5037
21. Zhu Y, Poyurovsky MV, Li Y et al (2009) Ribosomal protein S7 is both a regulator and a substrate of MDM2. Mol Cell 35:316–326
22. Wu CT, Lin TY, Hsu HY, Sheu F, Ho CM, Chen EI (2011) Ling Zhi-8 mediates p53-dependent growth arrest of lung cancer cells proliferation via the ribosomal protein S7-MDM2-p53 pathway. Carcinogenesis 32:1890–1896
23. Zhang W, Tong D, Liu F et al (2016) RPS7 inhibits colorectal cancer growth via decreasing HIF-1alpha-mediated glycolysis. Oncotarget 7:5800–5814
24. Wang Z, Hou J, Lu L et al (2013) Small ribosomal protein subunit S7 suppresses ovarian tumorigenesis through regulation of the PI3K/AKT and MAPK pathways. PLoS ONE 8:e79117
25. Levine AJ, Oren M (2009) The first 30 years of p53: growing ever more complex. Nat Rev Cancer 9:749–758
26. Fujimoto A, Furuta M, Totoki Y et al (2016) Whole-genome mutational landscape and characterization of noncoding and structural mutations in liver cancer. Nat Genet 48:500–509