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Protein phosphatase 5 promotes hepatocarcinogenesis through interaction with AMP-activated protein kinase

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ABSTRACT

The serine-threonine protein phosphatase family members are known as critical regulators of various cellular functions, such as survival and transformation. Growing evidence suggests that pharmacological manipulation of phosphatase activity exhibits therapeutic benefits. Ser/Thr protein phosphatase 5 (PP5) is known to participate in glucocorticoid receptor (GR) and stress-induced signaling cascades that regulate cell growth and apoptosis, and has been shown to be overexpressed in various human malignant diseases. However, the role of PP5 in hepatocellular carcinoma (HCC) and whether PP5 may be a viable therapeutic target for HCC treatment are unknown. Here, by analyzing HCC clinical samples obtained from 215 patients, we found that overexpression of PP5 is tumor specific and associated with worse clinical outcomes. We further characterized the oncogenic properties of PP5 in HCC cells. Importantly, both silencing of *PP5* with lentiviral-mediated short hairpin RNA (shRNA) and chemical inhibition of PP5 phosphatase activity using the natural compound cantharidin/norcantharidin markedly suppressed the growth of HCC cells and tumors *in vitro* and *in vivo*. Moreover, we identified AMP-activated protein kinase (AMPK) as a novel downstream target of oncogenic PP5 and demonstrated that the antitumor mechanisms underlying PP5 inhibition involve activation of AMPK signaling. Overall, our results establish a pathological function of PP5 in hepatocarcinogenesis via affecting AMPK signaling and suggest that PP5 inhibition is an attractive therapeutic approach for HCC.

1. Introduction

Protein phosphorylation/dephosphorylation plays an important role in signal transduction throughout the process of carcinogenesis. Reversible protein phosphorylation is achieved by the coordinated actions of protein kinases and phosphatases. Over the past decades, kinase inhibitors have been highly developed resulting in a new generation of cancer drugs [1]. Recently, increasing evidence suggests that protein phosphatases could also be potential drug targets for anti-cancer therapy [2,3].

Ser/Thr protein phosphatase 5 (PP5), along with PP1, PP2A, PP2B (calcineurin), PP4, PP6, and PP7, is a phosphoprotein

Abbreviations: HCC, hepatocellular carcinoma; NCTD, norcantharidin; AA, arachidonic acid; AMPK, 5′ AMP-activated protein kinase; p-AMPK, phosphorylated AMPK; PPP, phosphoprotein phosphatase; PP5, protein phosphatase 5; PI, propidium iodide; TPR, tandem tetratricopeptide repeat; IHC, immunohistochemistry; IP, immunoprecipitation; PARP, poly (ADP-ribose) polymerase; shRNA, short hairpin RNA.

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phosphatase (PPP). Previously, PP5 has been shown to regulate many cellular processes, including MAPK-mediated cell growth and apoptosis [4,5], cell cycle arrest and DNA damage repair via the p53 and ATM/ATR pathways [6–11], and steroid receptor signaling [12]. Interestingly, aberrant expression of PP5 has been reported in many different malignant diseases, such as breast cancer and mantle-cell lymphomas [13,14]. In breast cancer, overexpression of PP5 was shown to promote estrogen-independent growth of MCF-7 [15,16]. Conversely, ablation of PP5 impairs the proliferation potential of breast, and also other cancer types, including lung, glioma, colorectal, and hepatocellular carcinoma (HCC) [10,15,17–19]. Collectively, the above data suggest that aberrant expression of PP5 may contribute to the maintenance of cancer cells; however, the exact roles and molecular mechanisms responsible have not yet been fully discussed.

AMP-activated protein kinase (AMPK) is an evolutionarily conserved energy-sensing protein kinase that responds to numerous stimuli and regulates diverse physiological processes [20,21]. By regulating a variety of downstream targets (mTORC1, p53, FOXO3, etc.), AMPK can mediate various anti-tumor activities, including cell cycle checkpoint, senescence, autophagy, and apoptosis [22,23]. Recently, AMPK is emerging as a promising target for HCC treatment [24]. Compounds that stimulate AMPK activities have been shown to possess anti-HCC activities [25–28]. In the current project, we disclosed the oncogenic roles of PP5 in HCC and identified AMP-activated protein kinase (AMPK) as a bona fide substrate of PP5. PP5 inactivates AMPK and promotes cancer cell growth, while inactivation of PP5 significantly impaired HCC survival *in vitro* and *in vivo*. Notably, overexpression of PP5 was shown to be an important prognostic factor in our HCC patients. Taken together, our data suggest that PP5 is a novel oncoprotein and may be an attractive drug target worth pursuing.

2. Materials and methods

2.1. Reagents

Cantharidin (Cat. No. HY-N0209), dorsomorphin dihydrochloride (Cat. No. HY-13418) and metformin (Cat. No. HY-17471A) were purchased from MedChem Express (Monmouth Junction, NJ, USA) for *in vitro* studies. AICAR (no. 9944) was from Cell Signaling Technology (Danvers, MA, USA). Arachidonic acid (Catalog no. 1505-100) was from BioVision (Milpitas, CA, USA). Norcantharidin (N8784) was obtained from Sigma-Aldrich (St. Louis, MO, USA) for *in vivo* animal studies.

2.2. Plasmids and RNAi

Human PP5 cDNA was subcloned into pCMV-tag 2B (Agilent Technologies, Santa Clara, CA, USA) to express DDK-PP5. Deletional mutations of PP5 were made by PCR. PCR amplified fragments were cloned into pCMV-tag 2B, and confirmed by sequencing. pECE-HA-AMPKalpha2 and pECE-HA-AMPKbeta1 were gifts from Anne Brunet (Addgene plasmid #31654 & 31666). The plasmid expressing HA-AMPKgamma1 was made by fusing EcoRI/BamHI digested fragments from pFLAG-Cherry-N1-PRKAG1 (gift from Jay Brenman; Addgene plasmid #30308) and pcDNA3.0 HA vector. PP5-H304Q and AMPK-T172A constructs were made by site-directed mutagenesis (Agilent Technologies) and confirmed by sequencing. ON-TARGETplus Human AMPKα2 (PRKAA2) siRNA (L-005361-00-0005) and non-targeting pool (D-001810-10-50) were obtained from GE Healthcare Dharmacon (Lafayette, CO, USA). DharmaFECT 4 transfection reagent was used for siRNA transfection.

2.3. Cell culture and transfection

Hep3B and PLC/PRF/5(PLC5) cell lines were purchased from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). Huh7 cell line was obtained from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). Human HCC cell lines and HEK 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% (v/v) fetal bovine serum (FBS) and penicillin-streptomycin. Transfection for transient expression was performed using calcium phosphate co-precipitation for HEK 293T cells or Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) for HCC cells following the manufacturer's instructions. Recombinant lentiviruses were used for stable ectopic expression or knockdown cell line establishment. Lentiviral transfer vector pLAS2w.Ppuro carrying DDK-PP5 or DDK-PP5-H304 was used to generate stable PP5/PP5-H304Q ectopic expression cell pools. For PP5 stable knockdown cell establishment, pLKO.1-puro vector expressing non-targeting (NT) control shRNA (pLKO TRC025) or shRNAs targeting PP5 (TRCN0000002801 & TRCN0000002802) were used. The two PP5 shRNA oligo sequences: shPP5#1 (5′-CCGGCCACGAGACAGACAACATGAACTCGAGTTCATGTTGTCTGTCTCGTGGTTTTT-3′); shPP5#2 (5′-CCGGGAGACAGAGAAGATTACAGTACTCGAGTACTGTAATCTTCTCTGTCTCTTTTT-3′). The shRNA reagents and the recombinant lentiviruses were obtained from the National Core Facility for Manipulation of Gene Function by RNAi, Academia Sinica, Taiwan.

2.4. Colony-formation and sphere formation assay

HCC cells with PP5 overexpression or knockdown were seeded into a six-well plate (800 cells/well) and grown for 14 days. Colonies are fixed with glutaraldehyde (6.0% v/v), stained with crystal violet (0.5% w/v) and counted using Image J software 1.46e (Wayne Rasband, NIH, USA). For tumorsphere formation, 500 HCC cells were seeded into a 24-well Ultra-Low Attachment plate in serum free culture medium containing 10 ng/ml human EGF, 10 ng/ml bFGF, and B27 supplement. After incubation for one-week, tumorsphere numbers were counted under a phase-contrast microscope.

2.5. Western blotting and antibodies

HCC cells were harvested and lysed by RIPA buffer containing 1 mM of PMSF, NaF, Na_3VO_4, and protease inhibitor. After vortexing or sonication, the lysates were cleared by centrifugation at 13,500 rpm at 4 °C for 10 min. The supernatant was saved and the protein concentration of each sample was determined by the BCA method. Proteins (10–20 μg) were applied onto a SDS-PAGE gel and electrophorese. Following SDS-PAGE, the proteins were transferred to a PVDF membrane and detected with appropriate primary and horseradish peroxidase conjugated secondary antibodies. Enhanced chemiluminescence (ECL) was used for routine immunoblotting. The primary antibodies used in this study are listed below. Goat polyclonal anti-PP5 (C-20) (sc-32588) for immunoblotting and mouse monoclonal anti-PP5 (H-7) (sc-271816) for immunoprecipitation were obtained from Santa Cruz Biotechnology. A PP5 antibody (611020) from BD Transduction Laboratories (Bergen, NJ, USA) was used for immunohistochemistry. Mouse monoclonal anti-DDK (TA50011) and anti-HA (MMS-101P) antibodies were from OriGene Technologies (Rockville, MD, USA) and Covance (Princeton, NJ, USA), respectively. The following antibodies were from Cell Signaling Technology (Danvers, MA, USA): AMPKα (no. 2532/no. 2757), AMPKβ1 (no. 4178), AMPKγ1 (no. 4187), phospho-AMPKα (Thr172) (no. 2535), phospho-ULK1 (Ser317) (no. 12753), ULK1 (no. 8054), LKB1 (no. 3047), and Caspase 9 (no. 9502).