Original Article
Expression and function of protein phosphatase 5 in hepatocellular carcinoma

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Abstract: Ser/Thr protein phosphatase 5 (PPP5C) participates in signaling pathways in response to hormones or cellular stress, and elevated levels of this protein may be associated with development of some types of cancers by reversible phosphorylation at serine and threonine residues. The purpose of this study was to investigate the expression and function of PPP5C in human hepatocellular carcinoma (HCC) extensively. Real-time PCR and western blotting were used to quantify the expression levels of PPP5C in clinical HCC specimens. The relationship between PPP5C expression and typical clinical characteristics was statistically analyzed. MTT assay was performed to evaluate the cell growth capacity by PPP5C inhibition in HepG2 and/or overexpression in Hep3B cells. Annexin V/PI double staining followed by flow cytometry was carried to determine the apoptosis rate after doxorubicin treatment in control and PPP5C overexpressing cells. Xenograph model was finally used to investigate the function of PPP5C in HCC cells in vivo. PPP5C was found to be obviously upregulated in HCC specimens at mRNA and protein levels. Statistical analysis revealed that higher PPP5C levels correlated with TNM stage of patients. Functional assays demonstrated that PPP5C could promote cell growth, and its overexpression led to resistance to doxorubicin-induced both early and late apoptosis. We finally found that PPP5C inhibition could limit in vivo tumorigenesis in nude mice, reflected by reduced tumor volume and tumor weight. PPP5C may act as a potential tumor-promoting gene by enhancing cell growth and leading to doxorubicin resistance in HCC cells.

Keywords: PPP5C, HCC, cell growth, apoptosis, drug resistance

Introduction

Hepatocellular carcinoma (HCC), the most common primary liver malignancy, is one of the most common types of human solid tumor, and also a frequent cause of cancer-related death globally [1]. Most patients suffering from HCC are diagnosed at the advanced stage, and few effective therapeutic options are available at that time, hence chemotherapeutic or radiotherapy treatment is most appreciated and applied in the clinic. However, HCC cells are often refractory to general chemotherapy and resistant to radiotherapy, which contribute to bad outcome and poor prognosis in these patients [2, 3]. In spite of the keeping development and progress in understanding, diagnosis and treatment of this disease, HCC still remains as a malignant tumor with a very low 5-year survival rate, which is predominantly attributed to recurrence and metastasis [4]. Even in those patients undergoing liver cancer resection, the survival rate is as low as 30%-40% at 5 years postoperatively as a result of recurrence or metastasis [5, 6]. Besides, the precise mechanisms those governing the initiation and progression of HCC remain largely unknown. Keeping on study of the molecular mechanisms responsible for HCC is of great importance for drug discovery to treat this disease.

Protein phosphatases (dephosphorylation) and kinases (phosphorylation) cooperate to control cellular processes and signaling pathways [7, 8]. Also, reversible phosphorylation of serine, threonine and/or tyrosine residues in proteins is one of the essential events in signal transmission. For dephosphorylation regulation in eukaryotic cells, Ser/Thr phosphatases represent as one of the most important phosphatase families [9, 10]. Human PPP5C gene encodes a serine/threonine (Ser/Thr) phosphatase (also
named PP5) which is a member of the protein phosphatase catalytic subunit family. Proteins in this family participate in pathways regulated by reversible phosphorylation at serine and threonine residues. Along with PP1, PP2A, PP4, and PP6, they all belong to the phosphoprotein phosphatase (PPP) family of Ser/Thr phosphatases [11-13]. PP5 is ubiquitously expressed and a unique member of the PPP family based on the presence of tetratricopeptide repeat (TPR) domains within its structure.

PPP5C has been reported to be implicated in various cellular processes, including MAPK-mediated growth and differentiation, cell cycle arrest and DNA damage repair via the p53 and ATM/ATR pathways [12]. Meanwhile, there are a few studies showing that PPP5C is involved in cancer development. For instance, high level of PPP5C was observed in breast cancer, and it was found that PPP5C could promote proliferation of breast cancer cells and growth of tumors in a mouse xenograph model [14].
In HCC, our previous study had found that PPP5C was widely expressed in HCC cell lines, and short hairpin RNA (shRNA)-mediated inhibition of intrinsic PPP5C markedly suppressed the proliferation and colony formation ability of HCC HepG2 and Bel-7404 cells. We further demonstrated that PPP5C depletion in HepG2 cells led to G0/G1 phase and G2/M phase arrest [15]. Here, we carried a more comprehensively study in human HCC, including verifying the mRNA and protein levels of PPP5C in clinical HCC specimens, evaluating the clinical value, investigating the biological roles (cell growth and doxorubicin-induced apoptosis) of PPP5C in HCC cells in vitro and in vivo.

**Materials and methods**

**Cell culture and drugs**

Human normal liver cell lines QSG-7701, HL-7702, and HCC cell lines HepG2, Hep3B, BEL-7402, BEL-7404, HB661, HCC-LM3 were obtained from ATCC. Cells were cultured in DMEM or 1640 medium supplemented with 10% FBS and 1% Penicillin/Streptomycin (complete medium) in a humidified atmosphere of 5% CO₂ at 37°C. Doxorubicin and vehicle dimethylsulphoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

**Patients and tissue samples**

The human HCC tissues and their matching adjacent non-tumor tissues were obtained from the 2nd affiliated hospital of Harbin Medical University. All patients had provided the written consent forms before collection. All tissue samples were histologically confirmed with HE (hemotoxylin-eosin) staining. This study was approved by the Research Ethics Committee of the 2nd affiliated hospital of Harbin Medical University. All specimens were immediately snap-frozen in liquid nitrogen, and stored at -80°C.

**Vector construction and lenti-virus construction**

The PPP5C expression vector was constructed by PCR amplification of the open reading frame of PPP5C gene without the 5'- and 3'-UTR region. The amplified fragment was cloned into pCMV-c-flag, named flag-PPP5C. The lenti virus containing shRNA which targeted PPP5C and control virus was purchased from GenePharma (Shanghai, China).

**Transfections and infections**

The overexpression vector of PPP5C and control vector was transfected into Hep3B cells by

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Table 1. Correlation of PPP5C expression with clinico-pathological factors in 60 HCC specimens

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Increased (ΔΔCt&gt;1) n=34</th>
<th>Non-increased (ΔΔCt≤1) n=26</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>22</td>
<td>19</td>
</tr>
<tr>
<td>Female</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>( \kappa^2=0.4771, P=0.4897 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;50</td>
<td>26</td>
<td>21</td>
</tr>
<tr>
<td>≥50</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>( \kappa^2=0.1604, P=0.6888 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatitis status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>28</td>
<td>19</td>
</tr>
<tr>
<td>No</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>( \kappa^2=0.7469, P=0.3874 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor Size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;5 cm</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>≥5 cm</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>( \kappa^2=0.6671, P=0.4141 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor Number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>24</td>
<td>16</td>
</tr>
<tr>
<td>Multiple</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>( \kappa^2=0.5430, P=0.4612 )</td>
<td></td>
<td></td>
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<tr>
<td>TNM stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I/II</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>III/IV</td>
<td>24</td>
<td>10</td>
</tr>
<tr>
<td>( \kappa^2=6.1927, P=0.0128 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>No</td>
<td>29</td>
<td>20</td>
</tr>
<tr>
<td>( \kappa^2=0.2438, P=0.6215 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascular invasion</td>
<td></td>
<td></td>
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<tr>
<td>Yes</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>No</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>( \kappa^2=2.8671, P=0.0904 )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. PPP5C expression was upregulated in HCC cell lines and regulated cell proliferation. A: The expression of PPP5C was low in HCC cell lines compared with 7701 and 7702 normal hepatocytes by real-time PCR assay. GAPDH was used as a loading control. B: Promoting of cell proliferation in Hep3B cells after PPP5C overexpression. Cell proliferation was measured using MTT assay at 595 nm. Data had three repeats. **: P<0.01, compared to pCMV-c-flag.

C: Overexpression of PPP5C influenced cell morphology. 72 h after transfection, Hep3B cells were photographed under a microscope. D: Inhibition of cell proliferation in HepG2 cells after PPP5C knockdown. Cell proliferation was measured using MTT assay at 595 nm. Data had three repeats. **: P<0.01, compared to Lv-ShCon. E: After infection of lentivirus and selected by puromycin for two weeks, the cells were stained with crystal violet as described in Methods and Materials.

Lipofectamine2000 according to the manufacturer’s instruction. RNAi lenti-virus particles (sh-con and shPPP5C) were purchased from GenePharma (Shanghai, China). HepG2 cells
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were infected with the indicated lenti-virus particles of the same MOI=5 for 24 hours and then stable knockdown cells were selected with the medium containing puromycin for two week.

**RNA extraction and quantitative rt-pcr analysis**

Total RNA was extracted by TRizol and synthesized into cDNA using M-MLV Reverse Transcriptase. Primers for qRT-PCR were designed according to PPP5C sequence with GAPDH as control. The sequences of primers are as follows: PPP5C-forward: 5’-CCCAACTAC-TGCGACCAGAT-3’; PPP5C-reverse: 5’-CCCGTCACCTCACATCATTC-3’.

A two-step real-time PCR procedure was performed by SYBR system. The statistical method was $2^{-\Delta\Delta C_t}$. All the real-time qPCR experiments were performed with ABI7900 system.

**Western blot analysis**

The whole cell extracts were prepared by lysing cells in RIPA buffer (Beyotime Biotechnology). Protein concentration was determined using a BCA Protein Assay Kit (Beyotime Biotechnology), and 40 μg of protein was separated by 8% SDS-PAGE and transferred onto NC membranes. The membranes were then incubated by the PPP5C, PARP-1, caspase-3 anti-bodies, which were from Cell Signaling Technology. β-actin or GAPDH expression was evaluated as a loading control.

**MTT assay**

To measure the cell growth, 24 h after transfection or infection with vectors or lenti virus, cells were trypsinized and reseeded in a 96-well plate. 24 h, 48 h and 72 h later, 100 μl of MTT (5 mg/ml) was added into each well. Cells were then incubated for additional 4 h. After discarding the MTT-containing medium, 150 μl of DMSO was added. And absorbance OD490 was measured using a multi-well spectrophotometer.

**Crystal violet staining**

Growth of cells was determined by crystal violet staining. The infected cells grown in 6-well plates were fixed in 4% paraform aldehyde for 20 min. After wash, the cells were stained with 0.1% crystal violet for 15 min. The plates were aspirated, washed twice and allowed to air dry.

**Late and early apoptosis assays**

To assess the late apoptosis rate, Hep3B cells were transfected with flag-PPP5C or control vectors using lipofectamine2000 in 6-cm dishes. 24 hours later, cells were treated with 1 μg/ml doxorubicin. After another 24 h, cells were collected and fixed with 70% ethanol over night. The next day, cells were incubated with 100 μg/ml PI (propidium iodide) and 0.5 μg/ml RNaseA for 45 minutes at 37°C before subjecting to FACS analysis. To assess the early apoptosis rate, 24 hours after transfection and treatment with doxorubicin, the Hep3B cells were harvested and were analyzed using the Annexin V-PI apoptosis detection kit according to the manufacturer’s instruction.

**In vivo xenograft tumor growth**

Five-week-old male nude mice [BALB/c-CA-nu (nu/nu)] were obtained from Nanjing Experimental Animal Center and maintained in pathogen-free conditions. The mice were separated into two groups and were subcutaneously injected with control or shPPP5C lenti-virus-infected cells (2×10^6) at each flank. Tumor volumes were measured with a caliper, and tumor weighted were measured with a scales.

**Statistical analysis**

All data were analyzed by SPSS 18.0 software and presented as mean ± SEM. $\chi^2$ test was used to analyze the association between PPP5C expression and clinicopathological variables. Differences between any two groups were assessed by the Student’s t-test. $P<0.05$ was considered statistically significant.

**Results**

PPP5C expression was upregulated in clinical HCC specimens

In our previous study, we had showed that PPP5C mRNA was expressed in different HCC cell lines, in which HepG2 showing the highest expression [15]. However, whether it was also upregulated in clinical HCC specimens at mRNA and protein levels remained unclear. To give a more comprehensive impression of this query, we collected 60 pairs of HCC specimens from our hospital from 2014 Apr to 2015 Dec. Each HCC tissue was matched with para-HCC non-
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As shown in Figure 1A, among these 60 pairs of HCC specimens, the mRNA expression of PPP5C exhibited significant upregulation (fold change >2 fold) in more than half (34/60 = 56.67%, \( P < 0.001 \)) patients. Afterwards, we randomly selected 16 pairs protein extracts from these HCC specimens, and applied western blotting to determine the protein level of PPP5C. We observed that PPP5C was higher expressed in most of these tested HCC tissues than matched para-cancer tissues (Figure 1B). And the quantification of PPP5C protein expression also confirmed the tumoral higher expression of PPP5C (Figure 1C).

Elevated PPP5C levels were correlated with advanced HCC stages

From the above results, we had established that PPP5C was higher expressed in HCC tissues. However, the clinical significance of this increase remains unknown. Since all of these 60 HCC patients were recorded with detailed clinical data, including sex, age, tumor size, TNM stage and so on. Then the relationship between PPP5C gene expression and selected clinical characteristics was statistically analyzed by \( \chi^2 \)-test. As seen in Table 1, we found that elevated PPP5C was positively correlated with advanced TNM stages \( (P<0.05) \), which suggesting its potential to be used as a therapeutic target in HCC. There seemed to be no significant association of PPP5C expression with other clinical parameters, and this might attribute to our small scale of patients.

The tumor-promoting effects of PPP5C in HCC cells in vitro

To examine the function of PPP5C in HCC cells, we firstly evaluated its expression in a panel of HCC cell lines and immortalized hepatic cell line QSG-7701, HL-7702. We found that in accordance with our above results in clinical HCC specimens, PPP5C was also upregulated in HCC cell lines, such as HepG2 and HB661 cells (Figure 2A). We then performed PPP5C knockdown assay in HepG2 cells, and overexpressing PPP5C in Hep3B cells, another cell line in which PPP5C was relatively lower expressed. Knockdown and overexpression modulation were achieved by lentivirus containing shRNA targeting PPP5C and PPP5C coding sequences, respectively. MTT assay was performed to evaluate the cell growth capacity in these cells. As shown in Figure 2B and 2C, flag-PPP5C overexpression in Hep3B led to rapid...
growth rate than control cells, whereas PPP5C inhibition in HepG2 cells arrested cell growth (Figure 2D and 2E). Since drug resistance is a major actual difficulty in chemotherapy of this disease, we then tested the role of PPP5C in doxorubicin-induced apoptosis in HCC cells. PPP5C overexpressing HepG2 was used as the experimental cell line. The PI single staining followed by flow cytometry result shown in Figure 3A illustrated that PPP5C overexpressing HepG2 cells were more resistant to dox-induced apoptosis than control cells. In addition, annexin V/PI double staining results showed that both early and late apoptosis were reduced in PPP5C overexpressing cells (Figure 3B). The overexpression of PPP5C and flag-tag were validated in Figure 3C. Furthermore, we detected the protein levels of cleavaged PARP1 and caspase-3 by western blotting in control and PPP5C overexpressing cells with or without dox.
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treatment. The results shown in Figure 3D demonstrated that dox indeedly induced apoptosis, reflected by elevated cleaved PARP1 and caspase-3 levels. And PPP5C overexpression could impair this elevation, supporting its anti-apoptosis capacity in HCC cells.

**PPP5C knockdown inhibited the tumor formation in mice**

Since we had accessed the tumor promoting effects of PPP5C in HCC cells in vitro, we next sought to investigate the in vivo function of this molecule. The stable PPP5C knockdown HepG2 cells and the matched control cells were injected subcutaneously into nude mice. Every four days we measured the length and width of each xenograft and calculated the corresponding tumor volume. About a month later, mice were killed and the tumors were collected, photographed and weighed. As shown in Figure 4A-C, PPP5C inhibition significantly reduced the tumor formation ability of HepG2 cells in vivo, as assessed by mice photos, tumor volume and tumor weight. The knockdown efficiency was confirmed by real-time PCR and western blotting as shown in Figure 4D and 4E.

**Discussion**

HCC is one of the most malignant diseases in the world today, and HCC patients at their advanced clinical stages still have a poor prognosis although recent progress have been made at the anti-tumor therapy for HCC. Also, these advanced HCC patients are extremely resistant to most available chemotherapy drugs, which lead to a worse situation [16]. Therefore, there is an urgent need to explore and identify more and efficient agents for treating HCC, as well as to develop more predictive and exact diagnosis options.

In this study, we firstly retrospect the expression pattern in HCC cell lines and specimens particularly. We showed that PPP5C was obviously upregulated in clinical HCC specimens at both mRNA and protein levels. Importantly, our statistical analysis revealed that higher PPP5C levels were correlated with advanced TNM stages of HCC patients. Cellular functional assays were then performed and the results demonstrated that PPP5C could promote cell growth, and its overexpression led to resistance to doxorubicin-induced apoptosis. We finally found that PPP5C inhibition could limit in vivo tumorigenesis in nude mice, reflected by reduced tumor volume and tumor weight.

Our previous study [15] had reported that knockdown of PPP5C could block cell cycle progression of HepG2 cells. Specifically, the checkpoints between G0/G1 and S phase and S and G2/M phase were both impaired after knockdown of PPP5C [15]. Hence in our current study, we did not repeat this experiment. Instead, we focused on the effect of PPP5C on drug-induced apoptosis and more importantly, we performed the in vivo xenograft experiment to evaluate the tumor promoting effect of PPP5C.

Emerging evidence indicate that overexpression of PPP5C could promote cell proliferation and tumor progression from yeast to human [14, 17-19]. But only few studies show that PPP5C are directly related with the development of cancer. Our study together with our previous study [15] combined to demonstrate the critical role of PPP5C in HCC progression, especially impacting on cell growth and apoptosis. The internal mechanisms that corresponding for its oncogenic role require to be dug out in the long run.

As a Ser/Thr phosphatase, several substrates of PPP5C have been reported, such as Chk1 [17]. For our following mechanism investigation, we will pay more attention to identify its cellular substrates, thus to comprehensively understand the functions of PPP5C in HCC cells. Taken together, our results presented here provide a more systematic and overall understanding about the tumor promoting effects of PPP5C in HCC. We believe that our results provide a foundation for further study into the clinical potential of PPP5C RNAi therapy for the treatment of HCC.

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**Disclosure of conflict of interest**

None.

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