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Research paper

Downregulation of Peptidylprolyl isomerase A promotes cell death and enhances doxorubicin-induced apoptosis in hepatocellular carcinoma

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ABSTRACT

Peptidylprolyl isomerase A (PPIA) is a peptidyl-prolyl cis-trans isomerase that is known to play a critical role in the development of many human cancers. However, the precise biological function of PPIA in hepatocellular carcinoma (HCC) remains largely unclear. In this study, lentiviral overexpression vectors and small interfering *RNA* knockdown methods were employed to investigate the biological effects of PPIA in HCC. PPIA levels in HCC tissues and peritumoral tissues were detected by real-time Polymerase Chain Reaction (RT-PCR), Western blotting, and immunohistochemistry. Our results indicate that PPIA levels were significantly higher in the HCC tissues compared to the matched peritumoral tissues. Moreover, PPIA expression was significantly associated with tumor size in these tissues. Interestingly, serum PPIA (sPPIA) levels were significantly higher in healthy controls compared to the HCC patients. Knockdown or overexpression of PPIA was shown to downregulate and upregulate cell growth, respectively. Moreover, PPIA siRNA knockdown appears to promote doxorubicin-induced apoptosis in HCC cells, altering the expression of downstream apoptotic factors. In summary, our results indicate that PPIA may play a pivotal role in HCC by regulating cell growth and could serve as a novel marker and therapeutic molecular target for HCC patients.

1. Introduction

Hepatocellular carcinoma (HCC), one of the major cause of malignant tumor in China, is the fifth-most common cancer and the third cause of cancer-related mortality worldwide (Siegel et al., 2014). The relationship between chronic infection with hepatitis B virus and hepatocellular carcinoma is well established, and some 340,000 cases of HCC are attributable to hepatitis B infection (Parkin, 2006). The clinical characteristics of HCC include highly aggressive, recurring tumor formation that are frequently associated with metastasis to the lungs. To treat HCC patients, surgical resection, chemoembolization, radiation therapy, and liver transplantation have all been employed. However, even with these techniques, the incidence rate is still highly correlated to the death rate, with the five-year survival rate only being modestly improved to approximately 26% in the United States (Maluccio and Covey, 2012; Simard et al., 2012). Although progress has been made in recent decades to better understand and treat HCC, the underlying molecular mechanisms of this aggressive cancer remain elusive (El-Serag and Rudolph, 2007). Therefore, it is critical to explore the cellular signaling cascades operating during HCC pathogenesis in order to provide insights into other diagnosis and treatment options for HCC patients.

Peptidylprolyl isomerase A (PPIA), also known as CYPA, a member of the peptidyl-prolyl cis-trans isomerase (PPIase) family, catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides and accelerates protein folding. It was originally purified from bovine thymocytes and characterized as the primary cytoplasmic binding protein of the immunosuppressant cyclosporin A (CsA) (Handschumacher et al., 1984). Previous studies have also reported that PPIA is involved in several diseases, including viral infection, cardiovascular disease, and various inflammatory diseases (Franke et al., 1994; Jin et al., 2000; Gwinn et al., 2006). Moreover, this enzyme has also been shown to be a key molecule in multiple biological functions, including molecular chaperoning, protein folding, protein trafficking, immune modulation, and cell signaling (Kern et al., 1995; Galigniana et al., 2004; Syed et al., 2003; Colgan et al., 2004). Phosphorylation of apoptosis signaling-

Abbreviations: PPIA, Peptidylprolyl isomerase A; HCC, hepatocellular carcinoma; ELISA, Enzyme linked immunosorbent assay; Bcl-2, B-cell CLL/lymphoma 2; Bcl-xL, BCL2-like 1.

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regulating kinase 1 (ASK1) is also regulated by PPIA, which reduces the expression and function of ASK1 as well as its downstream kinases in the JNK and p38 signaling pathways (Kim et al., 2015). Recently, several studies have shown that PPIA is overexpressed in some human cancers, including non-small cell lung cancer, pancreatic adenocarcinoma, tongue squamous cell carcinoma, and head and neck squamous cell carcinoma (Campa et al., 2003; Howard et al., 2005; Qian et al., 2012; Li et al., 2005, 2006; Huang et al., 2012; Takahashi et al., 2012). Furthermore, PPIA also appears to participate in cancer proliferation, cell cycle progression, regulation of apoptosis, and cell migration/invasion, all of which play various pathophysiological functions during tumor progression (Li et al., 2008; Semba and Huebner, 2006; Choi et al., 2007; Calhoun et al., 2009). However, although there is a growing body of evidence that PPIA may play a pivotal role during tumor development, our understanding of the precise roles of PPIA in tumor cell proliferation and apoptosis is limited, particularly in HCC.

In the present study, we sought to determine the clinical significance of PPIA in HCC carcinogenesis and progression. To do so, we first established the expression level of PPIA in HCC tissue, followed by functional analysis in multiple liver cancer cell lines. With these data, we further clarified the molecular relationship between PPIA and HCC progression, during which downregulation of PPIA appears to promote cell death and enhance doxorubicin-induced apoptosis.

2. Materials and methods

2.1. Patients

Ninety HCC tissues and peritumoral tissues were randomly selected from patients undergoing hepatectomy between 2012 and 2014. Blood samples were collected between May 2014 and November 2014 from Fifty-four patients with histologically documented HCC who were candidated for surgical treatment. The control group consisted of one hundred and five healthy blood donors. All specimens were from our hospital (First Affiliated Hospital, Zhejiang University School of Medicine, Zhejiang, China). Sample collection, use, and storage procedures used in this study were approved by the Ethics Committee of Zhejiang University. Written informed consent was obtained from each patient prior to inclusion in this study, and all experiments were conducted according to our institutional guidelines and the Declaration of Helsinki. For animal experiments, we have the permission with animal approval board for permission of mouse images (Supplementary Fig. 1).

2.2. Cell lines and culture

The human liver cancer cell lines HepG2, SK-HEP-1, Huh7, and MHCC-LM3 were purchased from the Shanghai Institute of Cell Biology (Shanghai, China). All cell lines were cultured in Dulbecco's modified Eagle's medium (Gibco, CA, USA) supplemented with 10% fetal bovine serum (Gibco, CA, USA) in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Transfection with small interfering RNA and construction of PPIA-expressing lentivirus

For our transfection analyses, the various types of liver cancer cells were seeded in 6-well plates and transfected with negative control RNA or PPIA specific siRNA (Genepharma Corp, Shanghai, China). When cells were 30% confluent, transfections were carried out using Lipofectamine reagent (Invitrogen, CA, USA). The PPIA siRNA target sequence was used: 5′-GUCCCAAAGACAGCAGAAATT-3′. Furthermore, a replication-defective lentivirus encoding the complete PPIA open reading frame (Lenti-PPIA) was constructed by Invitrogen (Carlsbad, CA, USA). Lentivirus containing negative control RNA (designated Lenti-NC) was also constructed. The efficiency of interference and overexpression was assessed by Western blotting after these treatments.

2.4. Determination of PPIA serum levels

The concentration of serum PPIA were analyzed using a specific immunoassays (Human PPIA ELISA Kit, yuanmu biological technology, Shanghai, China). Enzyme linked immunosorbent assay (ELISA) were performed. Microtiter strips coated with a monoclonal antibody against PPIA were used. The PPIA antibody was purchased from the Proteintech company,Catalog number: 10720-1-AP. Optical densities was measured at 450 nm by absorbance Microplate Reader (BioTek Instruments, Winooski, VT,USA),which directly showed the concentration of PPIA presented in the samples.

2.5. Immunohistochemistry

Paraffin-embedded tissues were cut into sections (4 μm), deparaffinized, and rehydrated. After deparaffinization, antigen retrieval was performed in 10 mmol/L citrate buffer using a 750-W microwave. Endogenous peroxidase was inactivated by 3% H2O2/methanol for 15 min. The slides were then incubated with a PPIA-specific antibody (Proteintech company, Chicago, USA, Catalog number: 10720-1-AP) at 4 °C overnight. The slides were incubated with a streptavidin-biotin peroxidase staining kit (Histofine Simple Stain Max PO Multi, Nichirei, Tokyo, Japan). The immunoreactivity was visualized using DAB (3,3-diaminobenzidine), and the nuclei were counterstained with hematoxylin. The degree of immunostaining for PPIA was calculated using the staining intensity and the proportion of PPIA-positive cells. The former was graded as 0 (absent), 1 (weak), 2 (moderate), and 3 (strong). The proportion of positive cells was scored as 0 (no positive tumor cells), 1 (<10%), 2 (10–35%), 3 (35–70%), and 4 (>70%). A staining index score of 0–6 indicates low PPIA expression, while a staining index score of 7–12 indicates high PPIA expression.

2.6. Extraction of total RNA and real-time reverse transcriptase polymerase chain reaction (PCR) analysis

Total RNA was extracted from resected specimens and all cell lines using TRIZOL (Invitrogen, CA, USA). cDNA was synthesized from the resulting RNA samples using M-MLV Reverse Transcriptase reagent (Invitrogen, CA, USA). Real-time quantitative PCR was carried out on an ABI 7500fast instrument (Applied Biosystems, CA, USA) with SYBR Premix Ex Taq (Takara Bio Inc., Dalian, China). The housekeeping gene GAPDH served as an internal control, and the relative expression level of PPIA was calculated using the $2^{-\Delta\Delta CT}$ method. The primer sequences used were as follows: GAPDH-F:TGACTTCAACAGCGACACCCA,GAPDH-R:CACCCTGTTGCTGTAGCCAAA.PPIA-F: ACCGCCGAGGAAAACCGTGTA, PPIA-R:TGCTGTCTTTGGGACCTTGTCTGC.

2.7. Western blotting analysis

Following transfection with siRNA and incubation for 72 h, the liver cancer cells were washed with cold PBS. The cells were then collected and lysed with RIPA Lysis Buffer (Beyotime, Nanjing, China). The subsequent lysate was sonicated, followed by centrifugation for 15 min. Protein concentration was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). All samples were denatured by heating at 70 °C prior to electrophoresis. After separation with 10% SDS-PAGE, the proteins were blotted electrophoretically onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Furthermore, the membranes were then incubated with primary antibody (all purchased from Cell Signaling Technology, MA, USA and used at a 1:1000 dilution) for cleaved caspase-7, B-cell CLL/lymphoma 2 (Bcl-2), BCL2-like 1 (Bcl-xL), PPIA, and β-actin (loading control) at 4 °C overnight. After rinsing the membranes, horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000, Jackson Immuno Research, PA, USA) was used to develop the membrane. The images were captured with SuperSignal West Pico Chemiluminescent Substrate (Pierce, MA, USA).