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Oncogene RPA1 promotes proliferation of hepatocellular carcinoma via CDK4/Cyclin-D pathway

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ABSTRACT

As the sixth most prevalent cancer, hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide. Human replication protein A (RPA), a three-subunit protein, plays a central role in eukaryotic DNA replication, homologous recombination, and excision repair, including RPA1, RPA2 and RPA3. Recently, some studies focusing on the relation between RPA1 and carcinogenesis have demonstrated that RPA1 is a candidate oncogene and influences tumor biological behaviors in many cancers such as esophageal carcinoma, colon cancer, urothelial carcinomas, etc. However, the characteristic role of RPA1 in HCC and the detailed potential mechanism remain unknown. To identify the real effects of RPA1 on HCC and its potential pathway participating in the changes of liver cancer cells, we have conducted this study and demonstrated that RPA1 is up-regulated both in liver cancer cell lines and HCC tissues, which is associated with poorer prognosis, advanced TNM stage and larger tumor size. Stable knock-down of RPA1 by specific small hairpin RNA (shRNA) contributes to the impaired proliferate ability of SK-HEP-1 cells both in vitro and vivo. Consistently, upregulation of RPA1 in HuH-7 cells by specific adenovirus promotes tumor cells' proliferation. Furthermore, cyclin-dependent-kinase 4(CDK4)/Cyclin-D pathway is found to be well associated with RPA1 induced proliferation. In conclusion, RPA1 plays a pivotal role as a potential oncogene in HCC and promotes tumor proliferation via CDK4/Cyclin-D pathway.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most prevalent cancer worldwide. Despite advances in HCC therapy, it remains the third leading cause of cancer mortality, causing approximately one half million deaths each year [1,2]. Although there have been many advances in HCC therapy, such as targeted therapies and interventional radiological treatment, the overall treatment outcomes have not improved substantially [3–6]. New treatments (such as a novel gene therapy) and a better understanding of the underlying mechanisms of HCC tumorigenesis are urgently needed to advance HCC therapy [7].

Human replication protein A 1 (RPA1) is a subunit of RPA and proved to be amplified in many cancers like esophageal carcinoma, colon cancer, urothelial carcinomas, etc. [8–11] However, the characteristic role of RPA1 in HCC remains unknown.

Infinite proliferation is one of the most remarkable signs of cancers but the underlying mechanisms are of too much variety [12]. Cyclins and CDKs reflect tumor cell distribution in cell cycles, thus indicating tendency in overcoming restriction in cell phase change [13].

Here, we demonstrate that RPA1 is up-regulated both in liver cancer cell lines and HCC tissues, which is associated with a poorer prognosis, advanced TNM stage and larger tumor size. Stable knock-down of RPA1 by specific shRNA contributes to the impaired ability to proliferate in SK-HEP-1 cells. Consistently, up-regulation of RPA1 in HuH-7 cells by specific adenovirus promotes cell proliferation. Further, experiments both in vitro and vivo are well performed to validate oncogenetic role of RPA1 in HCC. Besides,

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CDK4/Cyclin-D pathway is found to be well associated with RPA1 induced proliferation. In conclusion, RPA1 plays a pivotal role as a potential oncogene in HCC and promotes tumor proliferation via CDK4/Cyclin-D pathway.

2. Materials and methods

2.1. Immunohistochemistry staining

121 cases of specimens from HCC patients who underwent surgical resection in the First Affiliated Hospital of Zhejiang University, School of Medicine from 2005 to 2012 were included in this research with complete postoperative follow-up information for 2–3.7 years. Besides, ethic of each operation and usage of the specimens for research was approved by the Ethical Committee of the First Affiliated Hospital of Zhejiang University, School of Medicine, in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. An anti-RPA1 antibody (ab79398, Abcam) was used as the primary antibody. Immunohistochemistry staining was performed as described previously [14]. All immunohistochemistry staining results were evaluated and scored by 2 independent pathologists blinded to clinical patient data. The IHC score (percentage of positively stained cells) ranged from 0 to 3 (0, 0%; 1, <5%; 2, 5%–50%; 3, >50% positively stained cells). Scores of 0 or 1 were considered to indicate "low" expression of RPA1, while those of 2 or 3 indicating "high" expression of RPA1.

2.2. Cell culture

The HepG2, SMMC-7721, HuH-7, and SK-Hep-1 HCC cell lines, as well as the normal liver cell HL-7702 were maintained in our institute. HepG2, HuH-7, and SK-Hep-1 were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA). SMMC-7721 and HL-7702 cells were cultured in Roswell Park Memorial Institute 1640 Medium (Gibco, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA). All cells were maintained in a humidified atmosphere containing 95% air and 5% CO2 at 37 °C.

2.3. Cell transfections

RPA1-specific shRNA and control shRNA were purchased from Hanbio (Shanghai, China). SK-HEP-1 cells were transfected with either RPA1-specific shRNA or control shRNA using Lipofectamine 2000 (Invitrogen, 11668500) and cultured in selection medium containing 5 μg/ml of puromycin (Gibco, A1113802) for 4–5 weeks to generate stable RPA1 knockdown cell lines. An adenovirus overexpressing RPA1 and negative control adenovirus were constructed by Hanbio (Shanghai, China). HuH-7 cells were transduced with either adenovirus at a multiplicity of infection of 50 pfu/cell in DMEM supplemented with 10% FBS. Cells were collected 48 h after transfection.

2.4. Western blot analysis

Western blot analysis was performed as described previously [14]. Briefly, the cells were first washed twice with cold PBS, and total cellular proteins were extracted after incubation in radioimmuno-precipitation assay (RIPA) lysis buffer (Cell Signaling Technology) supplemented with complete protease inhibitor cocktail (Roche, Basel, Switzerland) for 1 h on ice. The supernatants were collected after centrifugation at 15,000 × g at 4 °C for 20 min. Protein concentrations were measured using a Bicinchoninic Acid Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Equal amounts of denatured proteins were separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. After blocking non-specific binding for 1 h in 5% non-fat milk, the membranes were incubated overnight on ice with primary antibodies against RPA1 (Abcam) and β-actin (Sigma-Aldrich). Antibodies recognizing CDK-2, CDK-4, CDK-6, cyclin A2, cyclin D1 were acquired from Abcam. All antibodies were used in their recommended concentration.

2.5. Tumor formation in nude mice

Stable transfection by shRNA of SK-Hep-1 cells were suspended in 0.2 mL of phosphate buffered saline (PBS) and subcutaneously injected into the left flank of mice (five-week-old female BALB/c nude mice, 5×10^6 cells/mouse). The mice were monitored every 5 days, recording tumor size and calculating tumor volume via $L \times W^2/2$. The animals were sacrificed 50 days after injection. Tumor formed in vivo were collected and paraffin embedded for IHC evaluation. Ki-67, Caspase-3 and RPA1 staining were performed as described above.

2.6. Cell-proliferation analysis

Cell proliferation assays were performed using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) and absorbance measurements at 450 nm to determine the cell viability in each well. Cell proliferation was also evaluated using a colorimetric immunoassay (Cell-Light™ EdU Apollo567 In Vitro Imaging Kit; Ribobio, Guangzhou, China), according to the manufacturer's instructions. The cells were seeded in a confocal dish at a density of 1×10^5 cells/dish and cultured for 24 h before transfection and detection by immunoassay 48 h after transfection.

2.7. Cell cycle analysis

Cell cycle distribution was determined by flow cytometry. Briefly, 5×10^5 harvested cells were fixed overnight at 4 °C in 70% cold ethanol. After washing with cold PBS, cells were resuspended in a buffer contained in the Cell Cycle Staining Kit (MultiSciences, Hangzhou, China) and incubated for 30 min at room temperature. All cells were then analyzed by flow cytometry and analyzed using ModFit LT (Verity Software House, Inc., Topsham, Maine, USA).

2.8. Statistical analysis

Data were analyzed using GraphPad Prism 6 software. The results are presented as the mean ± standard error of the mean. The difference between groups was analyzed using Student's *t*-test. In all cases, $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. RPA1 is significantly up-regulated in HCC and correlates with poor prognosis

IHC staining of RPA1 in HCC samples shows significantly higher expression compared to paired non-tumor samples (Fig. 1A). To identify the correlation between RPA1 and the prognosis of HCC, we combined postoperative fellow-up data and found that patients with relatively high RPA1 expression had a poor prognosis compared to those with low RPA1 expression ($p < 0.01$; Fig. 1B). In addition, investigation of the correlation between the expression of RPA1 and clinicopathological features showed that high levels of RPA1 were significantly associated with advanced tumor-node-metastasis (TNM) stage and large tumor size, with p-value 0.041 and 0.034, respectively (Fig. 1C). In summary, our data indicate that