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Negative regulation of transcription factor FoxM1 by p53 enhances oxaliplatin-induced senescence in hepatocellular carcinoma

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ABSTRACT

Previous studies have demonstrated the involvement of transcriptional factor forkhead box M1 (FoxM1) in cellular senescence of hepatocellular carcinoma (HCC). In the present study, we revealed that oxaliplatin could induce senescence in HCC cells, since advanced HCC patients with lower expression of FoxM1 were more sensitive to oxaliplatin therapy. Our data indicated that due to the repression by p53, FoxM1 played a critical role in oxaliplatin-induced senescence via regulating cycle-related proteins p21, p27, cyclins B1 and D1. Furthermore, inhibition of FoxM1, combined with oxaliplatin treatment, could significantly promote the senescence of HCC cells. Taken together, our findings suggest that FoxM1 may represent a promising therapeutic target for the medication of the chemosensitivity to oxaliplatin in HCC patients.

1. Introduction

Hepatocellular carcinoma (HCC), the fifth most common cancer worldwide, is rarely detected early and is usually fatal within months of diagnosis, resulting in 500,000 deaths per year [1]. Despite implementation of potentially curative treatments such as liver resection and orthotopic liver transplantation, the prognosis is generally poor, as only 10–37% of patients are suitable surgical candidates because of advanced tumor or poor hepatic functional reserve [2]. Thus, chemotherapy is chosen to be the first-line treatment for advanced patients, despite the fact that HCC is a relatively resistant tumor with response rates ranging from 0% to 29% in randomized controlled trials [3].

Oxaliplatin, a third-generation platinum-derived chemotherapy agent, displays a wide spectrum of *in vitro* cytotoxic and *in vivo* antitumor activities. As an alkylating agent that causes DNA damage, oxaliplatin has been shown in clinical studies to have activity against advanced or metastatic HCC [4]. Although the precise mechanism of action is unknown, platinum compounds in general are thought to exert their therapeutic effects via induction of various signal-transduction pathways, including DNA-damage recognition and repair, cell-cycle arrest, and apoptosis [5]. Recently, researchers have demonstrated that cancer cells derived from solid tumors would readily undergo senescence when exposed to a wide variety of DNA-damaging drugs, and among which, platinum compounds have also been shown to promote accelerated senescence [6–8].

Although oxaliplatin has been proven to be a promising systemic and locoregional chemotherapeutic agent for treatment of advanced HCC, patient response to oxaliplatin varies greatly depending on different conditions and only a small fraction of patients benefit greatly from this antitumor drug. Interestingly, in our previous studies, we accidently found that patients with lower expression level of transcriptional factor forkhead box M1 (FoxM1), which is a proliferation-associated transcription factor that has important roles in cellular proliferation, organogenesis, aging and tumorigenesis, responded well to oxaliplatin therapy, while those with higher expression level of FoxM1 did not.

FoxM1, a member of the forkhead box transcription factor family, is characterized by the forkhead box domain. Previous studies have demonstrated that the overexpression of FoxM1 is associated with the development and progression of various cancers, such as lung, prostate, and pancreatic cancers as well as glioblastomas [9–12]. Recent studies have also suggested that FoxM1 plays a

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crucial role in oxidative stress-induced cellular senescence [13–15]. However, the role of FoxM1 in chemotherapy-induced senescence is still unknown.

In the present study, we employed HepG2 and SMMC-7721 cells to investigate the possible mechanism underlying the FoxM1-mediated response to oxaliplatin treatment in hepatocellular carcinoma patients. We found p53-depended senescence was a potential therapeutic effect of oxaliplatin and that high FoxM1 expression would counter this therapeutic effect.

2. Materials and methods

2.1. Patients, samples and follow-up

A total of 91 HCC patients who underwent oxaliplatin treatment at the First Affiliated Hospital of Xi'an Jiaotong University (Xi'an, China) were enrolled in this study. Forty-nine of the patients with advanced HCC underwent systemic oxaliplatin chemotherapy. Standard oxaliplatin treatment procedure was administered until unacceptable toxicity, disease progression, or intercurrent illness [4]. The other 42 HCC patients received preoperative hepatic arterial infusion (HAI) with oxaliplatin for reducing tumor size. When the suppressed tumor was within surgical indications, hepatectomy was performed. In addition, another 25 HCC patients who received hepatectomy without chemotherapy history were collected as control. HCC samples obtained from hepatectomy were mainly examined for senescence in liver tissues. The clinical characteristics of the patients were listed in Table 1. Ethical approval was obtained from the research ethics committee of First Affiliated Hospital, and written informed consent was obtained from each patient.

The follow-up data were summarized at the end of December 2011 with a median follow-up of 11.5 months (range 2–45 months). In this study, the HCC patients who underwent HAI with oxaliplatin were excluded from survival analysis due to the short period of follow-up. Time to recurrence (TTR) and overall survival (OS) were considered as the primary endpoints. TTR was calculated as the time from registration to the first observation of disease recurrence, whereas OS was from registration to death or the final follow-up.

2.2. Cell culture and transfection

Human HCC cell lines (Hep3B, HepG2, MHCC-97H and MHCC-97L) and immortal normal hepatocytes (L02) were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). SMMC-7721 cells were provided by the Molecular Biology Center of the First Affiliated Hospital, Xi'an Jiaotong University. FoxM1 siRNA (5′-GGACCACUUUCCCUACUUU-3′), p53 siRNA (5′-CUGGAAGACUCCAGUGGUA-3′) and p21 siRNA (5′-CGUCAGAACCCAUGCGGCA-3′) were synthesized by Shanghai Gene Pharma Co. (Shanghai, China). FoxM1 overexpression plasmid (pcDNA3.1-FoxM1) was donored by Dr. Qichao Huang from the Fourth Military Medical University (Xi'an, China). Transfection of siRNA (100 nM) or plasmid (4 μg) was carried out using a Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the procedure recommended by the manufacturer.

2.3. MTT assay for cell viability

HepG2 and SMMC-7721 cells (5.0×10^4 cells/well) were seeded in 96-well plates and incubated with oxaliplatin at various concentrations (0, 1.25, 2.5 and 5.0 μg/mL) for 24, 48 or 72 h at 37 °C. Then, 20 μL of MTT solution (5 g/L) was added into each well and incubated for another 4 h. Supernatants were removed and formazan crystals were dissolved in 200 mL dimethylsulfoxide (DMSO). Finally, optical density was determined at 490 nm using a POLAR star OPTIMA microplate reader (BMG Labtechnologies, Ortenberg, Germany).

2.4. Colony-forming assay

Cellular proliferation was determined using a colony-forming assay. Twenty-four hours after oxaliplatin treatment, HepG2 cells were plated in 6-well tissue culture plates with a cell density of 5×10^3 cells/well. After 2 weeks of incubation, colonies were stained with crystal violet dissolved in methanol. Only colonies containing more than 50 cells were counted. The results were reported as the mean number of colonies observed in five randomly chosen microscope fields.

2.5. Reactive oxygen species (ROS) assay

DCFH-DA was cleaved intracellularly by nonspecific esterases and transformed to highly fluorescent 2,7-dichlorofluorescein (DCF) upon oxidation by ROS. After adding carboxy-DCFDA at a final concentration of 10 μM to the culture medium, the cells were incubated at 37 °C for an additional 30 min, harvested, washed with PBS, and immediately measured by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.6. Senescence β-galactosidase (SA β-gal) assay

SA β-gal staining is widely used as a biomarker of cellular senescence *in vivo* and *in vitro*, with the positive green or blue-colored staining of β-galactosidase at pH 6.0 being remarkably increased in senescent cells. Senescent cells in frozen tissues or HCC cell lines were analyzed using a SA β-gal staining kit (Beyotime Inc., Nantong, China) according to the manufacturer's instructions. The percentage of SA-β-gal positive cells was calculated by counting the cells in 5 random fields (at least 100 cells) using bright-field microscopy. The staining results for HCC samples were recorded as "positive" or "negative", according to the method reported by te Poele et al. [16].

2.7. RNA isolation and quantitative reverse transcription–polymerase chain reaction (qRT–PCR) analysis

Total RNA was isolated from cells using the RNAfast200 Kit (Fastagen Biotech, Shanghai, China). Reverse transcription was performed using the PrimeScript® RT reagent Kit (TaKaRa Biotechnology, Dalian, China). The mRNA expression was assayed in triplicate and normalized to the β-actin mRNA expression. The relative levels were calculated using the Comparative-Ct Method (ΔΔCt method). The following primers were used for qRT–PCR: FoxM1:5′-AACCGCTACTTGACATTGG-3′(sense) and 5′-GCAGTGGCTTCATCTTCC-3′ (antisense); β-actin: 5′-ATCGTGCG TGTGACATTAAGGAG-3′(sense) and 5′-AGGAAGGAAGGCTGGAAGAGTG-3′(antisense); $p16^{INK4a}$: 5′-CATCGCGATGTCGCACGGTA-3′(sense) and 5′-TACGAAAGCGG GGTGGGTTGTG-3′(antisense); p53: 5′-ATGAGCCGCCTGAGGTTGG-3′(sense) and 5′-CAGCCTGGGCATCCTTGAGT-3′(antisense); p21: 5′-TGGCACCTCACCTGCTCTG-3′(sense) and 5′-GTTTGGAGTGGTAGAAATCTGTCAT-3′(antisense). All primer pairs were synthesized by TaKaRa.

2.8. Immunofluorescence and immunohistochemical assays

To examine the expressions of FoxM1 and p53, the cells or tissues were firstly fixed and blocked, and then incubated with anti-FoxM1 and anti-p53 monoclonal antibodies (Santa Cruz Biotechnology, CA, USA). For immunofluorescence assay, samples were incubated with FITC (green)- or rhodamine (red)-conjugated secondary antibodies (1:1000; Pierce, Rockford, IL, USA), followed by observation with a laser scanning microscope (Olympus, Tokyo, Japan).

Similarly for immunohistochemical assay, slides were incubated with biotin-label goat anti-mouse or anti-rabbit IgG, followed by horseradish peroxidase (HRP) to label streptavidin. The intensity of immunohistochemical staining was scored as 0 (negative), 1 (weak), 2 (moderate strong) or 3 (strong). The extent of staining was assessed based on the percentage of positive tumor cells: 0 (negative), 1 (1–25%), 2 (26–50%), 3 (51–75%), and 4 (76–100%). The final staining score for each sample was the mean of the sum of the intensity and extent scores from three fields. The expression was considered as low if the final score was 1–5 and as high if the final score was 6–12.

2.9. TUNEL assay

Apoptosis in HCC sections was measured and quantified with the use of the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. Tumor sections were stained with TUNEL agent (Roche, Shanghai, China) following the manufacture's instructions. Tissue sections included in the kit were stained and served as positive controls. The TUNEL-positive cells were counted under 5 randomly selected 400× microscopic fields. The apoptotic index was calculated as: the number of apoptotic cells/total number of nucleated cells × 100%.

2.10. Western blot assay

Cells were lysed in RIPA buffer (Beyotime Inc., NanTong, China). Protein concentration was determined with the Bradford reagent (Beyotime Inc.). Equal amounts of total proteins were separated and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were subsequently immunoblotted with the appropriate primary antibody at 4 °C for 12 h, and then incubated with HRP conjugated anti-goat or anti-rabbit antibody (Santa Cruz). Signals were detected using the ECL Kit (Pierce, Rockford, IL).

2.11. Statistical analysis

All data were expressed as mean ± standard error of measurement (SEM) and analyzed by SPSS 11.0 software (SPSS Inc., Chicago, IL, USA). Categorical data were analyzed by Fisher's exact test. The cumulative survival and recurrence rates were estimated by using the Kaplan–Meier method and the log-rank test. Comparisons of quantitative data between two groups were analyzed by Student's *t* test, with $P < 0.05$ or $P < 0.01$ considered significant. Mean values of three independent experiments were presented for all samples.