Experimental and Molecular Pathology 96 (2014) 207–211

Contents lists available at ScienceDirect

Experimental and Molecular Pathology

journal homepage: www.elsevier.com/locate/yexmp

Recombinant adenovirus encoding FAT10 small interfering RNA inhibits HCC growth in vitro and in vivo

Jingxiang Chen [a,1], Li Yang [b,1], Hongxu Chen [a], Tao Yuan [a], Menggang Liu [a], Ping Chen [a,*]

[a] Department of Hepatobiliary Surgery, Daping Hospital, Third Military Medical University, Chongqing 400042, China
[b] Department of Cardiology, Daping Hospital, Third Military Medical University, Chongqing 400042, China

ARTICLE INFO

Article history:
Received 6 December 2013
Available online 14 January 2014

Keywords:
HCC
FAT10
RNA interference
Carcinogenesis
Strategy

ABSTRACT

Hepatocellular carcinoma is an aggressive and rapidly fatal malignancy representing the common cancer worldwide. The specific cellular gene involved in carcinogenesis has not been fully characterized. The ubiquitin-like modifier FAT10, recently reported to be overexpressed in 90% of hepatocellular carcinoma carcinomas, was attributed to transcriptional upregulation upon the loss of p53 and induced chromosome instability in long-term in vitro culture. However, the exact function of FAT10 in hepatocellular carcinoma is not clear. In the present study, we utilized adenovirus-mediated RNA interference to knock down FAT10 expression in hepatocellular carcinoma cells and observed its effects on hepatocellular carcinoma cell growth in vitro and in vivo. The results demonstrated that interference of FAT10 could inhibit cell proliferation by inhibiting the cell cycle S-phase entry and inducing cell apoptosis. In addition, in vivo experiments showed that adenovirus Ad-siRNA/FAT10 significantly suppressed tumor growth and prolonged the lifespan of tumor-bearing mice. These results suggest that knockdown of FAT10 by adenovirus-delivered siRNA may be a promising therapeutical strategy for treatment of hepatocellular carcinoma.

Introduction

Hepatocellular carcinoma accounts for >90% of all primary liver cancers and has a dismal prognosis with a median life expectancy of 6 to 9 months. It ranks fifth in frequency among all malignancies worldwide and causes nearly 1 million deaths annually (El-Serag, 2002; Mulcahy, 2005; Yu and Keeffe, 2003). Surgery is the mainly choice for HCC treatment. However, the long-term prognosis after resection of HCC remains unsatisfactory as a result of a high incidence of recurrence (Mann et al., 2009; Nakamura et al., 2005; Ziparo et al., 2002). To solve this problem, many biologic therapies have been investigated.

The ubiquitin (Ub)–proteasome pathway is the main system for the targeted degradation of intracellular proteins (Dai and Li, 2001; Love et al., 2007; Murray and Norbury, 2000). Members of ubiquitin-related family have also been implicated to be involved in the regulation of cell cycle as well as apoptosis (Bayer et al., 1998; Reverter and Lima, 2005; Su and Li, 2002). One member of this family is FAT10, which is also known as diubiquitin. Role of FAT10 in cell-cycle regulation has been suggested by its ability to bind to MAD2, a spindle checkpoint protein (Ren et al., 2006; Zhang et al., 2006). In addition, it is recently reported that the FAT10 gene is upregulated in various cancers, implicating its role in tumorigenesis (Ji et al., 2009; Lim et al., 2006; Oliva et al., 2008; Zhang et al., 2006). Whether function of FAT10 contributes to cancer development remains unclear and warrant further research.

Therefore, in this study, we employed the adenovirus delivered small interfering RNA (siRNA) technique to study the effects of knockdown of FAT10 on HCC cell growth in vitro and in vivo.

Materials and methods

Mice, cells, and other reagents

Nude mice were purchased from the Laboratory Animal Institute of Beijing Medical University and were used at 6 weeks of age. Animals were bred in the Laboratory Animal Center and all studies were performed in agreement with the local ethics committee. Hepatocellular carcinoma cells Hep3B were purchased from the American Type Culture Collection. The cell line was maintained as monolayers in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, penicillin (200 units/mL), and streptomycin (100 μg/mL) and was kept at 37 °C in a humidified atmosphere of 5% CO_2 in air.

Recombinant adenovirus generation

The complementary DNA sequence of FAT10 was obtained from GenBank. The potential target sequence for RNA interference (RNAi) were scanned with the siRNA Target Finder and Design Tool available at the Ambion Web site. The target sequence selected 5′-GCUCAGUG

* Corresponding author. Tel./fax: +86 23 68757966.
E-mail address: pingchen1956@163.com (P. Chen).
[1] These authors contributed equally to this study.

0014-4800/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.yexmp.2014.01.001

GC ACAAGUGAATT-3′ (sense) and 5′-UUCACUUGUGCCACUGAGCTG-3′ (antisense). The target sequence was subcloned into shuttle vector pDC315 and sequenced. The desired replication-deficient adenovirus containing the full-length cDNA of RNAi was generated by homologous recombination through co-transfection of plasmids pDC315-RNAi and pBHG1oXE1, 3Cre in HEK 293 cells using the DOTAP liposome reagent (Roche, Mannheim, Germany). After several rounds of plaque purification, the adenovirus was amplified and purified from cell lysates by banding twice in CsCl density gradients. Viral products were desalted and stored at −80 °C in PBS containing 10% glycerol (v/v). The infectious titer was determined by a standard plaque assay.

Adenovirus infection

Transduction of cells with adenovirus was done in 6-well plates with 1×10^6 cells/well in 3 mL RPMI-1640 medium containing 10% FBS. Adenovirus was added to the wells at an MOI of 100 and the cells were harvested after 48 h of incubation.

Reverse transcription PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the protocol of the manufacturer. Two micrograms of RNA was subjected to reverse transcription. The polymerase chain reaction (PCR) primers used were as follows: for FAT10, 5′-CAATGC TTCCTG CCTCTGTG-3′ (forward), 5′-TGCCTCTTTGCCTCATCACC-3′ (reverse) and for β-actin, 5′-ATG ATATCG CCG CGC TCG TC-3′ (forward), 5′-CGC TCG GTG AGG ATC TTC A-3′ (reverse). PCR products were separated on a 1% agarose gel, visualized and photographed under ultraviolet light.

Western blot analysis

For Western blot assay, proteins of the cell extracts were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was incubated with 5% non-fat milk in PBS and then with anti-FAT10 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. After washing, the membranes were incubated with an alkaline phosphatase-conjugated goat antimouse IgG antibody (Amersham Biosciences, Little Chalfont, UK) for 1 h at room temperature. Immunoreactive bands were detected using the ECL Western blot analysis system (Amersham Biosciences). Densitometry analysis of protein levels was performed by using Scion-Image 4.0.2 software (Scion Corporation, Frederick, MD).

Inhibitory effect of cell proliferation

Cell proliferation was measured by a colorimetric assay using MTT. In brief, the Hep3B cells were seeded in 96-well plates in triplicate at 5×10^3 cells/well and incubated in culture medium overnight. Then the cells were treated with Ad-siRNA/FAT10 (1×10^9 pfu) or controls in a total volume of 0.2 mL each well for 24 h. Thereafter, 20 μL of the indicator dye MTT solution (5 mg/mL) was added to each well and cultures were continued for 48 h at 37 °C, 5% CO_2. After centrifugation, the supernatant was removed from each well. The colored formazan crystal produced from MTT was dissolved with 0.15 mL DMSO, then the optical density (OD) value A490 was measured by the multiscanner autoreader (Dynatech MR 5000; Dynatech Laboratories, Chantilly, VA, USA). The following formula was used: cell proliferation inhibited (%) = [1 − (OD of the experimental samples / OD of the control) × 100%].

Colony formation assay

For colony forming assay, 3×10^2 cells were seeded into 10 cm culture dishes, then the cells were treated with Ad-siRNA/FAT10 (1×10^9 pfu) or controls for 18 days of culture, cell colonies were fixed with methanol, and stained with 0.1% crystal violet and visible colonies were manually counted (the colonies containing at least 50 cells were considered viable).

Cell cycle analysis

Standard fluorescence-activated cell sorter analysis was used to determine the distribution of cells in cell cycle of the cells. Briefly, the cells were infected with Ad-siRNA/FAT10 (1×10^9 pfu) or controls for 2 days. Adherent cells then were collected by trypsinization and fixed with 70% ethanol overnight at 4 °C. After washing with phosphate-buffered saline (PBS), the cells were treated with 100 μg/mL RNase A (Roche Diagnostics), 50 μg/mL of propidium iodide (Sigma) and 0.05% (vol/vol) Triton X-100 and incubated for 45 min at room temperature. The samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The cell cycle distribution was established by plotting the intensity of the propidium iodide signal, which reflects the cellular DNA content. Findings from at least 1×10^4 cells were collected and analyzed with CellQuest software (Becton Dickinson).

Flow cytometric analysis of apoptosis

An Annexin V-fluorescein isothiocyanate kit (Oncogene) was used to detect apoptosis. Briefly, the cells were infected with Ad-siRNA/FAT10 (1×10^9 pfu) or controls for 2 days. The cells were seeded in 100 mL bottles and incubated until there was 80–85% confluence in RPMI-1640 containing 10% bovine serum. Then the cells were harvested, washed with ice-cold PBS twice, and resuspended in binding buffer (10 mM of HEPES, pH 7.4, 150 mM of NaCl, 2.5 mM of $CaCl_2$, 1 mM of $MgCl_2$, 4% bovine serum albumin). Annexin V-fluorescein isothiocyanate (0.5 mg/mL) and propidium iodide (0.5 mg/mL) were then added to a 250 mL aliquot (5×10^6 cells) of this cell suspension. After a 15 min incubation in the dark at room temperature, stained cells were immediately analyzed by Flow Cytometry (Coulter Biosciences). Apoptotic cell were determined by Annexin V positive and propidium iodide (PI) negative cells. All of the samples were assayed in triplicate, and the cell apoptosis rate calculated as: apoptosis rate = (apoptotic cell number / total cell number) × 100%.

Tumor challenge assay

6-Week-old nude mice were challenged with subcutaneous (s.c.) injection of 1×10^5 Hep3B cells into the left flank to induce primary tumors. Two weeks after tumor cell inoculation, mice were divided randomly into three groups (ten mice per group) and received an intratumor injection of Ad-siRNA/FAT10 (1×10^9 pfu) or Ad-siRNA/LacZ (1×10^9 pfu). The control mice received 100 μL PBS. Tumor volume and mean lifespan of the mice were observed. Tumor volume was measured in two dimensions and calculated as follows: length / 2 × width^2.

Statistical analysis

All the experiments were done in triplicate, and the results are given as Means ± S.E.M. of triplicate determinations. Statistical analyses were performed using the Student's t test. The difference was considered statistically significant when the P value was <0.05.

Results

Construction of Ad-siRNA/FAT10 and its effects on FAT10 expression

To examine whether adenovirus mediated siRNA can be used to specifically inhibit target gene expression, the adenovirus encoding siRNA targeted against FAT10 was used to infect HCC cell lines. As shown in Fig. 1A, the expression of FAT10 was inhibited 48 h after infection. However, the expression of FAT10 was not decreased in the control