Lentiviral vector-mediated doxycycline-inducible iASPP gene targeted RNA interference in hepatocellular carcinoma

Ming-Shu Pang¹, Xia Chen²,³,⁴, Bin Lu²,³, Jian Zhao²,³, Bo-Hua Li²,³,⁴, Yu-Quan Wei¹, Ya-Jun Guo²,³,⁴

¹ State Key Laboratory of Biotherapy and Cancer Center, West China Hospital, West China Medical School, Sichuan University, Chengdu, Sichuan 610041, P. R. China; ² International Joint Cancer Institute, the Second Military Medical University, Shanghai 200433, P. R. China; ³ PLA General Hospital Cancer Center, Chinese PLA Postgraduate Medical School, Beijing 100853, P. R. China; ⁴ National Engineering Research Center of Antibody Medicine, Shanghai 201203, P. R. China

Abstract

Background and Objective: iASPP, an inhibitory member of the apoptosis-stimulating proteins of p53 (ASPP) family, has been found to be up-regulated in various human tumor types. This study was to construct an efficient doxycycline-regulated, lentiviral vector-mediated knockdown system for iASPP that will allow for inducible down-regulation of iASPP gene expression and preliminary functional analysis. Methods: A pair of complementary oligos with hairpin structures targeting the iASPP gene and a negative control were synthesized, then ligated with pLVTHM vector and sequenced. The fragment containing the shRNA cassette was cloned to pLVCT-ITR-KRAB plasmid. The recombinant vectors were co-transfected with viral packaging mix into 293T cells, and viral supernatant was harvested to determine the titre. After treatment with or without doxycycline, HepG2 cells infected with virus were harvested and the expression of iASPP was detected by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. Its effects on tumor growth were characterized using MTS assay, soft agar colony formation, and flow cytometry analysis. Results: The lentiviral vector expressing shRNA that targets to the oncogene iASPP was constructed successfully. HepG2 Infected with the lentivirus expressing shRNA against iASPP inhibited the expression of iASPP in the presence of doxycycline, which resulted in the repression of tumor cell proliferation and anchorage-independent growth potential. Conclusions: The lentiviral vector-mediated tet-on system demonstrates efficient and inducible knockdown of iASPP in hepatocellular carcinoma cells. iASPP gene may be involved in tumorogenesis and progression of human tumors.

Keywords: iASPP, tet-on system, lentivirus, shRNA

The tumor suppressor protein, p53, regulating expressions of several genes that are related to the DNA repair protein, cell cycle arrest, and apoptosis induction, plays a pivotal role in cell proliferation. It has been found that the p53 gene is frequently inactivated in over 50% human malignancies by point mutation or allelic deletion. However, the tumor-suppression function of p53 is lost in most tumors that express wild-type p53. Therefore, understanding how wild-type p53 loses its tumor-suppression function is one of the most important goals in molecular oncology.

The apoptosis-stimulating proteins of p53 (ASPP) family, including ankyrin-repeat-, SH3-domain-, and proline-rich-region-containing protein, comprises three members, ASPP1, ASPP2, and iASPP, and has been identified as specific regulators of p53-, p63-, and p73-mediated apoptosis. Among them, ASPP1 and ASPP2 are common activators of p53 family members, and specifically stimulate the transactivation function of p53 family members by binding to their most conserved and homologous region, through which ASPP1 and ASPP2 promote the apoptosis function of p53 family members.
**Materials and Methods**

**Plasmid**

The plasmids pLVTHM, pLVCT-ITR-KRAB, psPAX2 and pMD2G-VSVG were obtained from Addgene.

**shRNA construction**

Nucleotide sequences of shRNA were selected using BLOCK-iTR RNAi designer (Invitrogen, Carlsbad, CA; https://maidesigner.invitrogen.com/maiepxress/). To generate shRNA-expressing plasmids, the stuffer DNA was removed from pLVTHM by *Mlu* I and *Cla* I digestion and replaced with double-stranded oligos encoding the desired shRNA downstream of the tetO-H1 region. pLVTHM containing shRNA was cut with *Msc* I and *Fsp* I, and then the fragment containing the shRNA cassette was cloned to pLVCT-ITR-KRAB plasmid opened with *Msc* I-*Fsp* I. Target sequences are listed as follows: shiASPP, 5'-CGCGAGACCGTTGAAATCCGCGG-TCAAGAGCGCGAATTCGAAGCTCTAT-3'- (forward), 5'-CGATAGACCGGCCTCTTGAACGCGCAATTCGAAGCTCTAT-3' (reverse); shNon, 5'-CGCGAATTCTCCGGAACGCGACGCGCAATTCGAAGCTCTAT-3' (forward), 5'-CGATATGCAACGCGATGGCAGATTCGGATTTTTTTAT-3' (reverse).

**Generation of lentiviruses**

Lentiviruses were generated by co-transfecting 293T cells with 4 μg of shRNA-encoding plasmid, 3 μg psPAX2, and 1 μg pMD2G-VSVG plasmids using Lipofectamine2000 (Invitrogen). Growth media was exchanged 8 h after transfection and lentivirus-containing supernatant was harvested 48 h and 72 h after transfection. Target cell lines were transduced with lentiviral vectors-mediated inducible RNAi at MOI = 5.

**Cell culture**

293T and HepG2 cell lines were purchased from ATCC and maintained at 37°C in an atmosphere containing 5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen). All shRNA-expressing stable cell lines were cultured in DMEM supplemented with 10% Tet-approved FBS (Clontech, Mountain View, CA). shRNA expression was induced by culturing cells in the presence of the indicated concentrations of doxycycline (Sigma, St. Louis, MO) dissolved in deionized water for the indicated periods of time.

**Quantitative RT-PCR**

Total RNA was isolated from samples using RNeasy columns (Qiagen, Hilden, Germany) and treated with DNase prior to cDNA amplification. cDNA was synthesized using PrimeScript RT reagent Kit (TaKaRa, Otsu, Japan) according to manufacturer’s instructions. mRNA levels were determined using SYBR Premix Ex Taq (TaKaRa) on ABI 7500 Real-Time PCR Systems (Applied Biosystems, Carlsbad, CA). The primers used for detecting iASPP mRNA were as follows: 5'-GCGCGAATTCTCCGGAACGCGACGCGCAATTCGAAGCTCTAT-3' (forward) and 5'-CGATATGCAACGCGATGGCAGATTCGGATTTTTTTAT-3' (reverse). Actin was used as an endogenous control to normalize for differences in the amount of total RNA in each sample.
5'­CGGAGACATCGAAAGACC­3' (forward) and 5'­ACATCTGCTGGAAGTGGAC­3' (reverse). Relative expression of genes was calculated and expressed as 2^{ΔΔCT}, as described previously[28].

**Western blot analysis**

Cells were harvested by scraping into ice-cold PBS and lysed using radioimmunoprecipitation assay (RIPA) buffer (150 mmol/L sodium chloride, 1% triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris-HCl, pH 7.5 and 2 mmol/L EDTA) supplemented with protease inhibitor cocktails (Shenneng Bocai Biotech, Shanghai, China). Following separation by denaturing gel electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes. After probing with anti-iASPP (1:2500, kindly provided by Dr. Xin Lu, Ludwig Institute for Cancer Research Oxford branch, London, United Kingdom) and anti-GAPDH (Kangchen Biotech, Shanghai, China), antigen-antibody complex was visualized by enhanced chemiluminescence reagents (Supersignal, Thermo, Rockford, IL).

**Cell proliferation assay**

A total of approximately 5.0 x 10^3 HCC cells infected with lentivirus were plated in 96-well plates. After 24 h of incubation, cell proliferation was assessed by MTS assay every day for 5 days using CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI) according to the manufacturer’s instruction. All experiments were performed in triplicate. The cell proliferation curve was plotted using the relative absorbance at each time point.

**Soft agar colony assays**

Seventy-two h after lentiviral infection, the cells were mixed with cell culture medium containing 0.5% agar to a final concentration of 0.25%. One mL of the cell suspension was immediately plated in 24-well plates coated with 0.5% agar (1 mL per well) in cell culture medium. The colonies were counted in triplicate 15 days after plating, and the number of colonies per 10^3 cells was calculated.

**Apoptosis analysis**

Detection of early apoptotic cells was performed with the annexin V-propidium iodide (PI) detection kit (Mai Bio, Shanghai, China). Briefly, 1 x 10^5 cells infected with lentivirus were treated with or without cisplatin (0.5 μg/mL) for 24 h, washed with phosphate buffered saline (PBS), incubated in the dark at 4°C with annexin V-FITC and PI for 15 min, and then analyzed by a dual-color flow cytometry (FACS-Calibur, Becton Dickinson, Sunnyvale, CA). Cells that were annexin V-FITC+ (with translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane) and PI− (with intact cellular membrane) were considered as early apoptotic cells.

**Results**

**The construction of lentiviral vector expressing shRNA targeting iASPP**

For the construction of iASPP targeted shRNA-expressing plasmid, the annealed double-stranded oligos encoding the desired shRNA was inserted into the pLVTHM between Mlu I and Cla I restriction sites. The recombinant plasmid was verified by restriction endonuclease and DNA sequencing. Then the fragment containing the shRNA cassette was cut from the correct clone and cloned to pLVCT-TR-KRAB plasmid opened with Msc I-Fsp I, which was designated as pLVCTH-shiASPP and pLVCTH-shNon, respectively (Figure 1).

![Figure 1](image_url)
Inducible down-regulation of iASPP gene expression in HepG2 cells infected with lentivirus

By subjecting HepG2 cells infected with the lentivirus LVCTH-shiASPP to doxycycline treatment, we demonstrated efficient drug-controlled knockdown of both endogenous iASPP mRNA and protein expression as shown in Figure 2A and 2B. In contrast, iASPP expression was not affected by a scramble shRNA-expressing lentivirus LVCTH-shNon.

Down-regulation of iASPP expression inhibits cell proliferation and colony formation in human HCC cells

To investigate whether or not iASPP expression is associated with human hepatocarcinogenesis, we first determined the effects of iASPP expression down-regulated by shRNA on cell proliferation of HepG2 cells. Cell proliferation was evaluated by MTS assay daily for 5 days. As shown in Figure 3A, after treatment with doxycycline, proliferation of HepG2 cells infected with LVCTH-shiASPP was inhibited and proliferation rate decreased by 59.1%.

Next, we explored whether repression of iASPP by shRNA affected colony formation of HCC cells in soft agar assays. HepG2 cells infected with LVCTH-shiASPP or LVCTH-shNon were plated into medium with soft agar at 72 h after transfection. Colony formation was assayed on the 15th day after seeding. As shown in Figure 3B, after the treatment of doxycycline, colony formation was significantly inhibited in the LVCTH-shiASPP infected group but no significant inhibition in untreated or LVCTH-shNon infected group. All these results showed that down-regulation of lentiviral vector-mediated doxycycline-inducible iASPP expression resulted in marked inhibition of HCC cell proliferation in vitro.

Down-regulation of iASPP promotes cell apoptosis in human HCC cells

Cell apoptosis analysis by flow cytometry showed that the apoptotic rate significantly increased by (10.2 ± 2.4)% in LVCTH-shiASPP group compared with LVCTH-shNon group in HepG2 cells infected with lentivirus after treatment with doxycycline in combination with cisplatin treatment (P < 0.05, Figure 4). The results suggested that the expression of iASPP gene in HCC cells may play an inhibitory effect on the cellular response to chemotherapy-induced apoptosis.
The expression of iASPP promotes cell proliferation and anchorage-independent growth in HepG2 cells

HepG2 cells were infected with lentivirus encoding shiASPP or shNon for 72 h, and then were cultured in the cell media with or without doxycyclin (1 μg/mL) as indicated. A, cell proliferation was assessed every day for 5 days using MTS assay according to the manufacturer’s instruction. The cell proliferation curve was plotted using the absorbance results relative to the value on day 1 for each group at each time point. B, soft agar colony assays were used to evaluate the potential of colony formation in vitro. The colonies were counted in triplicate 15 days after plating, and the columns show the mean number of colonies per 10^3 cells calculated (right). The numbers represent the mean of three independent experiments ± standard deviation (SD). Photographs are representative images from each group as indicated under microscope (left). * P < 0.05.

The knockdown of iASPP expression promotes cell apoptosis in human HCC cells treated with chemotherapeutic drug

HepG2 cells were infected with lentivirus for 72 h, then were cultured in the cell media with or without doxycyclin (1 μg/mL), as indicated, for 4 days before treated with 0.5 μg/mL cisplatin for another 24 h. The percentage of apoptotic cells were detected by fluorescence flow cytometry with annexin V and propidium iodide (PI) staining. The columns show the average number of apoptotic cells (right). The experiments were repeated three times. * P < 0.05.
Discussion

iASPP is an inhibitory member of ASPP family, which are unified by their protein structure, containing four ankyrin repeats, an SH3 domain, and a proline-rich region at their C terminus[16]. Recently, it was reported that the expression of iASPP is frequently up-regulated in various human tumors, such as breast carcinoma, acute leukaemia, HCC, and so on. As a negative regulator on p53-activating apoptosis, iASPP may be a good candidate target gene in cancer therapy[16]. As reported previously, inhibition of iASPP mediated by RNA interference technology enhanced the sensitivity of tumor cells to ultraviolet radiation and chemotherapeutic drugs[9,10].

Due to the potentially toxic effects of iASPP gene knockdown on host cells with wild-type p53, it has been difficult to establish a stable cell line with iASPP gene knockdown. In the present study, we developed a lentiviral vector that contained all components of the tet-on system and the iASPP gene targeted shRNA, whose expression was observed with low background activity unless the addition of doxycycline. This system will be particularly suitable for studies that examine the biological function of iASPP and other genes coding for proteins that are toxic to host cells.

Our data in this paper showed that lentiviral vector-mediated shRNA interference targeting iASPP suppressed the cell proliferation and reduced the number of colonies in a colony formation assay, which suggested that iASPP may contribute to the tumorigenesis. As a key inhibitor of p53, iASPP has been demonstrated to inhibit apoptosis mediated by p53 in several different types of cells[9,10]. Then the anti-apoptotic role of iASPP in our study system was examined in HCC cell lines, and the results also showed that the knockdown of iASPP gene increased cell apoptosis slightly in HCC cell line (data not shown), while the number of apoptotic cells significantly increased in the presence of chemotherapeutic drugs. Further studies will be required to dissect the growth inhibition and pro-apoptosis function and mechanism of iASPP knockdown in human cancer cells. Taken together, our results provide a novel lentiviral system for drug-inducible knockdown of iASPP gene, and also suggest that iASPP may be a potential target for gene therapy in the future.

Reference