p53-expressing conditionally replicative adenovirus CNHK500-p53 against hepatocellular carcinoma *in vitro*

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Abstract

AIM: To develop a conditionally replicative gene-viral vector system called CNHK500-p53, which contains dual promoters within the E1 region, and combines the advantages of oncolytic virus and gene therapies for hepatocellular carcinoma (HCC).

METHODS: CNHK500-p53 was constructed by using human telomerase reverse transcriptase (hTERT) promoter to drive adenovirus *E1*a gene and hypoxia response element (HRE) promoter to drive adenovirus *E1*b gene. p53 gene expressing cassette was inserted into the genome of replicative virus. Viral replication experiments, cytopathic effect (CPE) and methyl thiazolyl tetrazolium (MTT) assay were performed to test the selective replication and oncolytic efficacy of CNHK500-p53.

RESULTS: Immunohistochemistry verified that infection with CNHK500-p53 was associated with selective replication of adenovirus and production of p53 protein in telomerase-positive and hypoxia-inducible factor-dependent HCC cells. p53 protein secreted from HepG2, infected with CNHK500-p53 was significantly higher than that infected with nonreplicative adenovirus Ad-p53 *in vitro* (388 ± 34.6 μg/L *vs* 76.3 ± 13.17 μg/L). Viral replication experiments showed that replication of CNHK500-p53 and CNHK500 or WtAd5, was much stronger than that of Ad-p53 in tested HCC cell lines. CPE and MTT assay indicated that CNHK500-p53 selectively replicated in and killed HCC cells while leaving normal cells unaffected.

CONCLUSION: A more efficient gene-viral system is developed by combining selective oncolysis with exogenous expression of p53 against HCC cells.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common solid tumor worldwide, accounting for 500 000 new cases annually. The majority of patients presenting with advanced disease are not candidates for liver transplantation, surgical resection, or regional therapy. In 60% to 80% of patients with HCC, underlying liver cirrhosis and hepatic dysfunction complicate its treatment. Systemic treatments have minimal effects with significant toxicity, and cannot improve patient survival[1]. The search for alternative treatment modalities has revived the concept of using oncolytic viruses to treat cancer[2,3]. In this respect, conditional replicative adenoviruses (CRAds) appear to be attractive anticancer agents that are currently evaluated in clinical trials[4,5]. CRAds exert intrinsic anticancer activity through selective replication and lysis in cancer cells. In addition, release of CRAd progeny by infected tumor cells provides a potential to amplify the oncolytic effect by lateral spread through solid tumors.

Recent studies have shown that telomerase activity may serve as a general marker of cancer cells. Its activity in normal cells is restricted to fetal tissue, whereas it is elevated in tumors[6]. Although some tumors could activate a yet unknown alternative mechanism of telomere extension, the majority (> 85%) of human HCC cells acquire immortality by expressing telomerase reverse transcriptase (hTERT)[7]. It has been shown that hTERT expression is regulated at the transcriptional level, thereby providing a promising tool for tumor-specific gene expression.

Hypoxia occurs in virtually all solid tumors as they outgrow their blood supply. Hypoxia augments cellular levels of hypoxia-inducible factor (HIF), a transcription

factor that regulates target genes through the binding of hypoxia response elements (HRE). Activation of the HIF pathway enables cancer cells to survive and proliferate in a hypoxic environment and contributes to a more aggressive phenotype[8,9]. Therefore, the HIF/HRE system of gene regulation, which is active under hypoxia or as a result of genetic alterations during cell transformation, is particularly attractive to specific target solid tumors.

Besides viral oncolysis, CRAds can be exploited as vectors of gene therapies by advanced virology and viral vector design to enhance their oncolysis. Many malignant neoplasms have lost the function of p53. Although many oncolytic adenoviruses use p53-dependent pathways to cause cell death, several studies have shown that replicating adenoviruses kill cells more rapidly when expressing p53[10,11].

Here we have constructed a novel gene-viral vector system called CNHK500-p53, which uses hTERT promoter to drive adenovirus *E1*a gene and HRE promoter to drive adenovirus *E1*b gene. In addition, human p53 gene was cloned into the downstream of E1A of adenovirus. E1A gene is essential for adenoviral replication, and adenovirus can hardly propagate without it. Telomerase and hypoxia are two important features of human solid tumors. Making use of these two promoters, CNHK500-p53 will replicate only in telomerase positive cancer cells undergoing hypoxia in theory. We tested the replication ability and oncolytic activity of CNHK500-p53 in HCC cell lines *in vitro*.

MATERIALS AND METHODS

Vectors, cell lines and cell culture
pXC1 (wild-type adenovirus plasmid) and pBGHE3 (a plasmid-containing right arm of adenovirus type 5 with deletion of 188-1339 bp sequence.) were purchased from Microbix Biosystems Ltd (Toronto, Canada). pGEM-3ZF and pGEM-3ZF-p53 were purchased from Promega Ltd, USA. Human HCC cell lines HepG2, Hep3B, normal human liver cell line L02, normal human fibroblast cell lines MRC-5 and BJ were purchased from the American Type Culture Collection (Manassas, VA). Human HCC cell lines SMMC-7721, Bel-7402 and wild-type adenovirus 5 (WtAd5) were obtained from Second Military Medical University (Shanghai, China). Human embryonic kidney 293 cell line was obtained from Microbix Biosystems (Toronto, Canada). Hep3B, HepG2, SMMC-7721, Bel-7402 and human embryonic kidney 293 cells were cultured in DMEM (Life Technologies, Rockville, MD). L02 was cultured in RPMI 1640 medium. BJ was cultured in modified Eagle's medium (MEM). All the media were supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies), 4mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin and cultured under a 5% CO_2 atmosphere at 37℃.

Construction of adenovirus vectors
Complete cDNA sequence of p53 gene was amplified by PCR from plasmid pGEM-3ZF-p53 by using the upstream primer VT182 (5'CCG GAA TTC (*EcoRI*) GCC ATG GAG GAG CCG CAG TCA GA3') and downstream primer VT183(5'CGC GGA TCC (*BamHI*)

TTA TCA GTC TGA GTC AGG CCC TTC TG3'). Synthetic DNA sequence was released with endonucleases *EcoRI* and *BamHI* (New England Biolabs, Beverley, MA) and ligated into plasmid pClon15 (made by ourselves, which contains the sequence of mouse cytomegalovirus promoter + multiple clone site + SV40 poly A) to generate pClon15-p53. pClon15-p53 was digested with endonucleases *AgeI* and *NotI* (New England Biolabs), a 1917-bp fragment containing mouse cytomegalovirus promoter + p53 gene + SV40 polyA was excised and inserted into *AgeI* and *NotI* sites of pSG500, which was constructed in our previous study and contained hTERT promoter core sequence with three extra E-boxes downstream and HRE promoter[12]. The plasmid resulting from the insertion of p53 gene cassette into the pSG500 in orthograde orientation was designated as pSG500-p53. pSG500-p53 and pSG500 were transfected by Lipofectamine 2000 (Life Technologies) into 293 cells together with pBHGE3. Viral plaques appeared 9-14 d after cotransfection and were sublimated three times. Recombinant adenoviruses, extracted using QIAamp DNA blood mini kit (Qiagen, Valencia, CA), were verified by PCR and named CNHK500-p53 and CNHK500. Ad-p53 was used as a control, which is a nonreplicative adenovirus vector carrying a SV40 early promoter-driven human p53 expression cassette[13]. A similar procedure was used by replacing the p53 gene with a 1538-bp fragment of the green fluorescent protein (GFP) expression cassette, obtained from plasmid pCA13-GFP (Takara Ltd, Japan), to derive conditionally replicative adenovirus CNHK500-GFP and nonreplicative adenovirus Ad-GFP. The nonreplicative adenovirus Ad-blank was used as a control.

Production and purification of adenovirus
Viruses were purified by CsCl density purification and propagated in 293 cells. After 72 h, the detached cells were harvested by centrifugation at 1000 × *g* for 5 min at 4℃, resuspended in 10 mL cold PBS (free Ca^{2+} and Mg^{2+}), and then lysed with three cycles of freeze and thaw. Lysate was collected by centrifugation at 1500 × *g* for 10 min at 4℃, and the supernatant was placed on a gradient prepared with equal parts of CsCl in PBS and then centrifuged at 15000 × *g* for 2 h at 12℃. The virus band was removed and placed in a preformed CsCl gradient by ultracentrifugation for 18 h and dialyzed into 10 mmol/L Tris-HCl (pH 7.4) containing 10 mmol/L MgCl2 and 10% glycerol. Titers of the purified adenovirus were determined by plaque assays of the tissue culture infectious dose 50 methods and shown as plaque forming unit per milliliter (pfu/mL). All viral preparations were free of endotoxin.

Viral replication assay
Monolayer cells, including logarithmically growing Hep3B, HepG2, SMMC-7721 (10^5 cells/well), and contact-inhibition BJ, L02 (10^6 cells/well) were cultured in six-well dishes overnight and infected with CNHK500-p53, WtAd5, Ad-p53 at a multiplicity of infection (MOI) of 5.0 pfu/cell. Virus inocula were removed after 2 h. The cells then were washed twice with PBS and incubated at 37℃ for 0, 12, 24, 48, or 96 h. Lysates of cells were prepared with three cycles of freeze and thaw. Serial dilutions of

the lysates were titered on human embryonic kidney 293 cells with the tissue culture infectious dose 50 methods, normalized with that at the beginning of infection, and reported as multiples.

Western blot analysis of E1A and E1B protein

HepG2, Hep3B and BJ were seeded in 6-well plates at a density of 5×10^5 cells/well and infected with CNHK500-p53 or wtAd5 at a MOI of 1 after 24 h of incubation. Two days after viral infection, cells were harvested and lysed with M-PER mammalian protein extraction reagent (PI-ERCE, Rockford, IC). Concentration of the extracted protein was measured with a biophotometer (Eppendorf AG, Hamburg, Germany). Total proteins (20 μg) were separated on 10% SDS-polyacrylamide gel, electroblotted onto PROTRAN nitrocellulose transfer membrane (Schleicher & Schuell Inc, Dassel. Germany) and blocked with 5% fat-free milk in Tris-buffered saline (TBS: 10 mMTris, pH 7.5, 0.9% NaCl) containing 0.1% Tween-20 (TBST) at room temperature for 1 h. The membrane was incubated with either rabbit polyclonal antibody against Ad-E1A protein (Santa Cruz Biotechnology) or rat anti-Ad5 E1B 55k monoclonal antibody overnight at 4℃ and repeatedly washed in TBST. After incubation for 1 h with appropriate secondary horseradish peroxidase-conjugated anti-bodies and extensive washing with TBST, immunocomplexes on the membrane were detected with LumiGLOTM reagent and visualized with Kodak BiomaxMR film. To detect the expression of E1A and E1B under hypoxic condition, CNHK500-p53 infected cells were exposed to 0.1% hypoxia for 16 h before harvest.

ELISA determination of p53 gene expression

HepG2 cells were seeded in 24-well plates at a density of 5×10^4 cells/well and cultured for 24 h, followed by infection with CNHK500-p53 and Ad-p53 at a MOI of 0.1. On days 3, 5, 7, and 10 post-infection, the supernatants of cell cultures were collected and assayed for p53 gene expression levels using the ELISA kit of p53 (Chemicon International, Temecula, CA) and the manipulation was done according to the manufacturer's instructions.

Evaluation of oncolytic activity of virus vector

Cytopathic effect (CPE): Hep3B, HepG2, Bel-7402 (2×10^4 Cells/well) and BJ (6×10^4 cells/well) were dispensed in 24-well plates. The culture solution was removed on the second day, and 1 mL serum free DMEM and virus were added to each well. The multiplicity of infection (MOI) of each well was 0.01, 0.1, 1, 10 and 100, respectively. The culture plate was then incubated for 90 min in a 37℃ incubator under the condition of 5% CO_2 in DMEM containing 5% serum.

Methyl thiazolyl tetrazolium (MTT) assay: MTT assay was performed to determine cell viability at various viral MOIs. HepG2, Hep3B and BJ cells were plated at a density of 1×10^4 cells/well in 96-well plates (Falcon) and 24 h later, the cells were infected with CNHK500-p53 at serial MOIs from 0.001 to 100. After 7 d of incubation, cell viability was measured by MTT
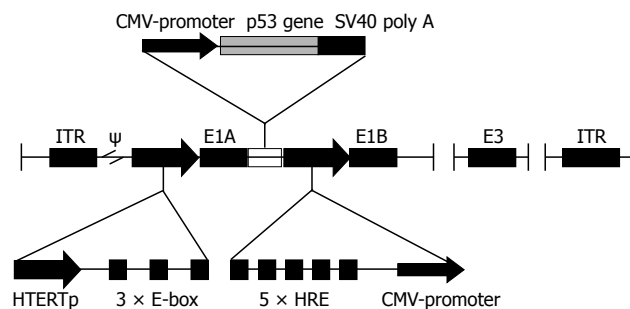
Figure 1 Schematic diagram of the CNHK500-p53 adenoviral construct. A 310-bp fragment of human telomerase reverse transcriptase (hTERT) promoter with three E-boxes (CACGTG) downstream of the core sequence replaced the endogenous E1A promoter (digested with *NotI* and *XhoI*) to control the expression of E1A. A 241-bp fragment of hypoxia response element (HRE) promoter replaced the endogenous E1B promoter to control the expression of E1B. A 1805-bp fragment of transgene expression cassette containing cytomegalovirus (CMV) promoter + p53 + SV40 poly A was inserted into the downstream of E1A (digested with *AgeI* + *NotI*) to generate CNHK500-p53. ITR, inverted terminal repeat; ψ, the adenovirus 5 packing signal.

assay using a non-radioactive cell proliferation kit (Roche Molecular Biochemicals) according to its protocol, and the spectrophotometrical absorbance of samples was measured with a microplate reader model 550 (BIO-RAD Laborato-ries, Tokyo, Japan) at 570 nm with a reference of 655 nm. Percentage of cell survival was calculated using the formula: % cell survival = (OD value of infected cells/OD value of uninfected control cells) × 100%. Eight replicate samples were taken at each MOI and each experiment was repeated at least 3 times. IC50 of CNHK500-p53, CNHK500, and WtAd5 was calculated in HepG2 and BJ 7 d after infection. Statistical analysis was performed using Student's *t* test for differences among groups. $P < 0.05$ was considered statistically significant.

Replication between CNHK500-GFP and Ad-GFP

HepG2, Bel-7402 (1×10^5 cells/well), and BJ (1×10^6 cells/well) were inoculated into six-well plates, respectively. When the cells were confluent, CNHK500-EGFP or Ad-EGFP was added to each well at the MOI of 1 and then washed with PBS 2 h later. Cells were then coated with 1.25% agarose. On days 3, 7 and 10, the cells were observed under a fluorescence microscope and significant changes were photographed. Fluorescence microscopy was performed with routine methods with fluorescence in isothiocyanate (FITC), with the excitement and emission wavelength being 475 nm and 490 nm, respectively.

RESULTS

To make a conditional replicative gene-viral vector, we adopted a design as shown in Figure 1, in which the adenovirus *E1*a gene was placed under the control of hTERT promoter plus three extra E-boxes, and *E1*b gene was controlled by HRE promoter, p53 gene-expressing cassette was inserted between E1A and HRE promoter. CNHK500-p53 was successfully made and verified by PCR. We were able to produce the adenovirus with high titers (2×10^{10} pfu/mL).

A

B

C
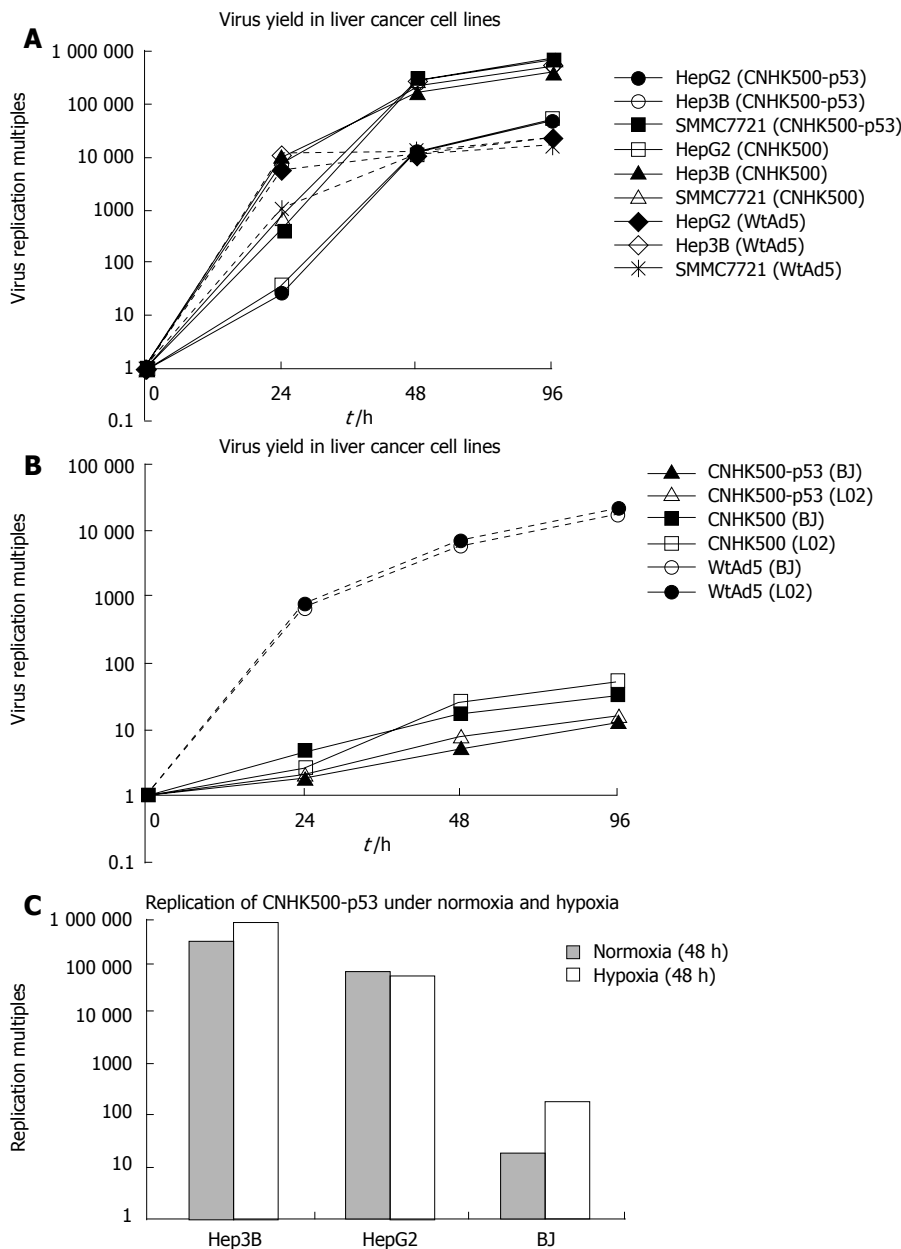
Figure 2 Selective replication of CNHK500-p53 *in vitro*. **A**: Human HCC cell lines HepG2, Hep3B, and SMMC-7721 were infected with CNHK500-p53 at a MOI of 5. Cells and media were harvested, and lysates were prepared from each group at diverse time points 0 h, 24 h, 48 h, and 96 h. Viral titers were measured with the tissue culture infectious dose 50 method, normalized with that at the beginning of infection, and shown as multiples. CNHK500-p53 replicated similarly as CNHK500 and WtAd5 in all of the tested telomerase-positive cancer cells; **B**: Comparison of replication capability of CNHK500-p53, CNHK500 and WtAd5 in telomerase-negative normal cell lines. At diverse time points 0 h, 24 h, 48 h, and 96 h after infection, cells and medium were harvested, and viral titers were measured as described previously. In all of the tested normal cell lines, the replication capability of CNHK500-p53 and CNHK500 was severely attenuated than that of WtAd5; **C**: Forty-eight hours after infection with CNHK500-p53 in HepG2, Hep3B and BJ, CNHK500-p53 showed enhanced replication ability both in HepG2, Hep3B and in BJ under hypoxia condition, but was higher in HepG2, Hep3B than in BJ.

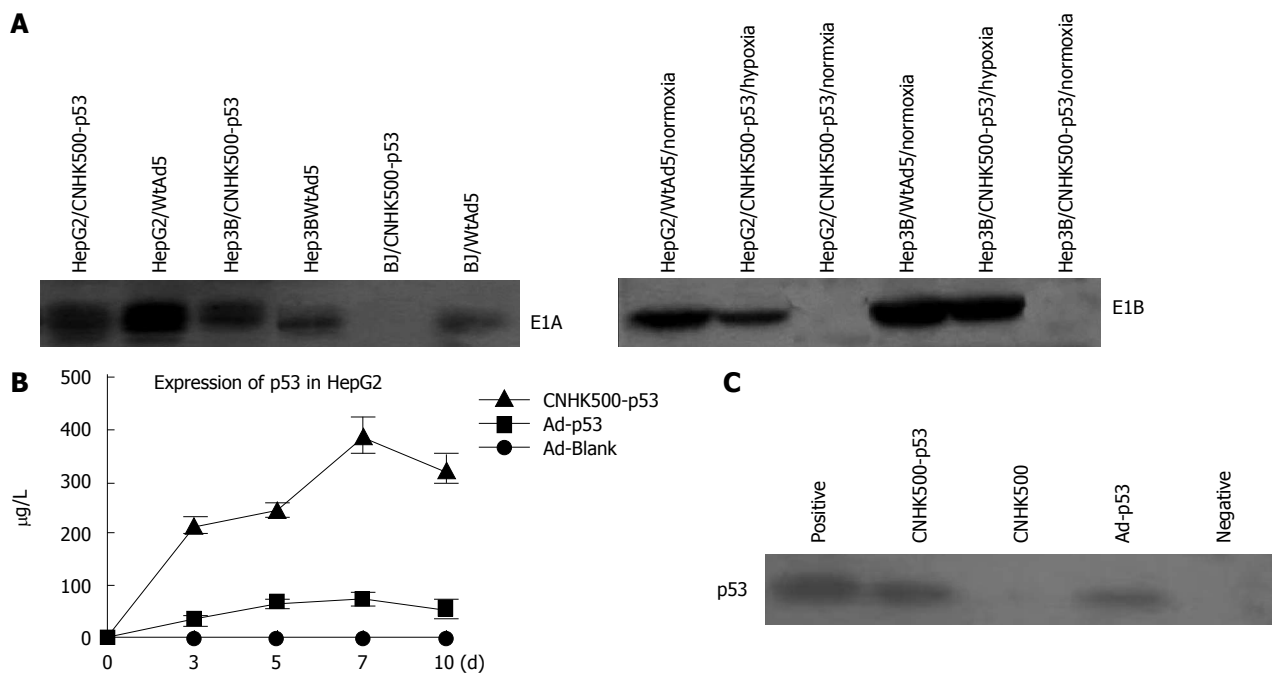### Selective replication of CNHK500-p53

Selective replication of the new recombinant adenovirus CNHK500-p53 was evaluated using telomerase-positive HCC cell lines Hep3B, HepG2, SMMC-7721 and telomerase-negative normal cell lines BJ, L02. In HepG2, Hep3B and SMMC-7721, the replicative multiples increased to 47 230-, 459 837- and 669 251- fold respectively after 96 h of CNHK500-p53 replication, similar to those of CNHK500 and WtAd5 (Figure 2A). However, in normal cell lines BJ, L02, the replicative multiples of CNHK500-p53 and CNHK500 were only 12.8-, 16.3- and 31-, 53- fold at 96 h, and attenuated as much as 1354-, 1325- and 559-, 407.5- fold when compared with WtAd5 (Figure 2B). CNHK500-p53 showed enhanced replication ability both in HepG2, Hep3B and in BJ under hypoxia condition, but was higher in HepG2 and Hep3B cells than in normal BJ cells (Figure 2C). As hTERT promoter regulates *E1*a gene, E1A protein could be detected in telomerase positive hepatocellular

cells HepG2 and Hep3B, but not in telomerase negative normal cells BJ. Under normoxic condition, E1B protein could hardly be detected due to poor activity of HRE promoter. When HCC cells were exposed to hypoxia, E1B protein was induced as a result of increased activity of HRE promoter (Figure 3A).

Expression of p53 produced by CNHK500-p53

To verify that the p53 expressed by CNHK500-p53 could secrete efficiently into the media, the conditioned media from 5×10^4 HepG2 cells infected with CNHK500-p53 or Ad-p53 at a MOI of 0.1 were collected and analyzed for the presence of p53 protein by ELISA. The quantity of p53 expressed by CNHK500-p53 and Ad-p53 on d 3, 5, 7, and 10 post-infection is shown in Figure 3B, indicating that p53 expression in CNHK500-p53 was 5.1 times more than that in Ad-p53 in HepG2 on d 7. Western blot analysis revealed a clear band of M_r 53 000 in the conditioned media after HepG2 cells were infected

A

Lane labels (left blot): HepG2/CNHK500-p53, HepG2/WtAd5, Hep3B/CNHK500-p53, Hep3BWtAd5, BJ/CNHK500-p53, BJ/WtAd5 — E1A

Lane labels (right blot): HepG2/WtAd5/normoxia, HepG2/CNHK500-p53/hypoxia, HepG2/CNHK500-p53/normxia, Hep3B/WtAd5/normoxia, Hep3B/CNHK500-p53/hypoxia, Hep3B/CNHK500-p53/normoxia — E1B

B

Expression of p53 in HepG2

■ CNHK500-p53
■ Ad-p53
● Ad-Blank

C

Lane labels: Positive, CNHK500-p53, CNHK500, Ad-p53, Negative — p53

Figure 3 A: E1A and E1B expression identified by Western blot demonstrating that all HCC cells infected with CNHK500-p53 or WtAd5 were positive for E1A expression, however, normal BJ cells were negative for E1A expression when they were infected with CNHK500-p53, and positive only when they were infected with WtAd5, while E1B of CNHK500-p53 was only expressed under hypoxia condition in HepG2 and Hep3B, and expressed both under normal and hypoxia condition with WtAd5; **B**: ELISA assay showing that p53 protein secreted from a HepG2, infected with CNHK500-p53, was significantly higher than that infected with nonreplicative adenovirus Ad-p53 and Ad-Blank *in vitro* ($P < 0.05$); **C**: Western blot showing enhanced p53 expression in HepG2 infected with CNHK500-p53.
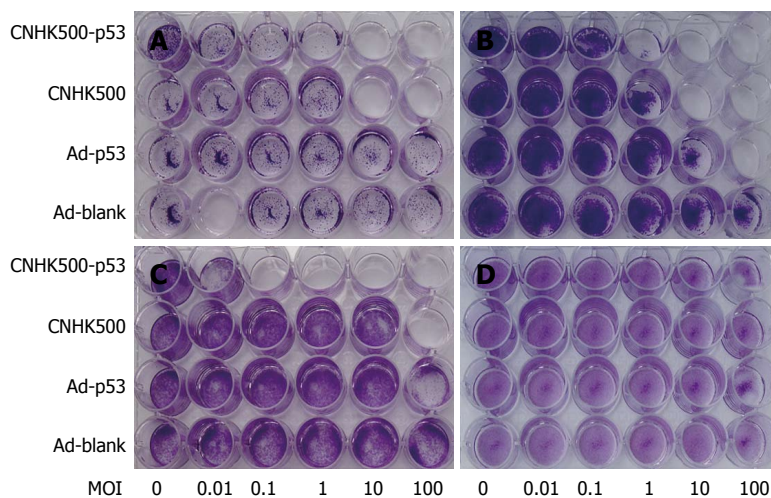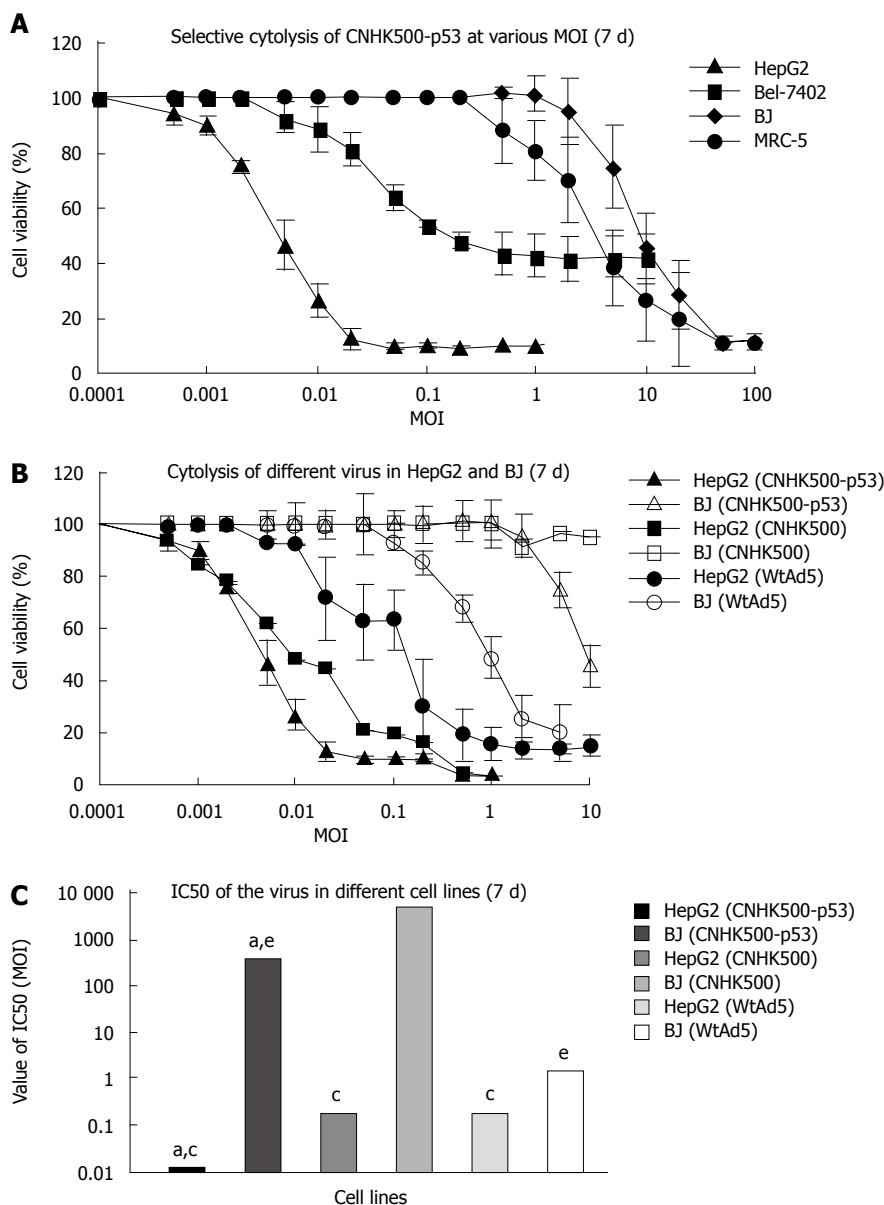

Figure 4 Cytopathic effects associated with CNHK500-p53, CNHK500, Ad-p53, and Ad-Blank infection in HCC cell lines HepG2 (**A**), Hep3B (**B**), Bel-7402 (**C**), and normal cell line BJ (**D**). Seven days after virus infection, all the cells were stained with crystal violet and photographed. Comparison with other viruses, CNHK500-p53 showed the strongest selective cytolysis against HCC cell lines. Infection with CNHK500-p53 at MOI of 0.1-1 was sufficient to induce its lytic effects in Bel-7402, HepG2, and Hep3B, although the sensitivity varied among the cell types. In contrast, no apparent cytopathic effects were observed in BJ even at MOI of 100 after CNHK500-p53 infection, which was similar to other viruses.

with CNHK500-p53 or Ad-p53 at a MOI of 1 on d 3, suggesting that the protein in the media was p53 protein (Figure 3C).

Selective cytolysis of CNHK500-p53

CPE was used to determine whether CNHK500-p53 infection induces selective cell lysis. HCC cell lines (HepG2, Hep3B, and Bel-7402) and the normal cell line (BJ) were infected with CNHK500-p53, CNHK500, Ad-p53, and Ad-Blank at various MOIs, fixed in methanol and stained with crystal violet 7 d after infection to visualize viable cells. CNHK500-p53 showed the strongest selective cytolysis effect among the viruses and killed all cancer cell lines in a dose-dependent fashion (Figure 4). Infection with CNHK500-p53 at a MOI of 0.1-1 was sufficient

to induce its lytic effects, although the sensitivity varied among the cell types. In contrast, no apparent CPE was observed in BJ cells 7 d after CNHK500-p53 infection. Cytotoxicity of CNHK500-p53 was also assessed by MTT assay. HepG2, Hep3B and MRC-5, BJ cells were infected with CNHK500-p53 at various viral MOIs. As shown in Figure 5A, CNHK500-p53 induced more rapid cell death in HCC cells than in normal BJ cells 7 d after infection. Furthermore CNHK500-p53 showed a different ability to kill HepG2 and Bel-7402 with the IC_{50} being 0.012 and 0.28 of MOI. On the contrary, the IC_{50} in normal BJ cells was as high as 352.1 of MOI, suggesting that more than 29 341- or 1257-fold of CNHK500-p53 was needed to kill half BJ compared with HepG2 and Bel-7402. Comparison with CNHK500 and WAd5 infection, CNHK500-p53 not

A Selective cytolysis of CNHK500-p53 at various MOI (7 d)

- ▲ HepG2
- ■ Bel-7402
- ◆ BJ
- ● MRC-5

B Cytolysis of different virus in HepG2 and BJ (7 d)

- ▲ HepG2 (CNHK500-p53)
- △ BJ (CNHK500-p53)
- ■ HepG2 (CNHK500)
- □ BJ (CNHK500)
- ● HepG2 (WtAd5)
- ○ BJ (WtAd5)

C IC50 of the virus in different cell lines (7 d)

- ■ HepG2 (CNHK500-p53)
- ■ BJ (CNHK500-p53)
- ■ HepG2 (CNHK500)
- ■ BJ (CNHK500)
- □ HepG2 (WtAd5)
- □ BJ (WtAd5)

Figure 5 Oncolytic efficacy induced by CNHK500-p53 infection evaluated by MTT assay. Statistical analysis was performed using Student's *t* test for differences among groups. Statistical significance was defined as *P* < 0.05. **A**: HCC cell lines HepG2, Bel-7402 and normal cell lines BJ, MRC-5 were infected at different MOI of 0.001-100 pfu/cell of CNHK500-p53. Cell viability was measured by MTT assay 7 d after infection. CNHK500-p53 induced a more powerful oncolysis in HCC cell lines than in normal cell lines (*P* < 0.05); **B**: Seven days after infection with virus, CNHK500-p53 not only showed more powerful cytolysis than CNHK500 and WtAd5 in HepG2, but also less profound cytolysis than WtAd5 in BJ (*P* < 0.05); **C**: IC50 of CNHK500-p53, CNHk500, and WtAd5 demonstrated significant differences among groups with [a,c,e]*P* < 0.05.

only demonstrated more apparent cytolysis against HepG2 than CNHK500 and WtAd5 but less profound cytotoxicity than WAd5 against BJ (Figure 5B and C).

### *Replication of gene-viral system and expression of GFP gene*

To determine the selective replication of CNHK500-GFP, HCC cell lines including HepG2, Bel-7402 and normal cell line BJ were observed under fluorescent microscope at different time points after being infected with CNHK500-GFP and Ad-GFP. By conducting GFP expression, CNHK500-GFP demonstrated a greater replicative ability than Ad-GFP in HCC cell lines, with no significant difference in normal cell line. After 3, 7 and 10 d of infection with CNHK500-GFP and Ad-GFP, only a few scattered cells emitted fluorescence in normal cell line. However, in HCC cell lines, CPE such as deformation and aggregation appeared 3 d after infection, and the fluorescence emission spread from a single cell to many cells within a large area 7 d after infection. Bel-7402 cells were particularly sensitive to CNHK500-GFP, many cells died with GFP degradation and fluorescence extinction 10 d after infection (Figure 6), showing selective replication of CNHK500-derivative and correct insertion of GFP gene.

DISCUSSION

Besides conventional approaches, CRAds specifically killing tumor cells while sparing normal cells have been introduced as new agents for cancer therapy in the past decade[14,15]. The efficacy of CRAds against cancer, including HCC, is however, limited by several factors, mainly including tumor specificity and oncolysis[16].

With the advancement in molecular biology and understanding of the function of viral genes, it has become possible to genetically re-engineer viruses to make them selectively kill tumor cells over normal tissue. At present, tumor specificity has been achieved in oncolytic adenoviruses mainly (1) by altering viral genes that attenuate replication in normal tissue but not in tumor cells such as ONYX015 with E1B 55KD deleted[15],
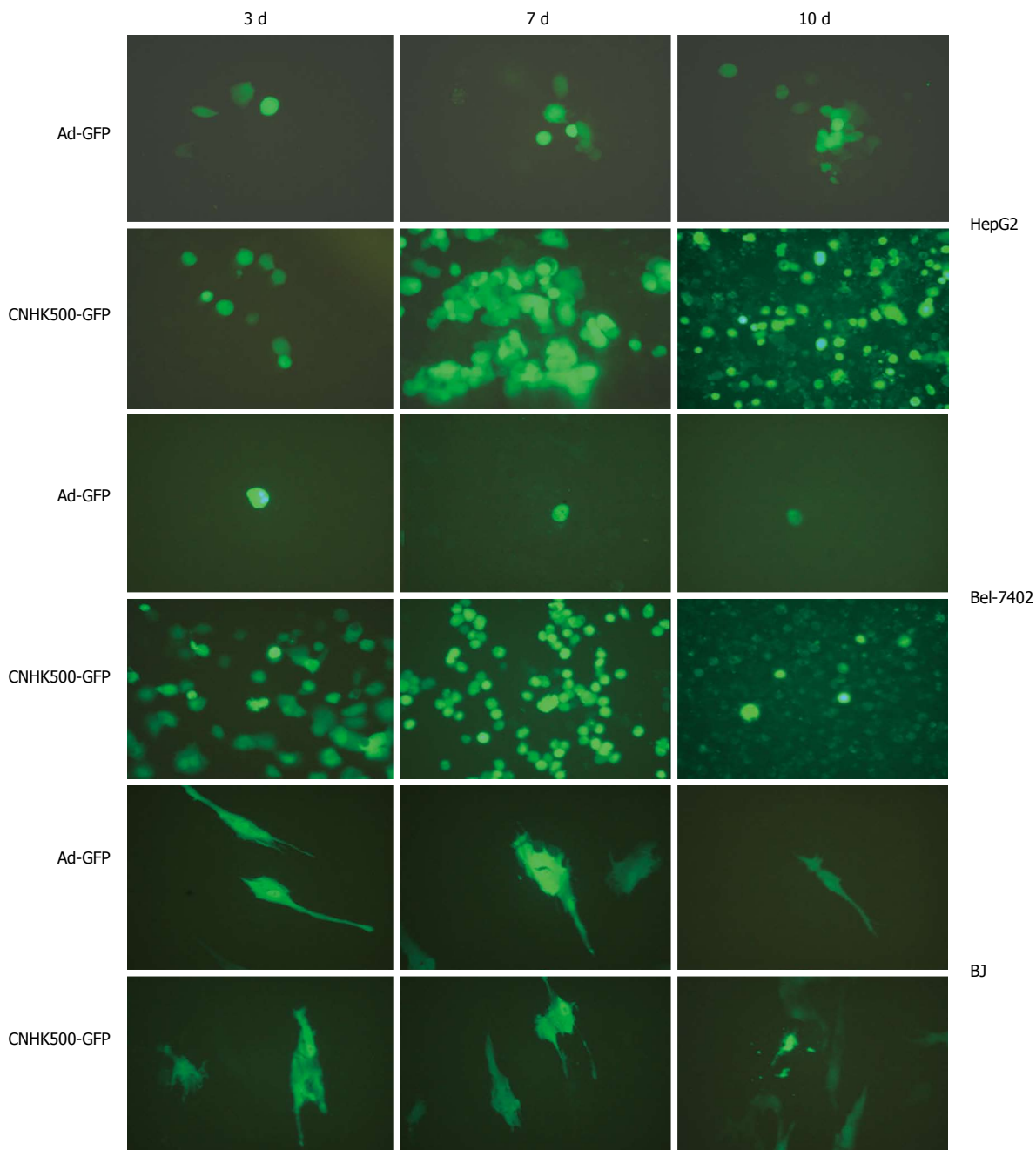
Figure 6 Fluorescent photos of HCC cell lines HepG2, Bel-7402 and normal cell line BJ infected with replicative virus CNHK500-GFP and nonreplicative virus Ad-GFP (200 magnifications).

(2) by placing viral genes that initiate viral replication under the control of promoter sequences that are active in tumor cells such as using AFP promoter to restrict viral replication in AFP-producing HCC[17,18], and (3) by modifing viral coat proteins that function in host cell infection.

Studies showed that adenovirus-induced oncolysis can benefit from combined gene therapy[11,19]. Cancer gene therapy typically involves delivery of tumor suppressor, enzyme/pro-drug gene, cytotoxic/pro-apoptotic gene, immunogene, anti-angiogenic gene directly into tumor cells[20]. After more than two decades of study, the tumor suppressor p53 gene is widely regarded as the "genome guardian." It has been estimated that at least half of all human malignancies, including HCC, are related to a mutation of the p53 gene[21].

In our previous study, we constructed a CRAd containing dual promoters within the E1 region, designated as CNHK500[12], in which the viral *E1*a gene is regulated by hTERT promoter and *E1*b gene by HRE promoter. Since telomerase is highly activated in most malignant tumors but inactive in normal somatic cells[22], CNHK500 can selectively replicate in telomerase-positive cancer cells. At the same time, its replication ability is further attenuated in normal cells as hypoxia microenvironment seldom exists among normal tissues. However, it may propagate very well in solid tumors because HRE promoter is transcriptally activated due to hypoxia, a unique feature

of human solid tumors[23]. A further study suggested that CNHK500 is tumor-selective *in vitro* and *vivo* when compared with CNHK300 and WtAd5[24]. To enhance the oncolytic potency of CNHK500, we constructed a new CRAd CNHK500-p53 by combining oncolytic virotherapy with gene therapy, which expresses functional p53 during viral replication in HCC cells as verified by PCR, Western blot, and ELISA assay. We evaluated the efficacy of CNHK500 and CNHK500-p53 against human HCC cell lines *in vitro*.

We found that exogenous expression of p53 by CNHK500-p53 could lead to enhanced oncolytic potency compared with its parent CNHK500 on most HCC cell lines, while the ability of selective replication was not significantly different between them. The superior efficacy of CNHK500-p53 was independent of the cellular p53 genetic background. It was reported that the expressed p53 gene appears to exert its anticancer activities by one or more of the following mechanisms: (1) simultaneously triggering apoptotic pathways in tumor cells by a transcription-dependent mechanism in cell nuclei[25,26] and by a transcription-independent mechanism in mitochondria[27] and Golgi apparatus[28]; (2) activating immune response factors such as natural killer cells[29] to exert "bystander effects"; (3) inhibiting DNA repair and antiapoptosis functions in tumor cells[30]; (4) down-regulating the expression of multidrug resistance genes[31] to revert the resistance of tumor cells against radio- and chemotherapies as well as the vascular endothelial growth factor gene[32] to block the blood supply to tumor tissue and matrix metalloproteinase[33] to suppress tumor cell adhesion, infiltration, and metastasis; (5) blocking the transcription of survival signals in tumor cells[34,35], thus inhibiting the growth of tumor cells in any stage of the cell cycle. Hence, dysfunctional p53 of HCC cells might delay conditionally replicative adenovirus-induced cell death, thus limiting conditionally replicative adenovirus efficacy.

In conclusion, CNHK500-p53 has the selective replicative ability in HCC cell lines and a higher oncolytic efficacy than its parent CNHK500 *in vitro*. The enhanced oncolytic efficacy may be related to the expression of p53 gene carried by CNHK500-p53. Further experiments are needed to warrant its potential therapeutic effect against HCC.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most frequent and lethal malignancies worldwide especially in China. According to the reports of American cancer society (ACS) in 2005, the 5-year survival rate of HCC is only about 8.3%. Therefore, development of effective alternative approaches is needed. Replication-selective virus-mediated gene therapy holds great promise for the treatment of cancer, including HCC.

Research frontiers

So far, 656 cancer gene therapy clinical protocols are in different phase of evaluation worldwide. Unfortunately, successful delivery and targeted expression of therapeutic gene into cancer cells still are very difficult to achieve. The main problem is due to the very low *in vivo* transduction and expression efficacy of available vectors. In this respect, conditionally replicative adenoviruses appear as attractive vectors. Combination of oncolytic virotherapy and gene therapy may be an effective alternative approach against cancer.

Innovations and breakthroughs

In order to construct tumor-specific conditionally replicative adenoviruses, adenovirus E1A gene expression was driven by the hTERT promoter and E1B gene by the hypoxia response (HRE) promoter through genetic engineering, which assures adenovirus replication only in telomerase-positive cells exposed to hypoxia. Besides, human p53 gene was cloned into the downstream of E1A of adenovirus to enhance oncolysis. It was different from other study in adenovirus reconstruction methods. Meanwhile, the recombinant adenovirus was verified by PCR assay, and showed tumor-specific replication and enhanced oncolysis against HCC cell lines *in vitro*.

Applications

The results of our present study demonstrate that p53-expressing conditionally replicative adenovirus has the selectively repictive ability in HCC cell lines and a higher oncolytic efficacy than non-p53-expressing conditionally replicative adenovirus *in vitro*. The enhanced oncolytic efficacy may be related to the expression of p53 gene. Further experiments are needed to warrant its potential therapeutic effect against HCC.

Terminology

CRAds: conditional replicative adenoviruses, recombinant adenoviruses modified to selectively replicate in cancer cells; Oncolytic virotherapy: one of the cancer therapies by obtaining a virus that replicates and preferentially kills cancer cells, leaving the surrounding normal tissues relatively intact; Cancer gene therapy: Cancer gene therapy can be defined as transfer of nucleic acids into tumor or normal cells to eradicate or reduce tumor mass by direct killing of cells, immunomodulation or correction of genetic errors, and reversion of malignant status. Initially started with lots of optimism and enthusiasm, cancer gene therapy has shown limited success in treatment of patients.

Peer review

This is an interesting manuscript. In this manuscript, Hing-Chuan Zhao *et al* take advantage of the selective expression of hTERT and HIF in tumoral cells to express under their respective promoters E1A and E1B assuring adenovirus replication only in telomerase cancerous cells exposed to hypoxia. The data are clearly presented. They show the selective replication and expression of adenoviral proteins as well as the selective cytopathic effect of their adenovirus.

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