Original Article

Inhibition of survivin expression to induce the apoptosis of hepatocarcinoma cells by adenovirus-mediated siRNA

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Summary **In order to provide more efficient transduction of plasmid siRNA into target cells, develop more susceptible transduction into cancer cell types, and more easily explore application in animal experiments, we examined development of an adenoviral vector-mediated siRNA expression system and inhibition of survivin gene expression to induce the growth and apoptosis of hepatocarcinoma cells. A system of adenoviral vector-mediated siRNA expression was constructed for the survivin gene. Survivin gene expression in HepG2 cells infected with recombinant adenovirus was detected by Western blot and RT-PCR, and apoptotic cells were investigated by FAC. Western blot analysis showed that the infection of adenovirus-mediated siRNA against survivin efficiently inhibited the expression of survivin in hepatocarcinoma cells with an inhibitory rate of 66.32%. Semi-quantitative RT-PCR showed that survivin gene mRNA transcription was reduced by nearly 72.34% with a peak at 72 h. The number of apoptotic cells increased. In conclusion, results demonstrated that this adenovirus-mediated siRNA system could serve as a useful tool for both basic research on the analysis of gene function and cancer therapy applications.**

Keywords: Adenovirus-mediated siRNA, Survivin, Hepatocarcinoma cells, Apoptosis

Introduction

The role of survivin, an inhibitor of apoptosis proteins (IAP), has gradually become apparent since 1997, when it was found to be specifically expressed in cancer cells (*1*). Using small interference RNA (siRNA) to inhibit survivin gene expression and induce the apoptosis of cancer cells is a new form of cancer gene therapy (*2-4*). However, most previous siRNA vectors were plasmids. The use of plasmid siRNA vectors was limited by its disadvantages such as limited transfection cell type, low and instable transfection efficiency, and difficult use in animal experiments. Virus vectors function *via* a recombinant virus that can infect cells. Since virus vectors can highly and stably infect various types of cells, they are more convenient for animal experiments. Therefore, virus vectors have recently enjoyed wide use, and much research describes siRNA vectors such as the adenovirus (Ad) and adeno-associated virus (AAV) (*5-9*). The present study, based on the siRNA construct (*4*), describes use of the adenoviral vector to construct an adenoviral vector-mediated siRNA (Ad-siRNA) vector of the survivin gene and discusses the recombinant virus's role in infecting HepG2 tumor cells, inhibition of survivin gene expression, and induction of apoptosis of tumor cells *in vitro*. This paper identifies the function of the Ad-siRNA vector system and establishes a foundation for animal experiments *in vivo* with AdsiRNA-survivin.

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Materials and Methods

siRNA sequences and plasmids

Survivin siRNA expression vectors were constructed as described previously by the authors (*4*). siRNA

sequences of survivin genes are as follows: Survivin-1 siRNA, 5'-TCGAG GAC CAC CGC ATC TCT ACA TTC TTCG GAA TGT AGA GAT GCG GTG GTC TTTT T-3' (sense) and 5'-CTAGA AAAA GAC CAC CGC ATC TCT ACA TTC CGAA GAA TGT AGA GAT GCG GTG GTC C-3' (antisense); Survivin-2 siRNA, 5'-TCGAG CTG AGA ACG AGC CAG ACT TG TTAGTACT CAA GTC TGG CTC GTT CTC AG TTTTT-3' (sense) and 5'-CTAGA AAAA CTG AGA ACG AGC CAG ACT TG AGTACTAA CAA GTC TGG CTC GTT CTC AGC-3'(antisense); Survivin-3 siRNA, 5'-TCGAG AAA GCA TTC GTC CGG TTG C GAGTACTG GCA ACC GGA CGA ATG TTT TTTTT-3' (sense) and 5'-CTAGA AAAA AAA GCA TTC GTC CGG TTG C CAGTACTC GCA ACC GGA CGA ATG CTT T C-3' (antisense). Transfection of plasmid DNA was carried out by lipofection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. The plasmids pAdTrack, pAdEasy-1, and *E. coli* BJ5183 were provided by Dr. Tong-chuan He of the University of Chicago Medical Center.

Cell culture

AD293 and HepG2 cell lines were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin at 37°C in a 5% CO_2 atmosphere. The cell growth state was observed periodically and cells were digested with 0.25% PentaZyme every 2-3 days to incubate generations.

Recombinant adenovirus

Plasmids of siRNA-pshRNA-survivin3 (constructed by the authors to efficiently inhibit survivin gene expression) and pTZU6+1 (as a control with no siRNA fragments inserted) were digested with Kpn I and Hind III and then separately cloned into the shuttle vector pAdTrack. For the generation of recombinant adenovirus, each plasmid was transfected into 293 cells by lipofection using Lipofectamine 2000 reagent (Invitrogen). The new constructs were named pAd-siRNA-survivin and pAd-control.

The plasmids pAd-siRNA-survivin and pAd-control were digested with Pme I to linearized them and then separately co-transfected with adenovirus frame vector pAdEasy-1 into *E. coli* BJ5183. Restriction analysis verified that the recombinant plasmids were pAd-Easy-siRNA-survivin and pAd-Easy-U6-control. The resulting plasmids were linearized with Pme I again and transfected into the adenovirus package 293 cell line for 7-13 days, virus formation was determined by GFP gene expression of the pAdTrack plasmid, and then the virus AdsiRNA-survivin and AdU6-control were harvested by alternately, freezing and thawing cells and then storing them at -70°C.

When AD293 cells were 80% confluent with fresh cells 24 h before infection, 5×10^5 cells were separately infected with 100 μL each of AdsiRNA-survivin and AdU6-control adenovirus original suspensions. The adenovirus original suspension was diluted to 1 mL with serum-free RPMI-1640 and replaced with fresh medium containing 10% FBS after 6 h. Virus formation was determined by GFP expression under fluorescence microscopy, and AD293 cells were harvested when virus was obviously formed or two-third of cells were floating. The cells were centrifuged at 800 rpm for 5 min, resuspended in 1 mL of PBS, and stored at -70°C.

Adenoviral vectors were purified by cesium chloride ultracentrifugation (*10*). Purified virus was dialyzed in phosphate-buffered saline (PBS) with 10% glycerol and stored at -70°C until use. Virus titer was determined according to the manufacturer's instructions (Stratagene).

Cell transfection

HepG2 cells were grown in RPMI-1640 with 10% FBS supplemented with 100 U/mL penicillin/streptomycin. The day before virus infection, HepG2 cells were plated in each well of 6-well plates. The following day, cells were incubated with recombinant virus (AdsiRNA-survivin and AdU6-control) at MOI of 10-20 at 37°C. After adsorption for 1-2 h, 2 mL of fresh growth medium was added and cells were placed in the incubator for an additional 2-3 days.

Immunoblot analysis

HepG2 cells were infected with AdsiRNA-survivin or AdU6-control virus suspension of the same titer (2.0×10^8 pfu/mL), harvested after 72 h, washed twice in PBS, and lysed in lysis buffer containing 1 mM PMSF (Merck, USA). The total protein was extracted and quantified with an ultraviolet spectrophotometer. Forty μg of the protein were subjected to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a NC membrane that was subsequently blocked for 2 h at room temperature. The membrane was first probed with rabbit anti-survivin (Santa Cruz, USA) (1:1,000 dilution) at 4°C overnight and then washed with PBST. The membrane was incubated with secondary antibody sheep anti-rabbit IgG-HRP (1:4,000 dilution) at room temperature for 1.5 h and visualized with an enhanced chemiluminescence (ECL) kit (Pierce, USA). The images were analyzed with the ImageMaster TotalLab System. The luminous intensity of the control protein served the reference to compare the relative proteins and ascertain weak intensity.

RT-PCR analysis

RT-PCR was performed to determine the relative quantity of survivin gene in hepatocarcinoma cells.

Cells were infected for 72 h, digested with 0.25% PentaZyme, and harvested. After cells were washed twice with PBS, total RNA was extracted according to instructions. There were two pairs of primers, the first of which was the GAPDH gene primer as the endogenous control: 5'-GGAAGGTGAAGGTCGGAGTC-3' and 5'-GACCACCTGGTGCTCAGTGT-3'; the second was the survivin gene primer: 5'-ATAGTCGACATGGGT GCCCCGACGTTG-3' and 5'-CTCGGATCCTCAAT CCATGGCAGCCAG-3'; procedures were performed according to the kit instructions. The quantity of added plates was expressed by density ratios to GAPDH. The degree of survivin mRNA silence was determined based on the quantity of plates.

TUNEL assays

Infected cells were grown for 72 h on coverslips, and TdT-mediated UTP end labeling (TUNEL) analysis was performed using the *in situ* Cell Death Detection kit (Roche Molecular Biochemicals). Apoptotic strand breaks were visualized by transmission epifluorescence microscopy.

Flow cytometric analysis (FCA) of cell apoptosis

HepG2 cells were seeded at a density of 1×10^6 cells/well into 6-well plates in which slides were previously laid, incubated for 24 h, and then separately infected with AdsiRNA-survivin (2.0×10^8 pfu/mL) and AdU6-control (2.0×10^8 pfu/mL) for approximately 72 h (according to the expression of fluorescence). The cells were harvested after absorbing all of the medium and the depositions were sufficiently mixed. The cells were kept in 75% glacial ethanol overnight and then analyzed by FCA.

Statistic analysis

The results of the tests were analyzed using SPSS10.0 software, and the difference between the two groups was compared using a student test. *P* values of less than 0.05 were considered to be significant.

Results

Construction of pAdsiRNA virus-vector

The pAdsiRNA vector was constructed on the basis of the constructs pshRNA-survivin3 plasmid and adenovirus vector pAdTrack (Figure 1). Results of restriction digest analysis of recombinant pAdsiRNA-survivin are shown in Figure 2, indicating that the U6+1 promoter and siRNA inverted repeat sequence fragment had been cloned into the vector pAdTrack. The linearized recombinant and pAdEasy-1 were transfected into *E. coli* BJ5183 to construct the recombinant pAdsiRNA-survivin. The pAdsiRNA-survivin was digested with Pac I, and the results of electrophoresis indicated that the recombinant yielded a 4.5 kb or 3.0 kb DNA fragment (Figure 3). The protocol to construct the plasmid pAdTrack-U6 was identical, but the digested vector pTZU6+1 had no siRNA fragment inserted, only including the U6+1 promoter.

The plasmids Ad-vector pAdsiRNA-survivin and pAdTrack-U6 were separately digested with Pac I and transfected into AD293 cells. The cells were harvested when virus spots and some floating cells were visible microscopically, and original virus suspensions were prepared. A large number of AD293 cells was infected with the original virus suspensions in order to provide a large amount of adenovirus. The virus was collected in accordance with GFP expression, and the process repeatedly yielded abundant recombinant virus *via* infected cells.

When the recombinant virus had infected AD293 cells for 36 h, almost 100% of cells displayed GFP expression under fluorescence microscopy. The titer of the recombinant virus was: AdsiRNA-survivin, 2.1

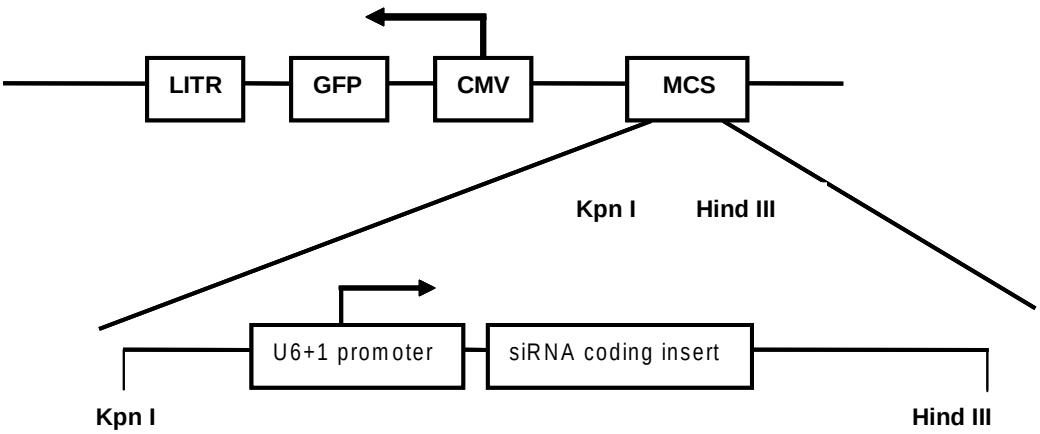

Figure 1. Schematic outline of recombinant adenovirus expression siRNA targeting survivin. The plasmid psiRNA-survivin was cut by Hind III and Kpn I and then inserted into plasmid pAdTrack to construct the vector pAdsiRNA-survivin.

× 10^8 pfu/mL; AdU6-control, 1.8 × 10^8 pfu/mL. After cesium chloride gradient centrifugation, the titer was: AdsiRNA-survivin, 2.4 × 10^9 pfu/mL; AdU6-control, 2.1 × 10^9 pfu/mL.

AdsiRNA efficiently inhibited survivin gene expression

Western blot analysis was performed to examine the levels of survivin expression after infection of the various vectors and to reveal the knockdown efficiency. The luminous intensity of survivin bands for cells infected with AdsiRNA-survivin was significantly weaker than that for cells that were not infected or were infected with AdU6-control, while the level of GAPDH expression as a control was not influenced (Figure 4). There was a significant difference ($P < 0.01$) in survivin expression between cells infected with AdsiRNA-survivin and those not infected, while no significant difference ($P > 0.05$) was found between uninfected cells and those infected with AdU6-control. These results indicate that the AdsiRNA construct efficiently inhibited survivin gene expression. Analysis with the ImageMaster TotalLab System showed that the intensities of survivin bands for AdsiRNA-survivin and AdU6-control were 33.68% and 99.20%, respectively ($P < 0.01$), in comparison to those for bands with no virus present.

As shown in Figure 5, semi-quantitative RT-PCR results for survivin and GADPH were similar to the results of Western blot analysis. The intensity of survivin amplification for cells infected with AdsiRNA-survivin was significantly weaker than that for uninfected cells or cells infected with AdU6-control ($P < 0.01$), while amplification of GAPDH as a control was not influenced. Analysis with the ImageMaster TotalLab System showed that the intensities of survivin mRNA for AdsiRNA-survivin and AdU6-control were 27.96% and 100%, respectively ($P < 0.01$), in comparison to the intensity with no virus present.

Apoptosis of cells infected by AdsiRNA

In order to analyze the cellular consequences of siRNA-mediated silencing of the survivin gene, TUNEL analyses were performed. As shown in Figure 6A and B, the uninfected HepG2 cells or cells infected with AdU6-control retained their normal shape and were closely packed; a small number of stained nuclei and lightly stained cytoplasms were observed. In contrast, many TUNEL-positive cells were observed among cells infected with AdsiRNA-survivin; HepG2 cell nuclei were stained brownish red and displayed karyopyknosis.

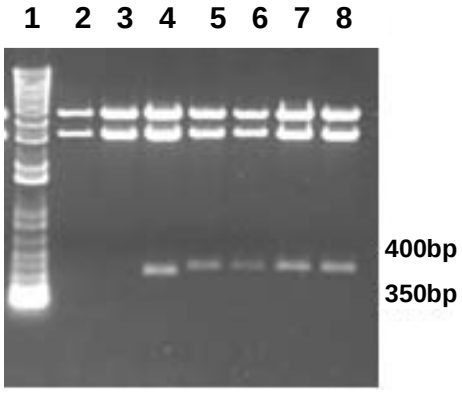

Figure 2. Restriction analysis of recombinant plasmids pAdTrack-survivin and pAdTrack-U6. Lane 1, Marker; lanes 2 and 3, pAdTrack + Kpn I + Hind III; lane 4, pAdTrack-U6 + Kpn I + Hind III; lanes 5-8, pAdTrack-survivin + Kpn I + Hind III.

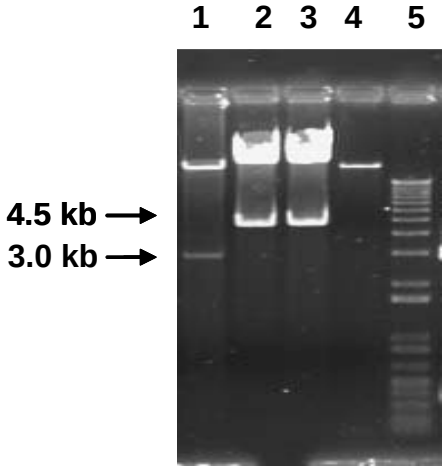

Figure 3. Restriction results for recombinant pAdsiRNA-survivin and pAd-U6-control. Lanes 1 and 2, pAdsiRNA-survivin + Pac I; lane 3, Ad-U6-control + Pac I; lane 4, pAdEasy-1 + Pac I, lane 5, Marker.

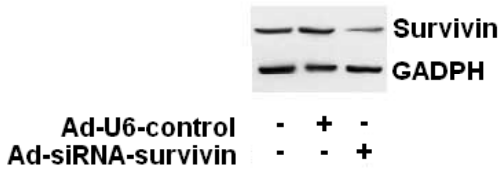

Figure 4. Western blot analysis of survivin expression in infected HepG2 cells.

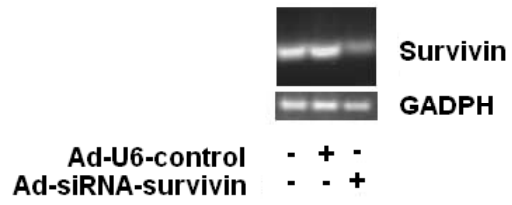

Figure 5. Semi-quantitative RT-PCR of survivin and GAPDH in infected HepG2 cells.

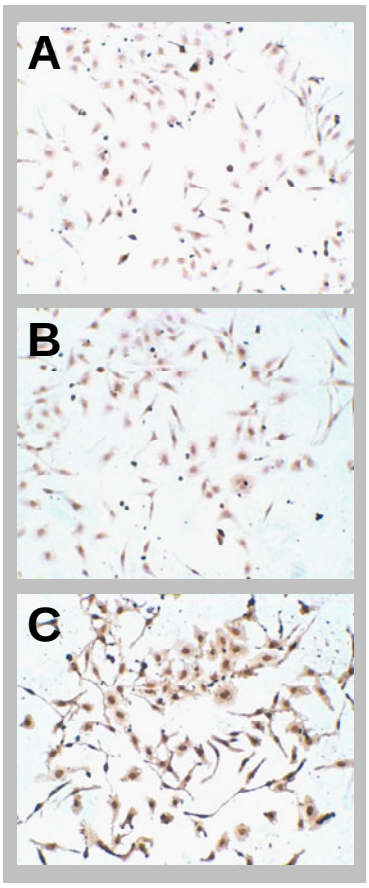

Figure 6. Detection of apoptotic cells in HepG2 as determined by TUNEL after infection of AdsiRNA. A, HepG2 cells; B, HepG2 cells + AdU6-control; C, HepG2 cells + AdsiRNA-survivin.

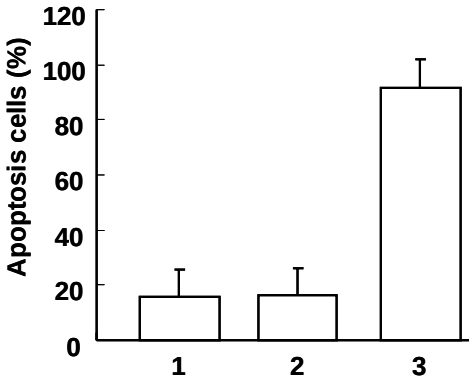

Figure 7. Detection of the effects of AdsiRNA-survivin on the apoptosis of HepG2 cells by TUNEL assay. 1, HepG2 cells; 2, HepG2 cells + AdU6-control; 3, HepG2 cells + AdsiRNA-survivin. The two stars indicate $P < 0.01$ in comparison to the control.

The fact that the number of apoptotic cells increased dramatically indicates that siRNA directed against survivin increases the spontaneous apoptosis rate of HepG2 cells. The number of apoptotic cells determined by TUNEL after infection with AdsiRNA is shown in Figure 7.

Figure 8 shows flow cytometrical detection of apoptotic cells. An obvious hypodiploid peak in the G0-G1 period, suggesting cell apoptosis, was observed in HepG2 cells infected with AdsiRNA-survivin, while the corresponding peaks in uninfected cells or cells infected with AdU6-control were significantly weaker. The apoptosis ratios for uninfected cells, cells infected with AdU6-control, and cells infected with AdsiRNA-survivin were 17.19%, 18.05%, and 87.68%, respectively ($P < 0.01$).

Discussion

RNA interference (RNAi) is an emerging method that provides a new platform for genome study and has been used in several fields, including gene therapy for viral disease and tumors (*11-13*). A number of studies have indicated that plasmid siRNA vectors have many shortcomings when used in experiments to inhibit target gene expression such as instable transfection efficiency and limited transfection cell types; moreover, their failure to act on target cells in animal experiments resulted in their limited use to experiments *in vivo* (*6*). The current study established an adenoviral vector-mediated siRNA expression system because the adenovirus has a wide-ranging host choice, including stationary and division-phase cells, and has a high and stable infection efficiency, so it can easily be used in animal experiments. Hepatocarcinoma cells were infected with AdsiRNA in order to examine the inhibition efficiency and influence of AdsiRNA on survivin gene expression in tumor cells. The results proved that this AdsiRNA system efficiently inhibited target gene expression at both gene and protein levels and can be used in other gene research. This study selected the IAP factor survivin as the target gene and found inhibition of survivin gene expression and obvious promotion of hepatocarcinoma cell apoptosis. Additionally, these results revealed survivin's anti-

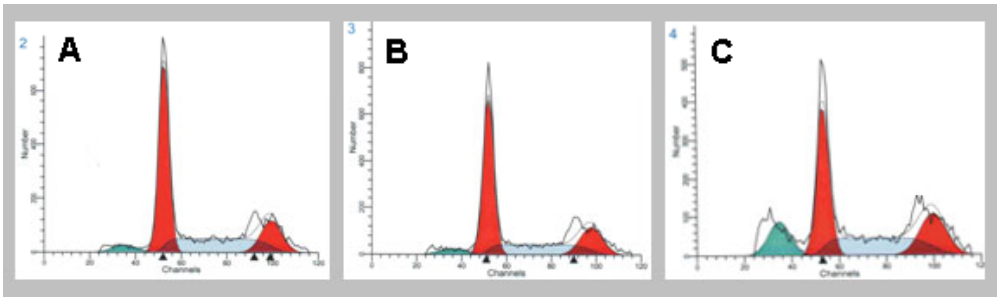

Figure 8. Detection of the effects of AdsiRNA-survivin on the apoptosis of HepG2 cells by cytometry. A, HepG2 cells; B, HepG2 cells + AdU6-control; C, HepG2 cells + AdsiRNA-survivin.

apoptotic role, which is consistent with previous studies (*3*). The current adenovirus siRNA system may provide technology to support future animal experiments with survivin, a popular and important tumor inhibitor, and at the same time offer an experimental base for research on the survivin gene signaling pathway.

This adenovirus siRNA vector system may be used in gene function research not only of the survivin gene but also of other target genes. An AdsiRNA-RhoA vector was also constructed and the same specific and efficient inhibition effect on the RhoA gene was achieved (paper in publication), indicating that this adenovirus siRNA vector may be widely used in siRNA construction and target gene research. The adenovirus siRNA vector system may provide a new investigation platform for gene function study, gene therapy for viral disease and tumor diseases, and drug screening tests, offering technological support for siRNA methods of studying target genes *via* animal experiments.

Acknowledgements

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