Brief Report

Adenovirus-mediated siRNA inhibited survivin gene expression induces tumor cell apoptosis in nude mice

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Summary	**In order to research the survivin gene's action on an animal tumor, we used an adenovirus-mediated siRNA system to inhibit the expression of survivin in an animal model of hepatocarcinoma using nude mice. We constructed a hepatocarcinoma model with nude mice using the hepatocarcinoma cell line HepG2 and divided the mice into four groups depending on the injection dose of AdsiRNA-survivin. We injected the constructed survivin-siRNA adenovirus into tumor-bearing nude mice, observed tumor growth, and determined the tumor growth curve. We then detected tumor cell apoptosis using a TUNEL kit that can assay sliced DNA in tumor cells. The growth of tumors injected with a high or low dose of AdsiRNA-survivin was obviously inhibited, and this level of inhibition was positively correlated with the injected dose of adenovirus. Results of the TUNEL test showed that many of the apoptotic cells were brown in color with concentrated nuclei and an irregular cell shape for both the high and low injection doses. The number of apoptotic cells decreased by group in the order of the high dose group, the low dose group, the AdsiRNA-U6 group, and the PBS group. In conclusion, our results demonstrated that an adenovirus-mediated siRNA system can be used for animal experiments *in vivo*. AdsiRNA-survivin efficiently inhibited tumor growth and induced tumor cell apoptosis, and it did so in a dose-dependent manner.**

1. Introduction

Tumors are a serious threat to human health. Every year in China, over 2.5 million people are diagnosed with cancer and more than 150 billion RMB is spent to treat over 6 million patients. At present, treating tumors with gene therapy is a focus of biomedicine, as inhibitors of programmed cell death (apoptosis) aberrantly prolonging cell viability may contribute to cancer by facilitating the insurgence of mutations and by promoting resistance to therapy.

Survivin is a recently discovered inhibitor of apoptosis (IAP) (*1*) that obviously counteracts apoptosis and is highly conserved between different species. The full length of the survivin gene is 14.7 kb, coding a 16.5

ku cytoplasmic protein including 142 amino acids. In order to research survivin's action on mammal tumors by RNAi *in vivo*, an animal model of hepatocarcinoma was created using nude mice and a recombinant adenovirus named AdsiRNA-survivin (adenoviral vector-mediated siRNA of the survivin gene) was constructed with highly efficient infectivity and tumor inhibition (*2*). Survivin gene expression was detected in hepatocarcinoma cells and the growth of transplanted human hepatocarcinoma was obviously inhibited by AdsiRNA-survivin *in vivo*, as indicated here, since many of the human hepatocarcinoma cells were apoptotic.

2. Materials and Methods

2.1. *Plasmids, adenovirus, cell lines, and nude mice*

The plasmids pAdTrack and pAdEasy-1 were provided by Dr. Tong-Chuan He of the University of Chicago Medical Center, USA. The adenovirus

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vector pAdsiRNA-survivin and pAdU6-control and the recombinant adenovirus AdsiRNA-survivin and AdU6-control were constructed at this lab; adenovirus titers were 2.4×10^9 pfu/mL for AdsiRNA-survivin and 2.1×10^9 pfu/mL for AdU6-control. The HepG2 cell line was purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Science (SIBCB) and kept at this lab. Four-week-old BALB/c nude mice were purchased from SIBCB; half were male and half were female.

2.2. *Construction of an animal model of hepatocarcinoma using nude mice*

The HepG2 cell line was maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin. Cells were incubated at 37°C with CO_2 saturation of 5%. Cells were harvested until 70-80% confluence was observed. They were then washed with PBS, suspended, and prepared in a 2×10^6/mL single-cell suspension. Mice were given a hypodermic injection of 2 mL suspension in the back and the growth of transplanted human hepatocarcinoma was observed.

2.3. *Animal experiments*

The recombinant adenovirus was used in 2 working doses: 2×10^9 pfu/mL and 2×10^8 pfu/mL. The control groups were an AdU6-control (2×10^9 pfu/mL) and a PBS control. The BALB/c nude mice were randomly divided into four groups when the tumor diameter reached 1 cm with 3 mice per group. These four groups were identified as the high survivin dose group, the low survivin dose group, the AdU6-control group, and the PBS control group. One hundred µL of adenovirus were injected straight into tumor tissue on days 1, 2, 10, 20, and 30, respectively, and mice were sacrificed 5 days after the injection was complete.

2.4. *Depiction of the tumor growth curve*

Tumor size was measured 2 days before injection and 5 days afterwards. Tumor volume was determined by the formula tumor length × tumor width^2 × 0.4 (tumor length, the tumor's longest diameter; tumor width, the shortest diameter perpendicular to the tumor length). A tumor growth curve was determined according to the tumor volume.

2.5. *Detection of DNA segments in hepatocarcinoma cells*

DNA segments in hepatocarcinoma cells were detected by TUNEL assay, which was performed in accordance with the in situ Cell Death Detection Kit (Roche Molecular Biochemicals) instruction manual.

2.6. *Statistical analysis*

The results of the tests were analyzed using SPSS10.0 software. The difference between groups was compared using ANOVA. A *p* value of less than 0.05 was considered to be significant.

3. Results

3.1. *Observation of AdsiRNA-survivin therapeutic effectiveness against transplanted human hepatocarcinoma in nude mice*

The growth of tumors in nude mice injected with HepG2 cells was inhibited with the greater duration of injection with AdsiRNA-survivin for both the high and low dose groups, but inhibition was more apparent in the high dose group. The tumor volume was larger in the PBS and AdU6-control groups. The tumor volume in the four groups was as follows: high dose of AdsiRNA-survivin > low dose of AdsiRNA-survivin > AdU6-control > PBS control (Figure 1). The tumor growth curve (Figure 2) depicted by the tumor volume indicated that the AdsiRNA-survivin adenovirus obviously inhibited transplanted human hepatocarcinoma in nude mice. This effect was directly proportionate to the injected dose and adenovirus concentration, but tumor growth in the AdU6-control group was similar to that in the PBS control group.

3.2. *Detection of apoptosis in animal tumor cells by TUNEL assay*

Results of the TUNEL assay are shown in Figure 3. Most nuclei in the mouse tumor cells infected with either a high or low dose of AdsiRNA-survivin were brown in color and had a high nucleo-cytoplasmic concentration and irregular cell shape. A great deal of apoptotic cells appeared in these two groups but few appeared in the AdU6-control and PBS control groups. The apoptotic cell count of the four groups was as follows: high dose of AdsiRNA-survivin > low dose of AdsiRNA-survivin > AdU6-control > PBS control.

Six fields of vision (400× high power filed, 100 cells per filed) under a high-powered microscope were selected to count the number of cells that were apoptosis-positive, and results were calculated as the mean ± standard deviation. Numbers of apoptotic human hepatocarcinoma cells are shown in Table 1. Results indicated that the number of apoptotic cells in the groups with a high or

**Table 1.** The number of apoptotic cells in three groups

Group	Apoptotic cell count
High dose of AdsiRNA-survivin	34.67 ± 2.70
Low dose of AdsiRNA-survivin	27.41 ± 2.42
AdU6-control	3.57 ± 1.61
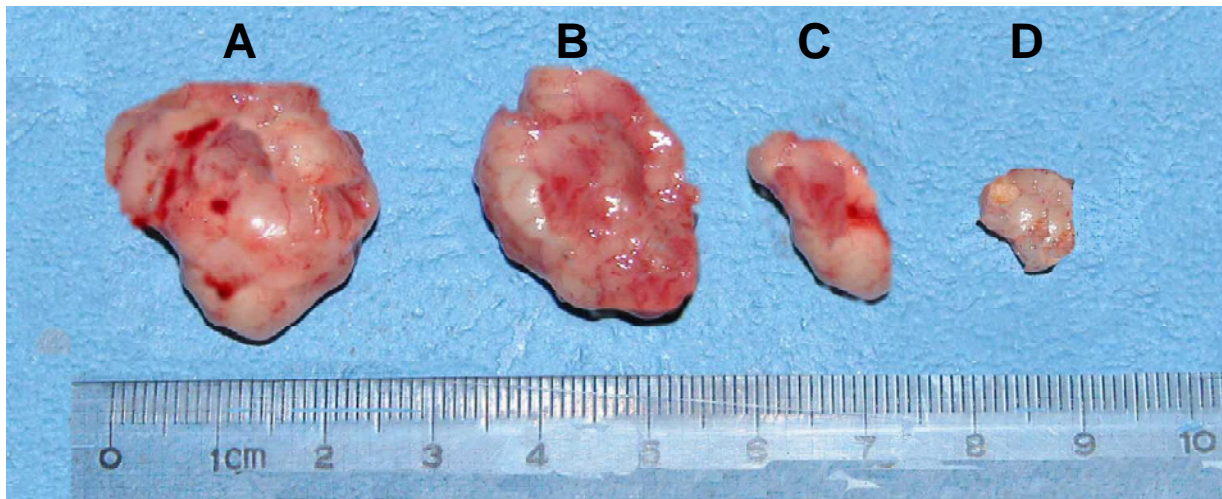
Figure 1. The size of tumors in mice after treatment with recombinant adenovirus-siRNA. A) PBS control, B) AdU6-control, C) low dose of AdsiRNA-survivin, D) high dose of AdsiRNA-survivin.
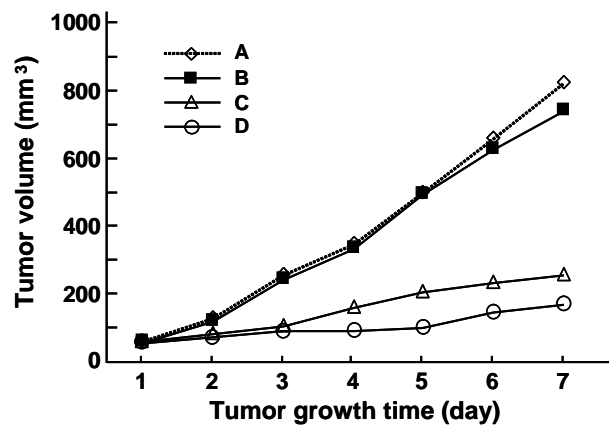
Figure 2. Growth curve of tumors. A) PBS control, B) AdU6-control, C) low dose of AdsiRNA-survivin, D) high dose of AdsiRNA-survivin.
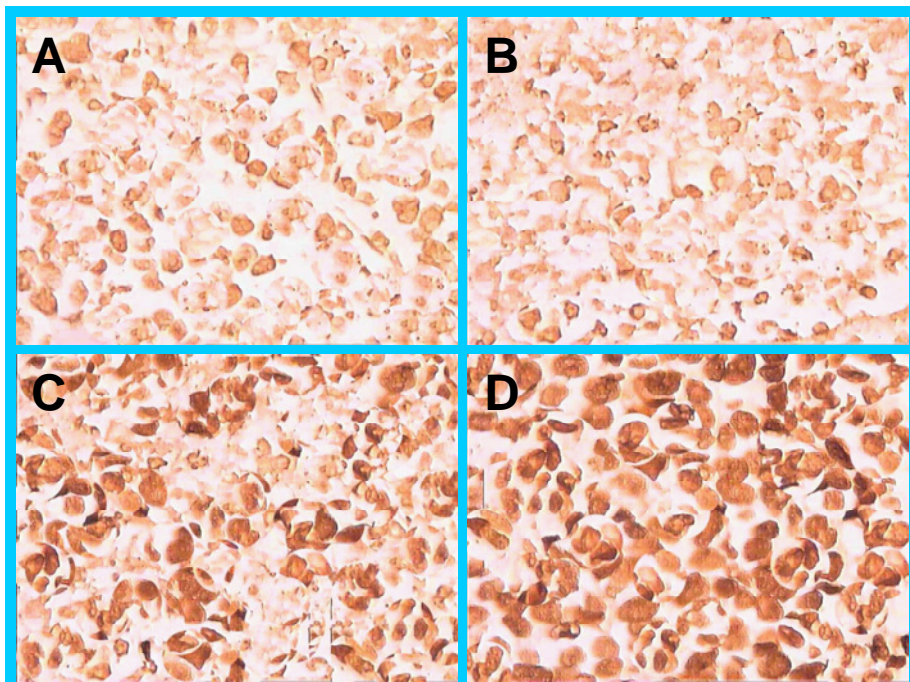
Figure 3. Detection of apoptotic cells in tumors by TUNEL after infection with AdsiRNA. A) PBS control, B) AdU6-control, C) low dose of AdsiRNA-survivin, D) high dose of AdsiRNA-survivin.

low dose of AdsiRNA-survivin differed significantly from that in the AdU6-control group ($p < 0.01$).

4. Discussion

In recent years, the new technology of RNAi has been used to inhibit target genes and research their effects and mechanism of action, but most RNAi experiments have used a plasmid-mediated siRNA vector. As the plasmid-mediated siRNA vector has some limitations such as its problematic use in animal trials and difficulty in demonstrating its effect on the target gene, a new RNAi system was established using adenovirus as the siRNA vector. In this study, the construction of the AdsiRNA-survivin was based on a plasmid-mediated siRNA-survivin vector harvested by cell infection *in vitro*. The AdsiRNA-survivin adenovirus is highly infective to various cell lines and host types and can effectively inhibit survivin expression in hepatocarcinoma cells *in vitro* (*2*). In the current study, it was used in an animal trial *in vivo* to prove that a viral-mediated siRNA vector could be easily used in animal experiments.

In this study, IAP survivin was selected as the target gene. Survivin obviously counteracted apoptosis in tumor cells. A feature readily distinguishing it from other IAPs is that it is not expressed in normal tissue (except for thymus gland) but is commonly expressed in human tumor cell lines. Tamm *et al*. (*3*) investigated the antiapoptotic mechanism of survivin and as its expression in 60 human tumor cell lines; they indicated that survivin was expressed in all 60 cancer cell lines analyzed, with the highest levels in breast and lung cancer and lowest levels in renal cancer. As demonstrated by immuno-histochemistry, Western blot, and in situ hybrid, survivin is prominently expressed *in vivo* in all the most common human cancers of the lung, colon, pancreas, prostate, and breast but is not detected in normal cells, suggesting that survivin is a potential new target for apoptosis-based therapy for cancer (*4*). Studies have reported that cells infected by various means such as plasmid siRNA, manual synthetic antisense oligonucleotides, antisense RNA, and negative dominance mutants displayed inhibited survivin expression and apoptosis (*5-7*). In the current study, an animal model of hepatocarcinoma using an immunodeficient mouse was created and adenovirus-mediated RNAi technology was used in a mammal; results demonstrated that the growth of the transplanted hepatocarcinoma was obviously inhibited and that apoptosis was induced in a number of cells by inhibiting survivin expression. These results represent credible trial experience with hepatocarcinoma gene therapy and establish a trial basis for subsequent research into survivin's inhibition mechanism and its role in the apoptosis signaling pathway.

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