A dual-regulated oncolytic adenovirus carrying TAp63 gene exerts potent antitumor effect on colorectal cancer cells

Qifeng Luo1, Heying Liu2, Zhenyu Zhang1, Shiva Basnet1, Zhenling Dai1, Shuping Li3, Yuxiang Wang4, Bin Xu4, Haiyan Ge1,5

Departments of 1Gastrointestinal Surgery, 3Research Administration, Shanghai East Hospital, School of Medicine, Tongji University, Shanghai 200120, P. R. China; 2Department of Respiratory Medicine, The 85th Hospital of Chinese People’s Liberation Army, Shanghai 200052, P. R. China; 4Department of General Surgery, Shanghai Tenth People’s Hospital, School of Medicine, Tongji University, Shanghai 200072, P. R. China; 5Department of General Surgery, Pinghu Second People’s Hospital, 314200, Zhejiang Province, P. R. China

Received February 20, 2017; Accepted May 18, 2017; Epub June 15, 2017; Published June 30, 2017

Abstract: The purpose of this study is to evaluate possible antitumor activity of a dual-regulated oncolytic adenovirus carrying the TAp63 gene on colorectal cancer. The recombinant virus Ad-survivin-ZD55-TAp63 was constructed by inserting the TAp63 gene into the dual-regulated pshuttle-survivin-ZD55 vector. RT-PCR and western blot assays were used to verify the recombinant virus Ad-survivin-ZD55-TAp63. Crystal violet staining was carried out to detect the cytopathic effect of Ad-survivin-ZD55-TAp63 in human colorectal cancer cell line HCT-116 and normal liver cell line L-02. MTT and cell apoptosis assays were applied to explore the biological functions of Ad-survivin-ZD55-TAp63 within HCT116 cells. To further identify the antitumor effects of Ad-survivin-ZD55-TAp63 on HCT116 xenograft in BALB/C nude mice, tumor volumes were calculated and tumor tissues from the xenograft models were examined by TUNEL assays. The results showed that Ad-survivin-ZD55-TAp63 was successfully constructed, and could selectively replicate in HCT116 cells without significant toxicity to L-02 cells. Furthermore, Ad-survivin-ZD55-TAp63 dose- and time-dependently inhibited cell proliferation and induced cell apoptosis in vitro. In HCT116 xenograft models, intratumoral injection of Ad-survivin-ZD55-TAp63 significantly suppressed tumor growth and caused tumor cell apoptosis. Therefore, these results suggest that the recombinant virus Ad-survivin-ZD55-TAp63 exhibits specific antitumor effects, and may be used in the future for the treatment of colorectal cancer.

Keywords: Colorectal cancer, oncolytic adenovirus, survivin, TAp63

Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed malignancies and leading causes of death in the world [1, 2]. The standard therapy for advanced CRC includes surgery followed by chemotherapy or other effective therapeutic regimens, but the five year relative survival rate for patients remains below 30% [2]. In addition, cancer recurrence and resistance to molecular-targeted therapies are not uncommon among CRC patients, and no ideal therapeutic options are currently available for these patients. Therefore, alternative therapeutic approaches are urgently needed to eradicate this aggressive disease.

Viral therapy is an attractive platform for the development of novel cancer treatments [3]. For example, a modified herpes simplex virus type 1 (termed talimogene laherparepvec, T-VEC) is the first oncolytic virus therapy to be approved by the US Food and Drug Administration (FDA) for the treatment of melanoma, and demonstrated therapeutic benefit in a phase III clinical trial [4]. The adenovirus (Ad) can be genetically modified to confer greater infectivity and a greater replication capacity in tumor cells than in non-tumor cells. For example, cell- or tissue-specific promoters can control the expression of early adenoviral genes essential for replication of the genetically modified recombinant virus [5, 6]. Survivin is fre-
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The gene therapy for the treatment of malignancies has progressed significantly in recent years [10, 11]. However, gene therapy with replication-competent Ad might be a useful strategy to improve the prognosis of patients. This strategy was also called Cancer-Targeting Gene-Virotherapy (CTGVT) strategy by Xinyuan Liu [12], which combined the advantages of both gene therapy and virotherapy by inserting an antitumor gene into a genetically modified adenoviral vector. TAp63 gene, the p53 family member, is a critical tumor suppressor gene in many malignancies [13, 14]. Further investigation found that TAp63 was a robust mediator of senescence that inhibited tumorigenesis in vivo [14]. In addition, published studies have also demonstrated that over-expression of TAp63 could inhibit cell migration and invasion in human colorectal cancer cell line HT-29 and SW-620 [15]. Thus, TAp63 is an effective tumor inhibition factor and could be a potential candidate gene for CTGVT.

In this study, the pZD55 plasmid was constructed in our laboratory with the E1B55-kDa encoding gene deleted to restrict the viral replication only to tumor cells [16]. We replaced the E1A promoter of pZD55 plasmid with the tumor-specific survivin promoter. So, the replication of the new vector, pshuttle-survivin-ZD55, is controlled by both genetic deletion of E1B55kDa and survivin promoter-controlled E1A. The antitumor gene TAp63 was then inserted into the double regulated new vector to generate the recombinant virus Ad-survivin-ZD55-TAp63. The results of this study showed that Ad-survivin-ZD55-TAp63 had selectively targeted CRC cells, inhibited cell growth and induced cell apoptosis in vitro. We also found Ad-survivin-ZD55-TAp63 effectively suppressed xenografted CRC cancer in nude mice. Taken together, the recombinant virus Ad-survivin-ZD55-TAp63 might be considered as a novel therapeutic agent for colorectal cancers.

Materials and methods

Cell lines and cell culture

Human colorectal cancer cell line HCT-116 and normal liver cell line L-02 were purchased from Chinese Academy of Sciences (Shanghai, China). The HEK293 cells were obtained from American Type Culture Collection (ATCC, Manassas, USA). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Enpromise, Hangzhou, China). Cells were incubated at 37°C in a humidified chamber supplemented with 5% CO2.

Expression of survivin in HCT116 and L-02 cells

The total RNAs were extracted using TRizol Reagent kit (Invitrogen, USA) according to the manufacturer’s instruction. Reverse transcription polymerase chain reaction (RT-PCR) was used to detect survivin expression in HCT116 and L-02 cells. The primers were designed according to the survivin cDNA sequence and GAPDH was taken as the internal control: for survivin: forward primer 5’-CGGAATTCACCATGGGTGCCCCGACG-3’, and reverse primer 5’-GAAGATCTTCAATCCATGGCAGCCAG-3’; for GAPDH: forward primer 5’-AAGGTCCGAGTCAA-CGGATT-3’, and reverse primer 5’-CTGGGAGA-TGGTATGGGATT-3’. cDNA was synthesized according to the manufacturer’s instruction, with the following amplification condition: 50°C for 30 min; 30 cycles of 94°C for 2 min, 94°C for 30 sec, 60°C for 30 sec, 72°C for 60 sec; and 72°C for 2 min.

Construction of the recombinant virus

The pCDNA3-TAp63 plasmid, pZD55 plasmid and pshuttle-survivin-ZD55 plasmid were constructed in our laboratory. The pCDNA3-TAp63 was digested with the Bg1 II restriction enzyme to obtain an expression cassette. Then, this cassette was sub-cloned into pshuttle-survivin-ZD55 plasmid to produce a pshuttle-survivin-ZD55-TAp63 plasmid. All plasmid constructs were confirmed by restriction enzyme digestion, PCR and DNA sequencing. Ad-survivin-ZD55-TAp63, Ad-survivin-ZD55 and Ad-ZD55 were produced in HEK293 cells by homologous
recombination between pshuttle-survivin-ZD55-TAp63, pshuttle-survivin-ZD55 or pZD55 and the adenovirus packaging plasmid pAdEasy, respectively. The recombinant Ads were then purified using conventional cesium chloride gradient centrifugation, and virus titers were measured by a plaque assay on HEK293 cells.

Western blot analysis assay

Cells were harvested and re-suspended in a lysis buffer. After centrifugation at 12,000 g for 15 min at 4°C, the supernatant containing cytoplasmic fractions was used to test for E1A and TAp63 proteins. Protein samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Be-yotime, China). Immune complexes were formed by incubation of membranes with primary antibody (E1A and TAp63 from Abcam, USA) overnight at 4°C. Blots were then washed and incubated for 1 h with secondary antibodies. After washing with PBST, immunoreactive protein bands were detected using the Odyssey scanning system (LI-COR, Lincoln, NE, USA).

Cytopathic effect assay

6×10⁴ cells (HCT116 cells and L-02) were seeded in 24-well plates and infected by Ad-ZD55, Ad-survivin-ZD55 and Ad-survivin-ZD55-TAp63 at various multiplicity of infections (MOIs). After four days, the culture medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS). Then, a 2% crystal violet solution in 20% methanol was added to cells for 15 min. The 24-well plates were washed with distilled water and photographed.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cells were plated in 96-well plates at a density of 3,000 cell/well and treated with various recombinant Ads. After 8 h, serum-free DMEM containing the Ads was replaced with normal growth medium. The cell survival rate was evaluated by a standard MTT assay (Sigma, USA). Briefly, 20 μl (5 mg/ml) MTT solution was added to each well. After cells were incubated at 37°C for 4 h, the supernatant of each well was carefully removed, and 150 μl DMSO was added to each well and mixed thoroughly on a shaker for 10 min. The optical density (OD) of each well was measured with a microplate spectrophotometer at 490 nm.

Apoptosis assay

The Annexin V-FITC/propidium iodide(PI) kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). HCT116 cells were infected with Ad-survivin-ZD55-TAp63, Ad-survivin-ZD55 and Ad-ZD55 viruses at an MOI of 10 for 20 h. Cells were washed three times with PBS and disassociated with trypsin. After adding Annexin V-FITC reagent, the cells were incubated in the dark for 15 min at room temperature. Subsequently, PI was added and the cells were incubated in dark for 5 min at room temperature. All samples were processed by flow cytometry (FACSCanto™ II, BD Biosciences, USA). The percentage of early and late apoptotic cells was obtained for statistical analysis.

Tumor xenografts in nude mice

The animal experiments were approved by the Ethics Review Board of Tongji University, Shanghai, China. Thirty healthy 4-week-old purebred BALB/C nude mice were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China). Mice were subcutaneously injected on the right side of axilla with HCT116 cells; 5×10⁶ cells were injected per mouse. Xenografts appeared at the injecting sites about a week after injection. Mice with too big or too small tumors were excluded and the rest of the mice were randomly divided into four equal groups: Ad-ZD55, Ad-survivin-ZD55, Ad-survivin-ZD55-TAp63 and control. In each group, the mice received multisite intratumor injections of the corresponding recombinant virus at 10⁹ plaque forming units (pfu)/mouse in 100 μl PBS, once every other day, through 5 injections. Mice in the control groups were injected with normal saline in the same manner. Tumor growth was monitored regularly using vernier calipers to measure the tumor size, and the tumor volumes were calculated using the formula: tumor volume = maximal diameter × minimal diameter²×0.5. Tumor growth curves were then plotted using these measurements.

TdT-mediated dUTP-biotin nick end-labeling (TUNEL) assay

Tumor specimens were fixed, processed, and embedded. Deparaffinized tumor sections were...
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The TUNEL assay (Roche, Palo Alto, USA) was used to detect apoptotic cells. Cells were infected with the recombinant virus.

Figure 1. Survivin expression and confirming recombinant virus Ad-survivin-ZD55-TAp63. A. Survivin was expressed in HCT116 cells but not in L-02 cells. B. Ad-survivin-ZD55-TAp63 mediated E1A and TAp63 expression specifically in HCT116 groups. ^Cells were infected with the recombinant virus.

Figure 2. Ad-survivin-ZD55-TAp63 showed tumor-specific cytopathic effect. HCT116 and L-02 cells were infected with recombinant viruses at different MOIs. A. The cytopathic effect of Ad-survivin-ZD55-TAp63 was stronger than Ad-ZD55 and Ad-survivin-ZD55. B. All the recombinant viruses produced similar effects in L-02 cells. MOI, multiplicity of infection; ^Cells were infected with the recombinant viruses.

Tissue sections in the normal saline-treated group were served as control groups. The staining was performed according to the manufacturer’s instructions. The TUNEL reaction preferentially labels DNA strand breaks generated during apoptosis and discriminates apoptosis from necrosis and primary DNA strand breaks induced by apoptotic agents.

Statistical analysis

Data from at least three separate experiments are presented as mean ± standard error of the mean (SEM). Independent sample t-test was used to draw a comparison between groups. Differences were considered statistically significant only when a two-tailed P-value was less than 0.05.

Results

Characteristics of the recombinant virus Ad-survivin-ZD55-TAp63

HCT116 and L-02 cells were collected, and total RNA was extracted using TRIzol reagent. As shown in Figure 1A, RT-PCR analyses confirmed that survivin was expressed in HCT116 cells, but not detected in normal L-02 cells. This is consistent with the results of previous study [17]. HCT116 and L-02 cells were then infected with Ad-survivin-ZD55-TAp63 at an MOI of 1 pfu/cell. 48 h after infection, the cells were collected, and western blot was used to detect the expression of E1A and TAp63 genes. E1A and TAp63 were positively expressed in HCT116 groups, but were not detected in L-02 groups (Figure 1B). Based on these data, the dual-regulated recombinant virus was selected for further experiments.
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Virus Ad-survivin-ZD55-TAp63 was successfully constructed.

Ad-survivin-ZD55-TAp63 mediated the tumor cell-specific cytotoxicity

The cytopathic effect was measured by crystal violet staining. HCT116 and L-02 cells were infected with Ad-ZD55, Ad-survivin-ZD55 and Ad-survivin-ZD55-TAp63 at MOIs of 0.01, 0.5, 1, and 10. Cells were stained with 2% crystal violet and photographed four days later. The cytopathic effect was observed in HCT116 cells in a time-dependent manner compared with other groups. Graph represented OD 490 nm ± SEM, *P*<0.05. OD, optical density.

Growth inhibition of Ad-survivin-ZD55-TAp63 in HCT116 cells

HCT116 cells were infected with Ad-ZD55, Ad-survivin-ZD55 and Ad-survivin-ZD55-TAp63 at MOIs of 0.01, 0.5, 1, 5, and 10. The uninfected HCT116 cells acted as controls. After 36 h, MTT assays were used to measure the cell proliferation. As depicted in Figure 3A, the cell proliferation ability of Ad-ZD55, Ad-survivin-ZD55 and Ad-survivin-ZD55-TAp63 groups were obviously decreased in a dose-dependent manner compared with control groups. Furthermore, Ad-survivin-ZD55-TAp63 produced a stronger growth inhibition than Ad-ZD55 and Ad-survivin-ZD55 at each infected concentration gradient. According to the results, we chose to perform subsequent experiments with the dose of 10 MOI. As shown in Figure 3B, the recombinant virus groups inhibited growth of HCT116 cells in a time-dependent manner compared with control groups when HCT116 cells were infected with the corresponding recombinant virus at an MOI of 10 pfu/cell. In addition, Ad-survivin-ZD55-TAp63 significantly inhibited the growth of HCT116 cells compared with Ad-ZD55 and Ad-survivin-ZD55 groups. These results indicated that the growth inhibition of Ad-survivin-ZD55-TAp63 groups was consistently and significantly higher than Ad-ZD55, Ad-survivin-ZD55 and control groups in a time- and dose-dependent manner.

Cell apoptosis was induced by Ad-survivin-ZD55-TAp63 in HCT116 cells

HCT116 cells were infected with different recombinant viruses at an MOI of 10 pfu/cell. 36 h after infection, the apoptotic rate was evaluated using flow cytometric analysis of Annexin V-FITC/PI staining. As shown in Figure 4, the proportion of apoptotic cells in the recombinant viruses was much higher than control groups. The percentage of the early and late apoptotic cells of Ad-survivin-ZD55-TAp63, Ad-survivin-ZD55, Ad-ZD55 and control is
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17.25±0.55%, 13.75±0.37%, 5.22±0.51% and 0.70±0.16% respectively (P<0.05). All the recombinant viruses produced a significantly greater percentage of early and late apoptotic cells compared with the control. The apoptotic rate of Ad-survivin-ZD55-TAp63 was highest among the recombinant viruses (Figure 4E).

Antitumor efficacy of Ad-survivin-ZD55-TAp63 in tumor xenografts

The in vivo oncolytic potential of Ad-survivin-ZD55-TAp63 was evaluated in HCT116 tumor xenografts model. The BALB/C nude mice were divided into four groups when the diameter of the tumors reached approximately 5 mm. Multisite intratumor injections were then administered to the mice at totally 1×10⁹ pfu dose of the recombinant virus per mouse, once every other day for 5 times. The tumor growth was monitored regularly using vernier calipers to measure tumor size. As shown in Figure 5A, the tumors grew rapidly in control groups. The therapeutic effects were clearly observed in the recombinant virus treatment groups by 14, 21 and 28 days. Furthermore, the Ad-survivin-ZD55-TAp63 group achieved a significantly greater therapeutic effect than the Ad-P53 and Ad-survivin-ZD55 groups. The TUNEL analysis showed HCT116 cells treated with the recombinant Ads were undergoing apoptosis in cancer cells, in contrast, the control groups showed no signs of apoptosis. Furthermore, Ad-survivin-ZD55-TAp63 group induced more severe apoptosis than Ad-ZD55 and Ad-survivin-ZD55 groups (Figure 5B-E).
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Discussion

The CTGVT strategy [12] combines gene therapy with oncolytic adenoviral therapy by inserting an antitumor gene into the oncolytic adenoviral vector. The most important thing is to guarantee the specificity and safety of adenoviral vector. There are three main strategies to improve the specificity and safety. The first strategy involves the modification of adenoviral capsid proteins that improve the affinity of Ad and tumor cells. For example, a short peptide sequence with an RGD motif was inserted into the HI loop of the adenoviral knob, which significantly raised the infection efficacy of the recombinant virus [18]. The second strategy is to delete adenoviral genes that are necessary for replication of normal cells, but unnecessary for viral replication in tumor cells. ONYX-015, deletion of adenoviral E1B55kDa, resulted in the loss of replication capacity of virus in normal cells but not in tumor cells. But, the antitumor effect of single application of Onyx-015 was not great for tumor treatment [19]. The last strategy is to use tumor- or tissue-selective promoters to control the expression of early viral genes essential for replication. A number of these promoters have been identified and some of them have been used for oncolytic Ad, such as the carcinoembryonic antigen (CEA) promoter [20], mucin-like glycol protein episialin (MUC1) promoter [21] and survivin promoter [9]. In addition to the specificity and safety of adenoviral vector, the appropriate antitumor genes are also an important factor for the CTGVT strategy. Multiple studies on tumourigenesis found TAp63 was a critical tumor suppressor gene. The ability of TAp63 to trigger senescence and halt tumourigenesis identified TAp63 as a potential target of antitumor therapy for human malignancies.

In this study, the recombinant virus Ad-survivin-ZD55-TAp63 was constructed and the modification of this Ad included: survivin promoter is driving E1A gene; deletion of E1B55kDa gene and carrying antitumor gene TAp63. Firstly, we analyzed the expression level of survivin by RT-PCR. The results of RT-PCR confirmed that survivin was only expressed in HCT116 cells, but not in L02 cells. Then, after Ad-survivin-ZD55-TAp63 infection, high E1A and TAp63 expression were exhibited in HCT116 cells, whereas negative expression of E1A and TAp63
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were observed in L-02 cells. At the same time, the results of crystal violet staining assays showed that HCT116 cells were susceptible to Ad-ZD55, Ad-survivin-ZD55 and Ad-survivin-ZD55-TAp63, whereas the normal L-02 cells showed much lower sensitivity to all recombinant viruses. In addition, HCT116 cells were exceptionally sensitive to Ad-survivin-ZD55-TAp63 from 1 MOI compared with Ad-ZD55 and Ad-survivin-ZD55 groups. The above results indicated that a specific, safe and effective antitumor gene therapy recombinant virus Ad-survivin-ZD55-TAp63 was successfully established.

The ability of cell proliferation was measured by MTT assays. Ad-survivin-ZD55-TAp63 significantly repressed the growth of HCT116 cells which followed a dose- and time-dependent manner compared with the Ad-ZD55, Ad-survivin-ZD55 and control groups. HCT116 cells were infected with 10 MOI of different recombinant viruses to conduct apoptosis assays. The percentage of the early and late apoptotic cells was highest in the Ad-survivin-ZD55-TAp63 groups compared with the Ad-ZD55, Ad-survivin-ZD55 and control groups. Lastly, the in vivo study in nude mice bearing CRC cancer also found that the recombinant virus Ad-survivin-ZD55-TAp63 could inhibit the growth of tumor and induce much apoptosis in cells, and it has a stronger tumor inhibitory effect than Ad-survivin-ZD55, Ad-ZD55 and control groups.

Ad-survivin-ZD55 and Ad-ZD55 groups showed antitumor effects in vitro and in vivo compared with control groups. This may be because they were controlled by both genetic deletion of E1B55kDa and survivin promoter-controlled E1A, which enhances their antitumor effects. Our study also found that Ad-survivin-ZD55-TAp63 exhibited greater antitumor effect than Ad-survivin-ZD55 and Ad-ZD55. This may be because Ad-survivin-ZD55-TAp63 combines the advantages of both gene therapy and virotherapy by using the dual-regulated oncolytic adenoviral vector harboring TAp63.

Collectively, our findings suggest that Ad-RGD-survivin-ZD55-TAp63 exhibits specific antitumor effect in CRC cells. Therefore, we hope gene therapy based on oncolytic Ads could offer a promising new direction for future CRC treatment.

Acknowledgements

This work was supported by the International Exchange Program for Graduate Students, Tongji University (no. 2016020035); The Project of Shanghai Science Committee (no. 134119b0600; no. 16411970800); Project of Shanghai Municipal Health Bureau (no. 2013-4194); Jiaxing Science Committee Foundation of Zhejiang Province (no. 2015AY23071); the Technology Plan Project of Medicine and Health of Zhejiang Province (no. 2016KYB295) and the Scientific Research Foundation for the Returned Overseas Chinese Scholars, Chinese Ministry of Education (no. 020114001).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Haiyan Ge, Department of Gastrointestinal Surgery, Shanghai East Hospital, School of Medicine, Tongji University, No. 150, Jimo Road, Pudong New District, Shanghai 200120, P. R. China. Tel: +86-21-38804518; E-mail: ge_east150@163.com; Dr. Bin Xu, Department of General Surgery, Shanghai Tenth People’s Hospital, School of Medicine, Tongji University, Shanghai 200072, P. R. China. Tel: +86-21-66303482; E-mail: pfdsbxubin@163.com

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