Original Article
Inhibition of renal cell carcinoma cell growth by a novel conditionally replicative adenovirus in combination with radiotherapy

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Abstract: The most common kidney malignancy is renal cell carcinoma (RCC). Conditionally replicative adenoviruses, which are able to replicate in tumor cells, constitute an effective and novel approach for treating malignant tumors. Based on the Ad5-ZD55 adenoviral vector with the E1B55 K gene deleted, we constructed a 5/35 fiber chimeric conditionally replicative adenovirus, in an attempt to evaluate the antitumor activity in RCC cells. RCC cells were independently treated with F5/35-ZD55 + X-ray, F5/35-ZD55 alone, X-ray alone, or PBS as a control. Detection of the expression of reporter gene was examined in RCC cells. The cytotoxic potential of each treatment was assessed using cell viability measurements. Apoptosis was assayed using the Western Blotting, Hoechst 33258. Differences in tumor growth were also assayed in the Ketr-3 xenograft model. Our data show that F5/35-ZD55 exhibited excellent transfection ability and efficient delivery of E1A in the Ketr-3 cells. In comparison with F5/35-ZD55 or radiochemotherapy alone, the combined F5/35-ZD55 and radiotherapy treatment synergistically enhanced the loss of cell viability, induced apoptosis in the Ketr-3 cells, and suppressed Ketr-3 tumor growth in vivo. F5/35-ZD55 combined with radiotherapy is a potential treatment strategy against RCC.

Keywords: Renal cell carcinoma, fiber chimeric conditionally replicative adenoviruses, radiation, apoptosis

Introduction

Renal cell carcinoma (RCC) is the most common type of kidney malignancy and with metastatic RCC having a poor prognosis [1]. In metastatic RCC, the estimated five-year survival rate is < 10% [2]. RCC is highly chemoresistant; only ≤20% of patients benefit from high-dose interleukin-2 (IL-2) and/or interferon-alpha (IFN-α) cytokine therapy [3]. Following cytokine therapy, the median overall survival is about 10-13 months, and there is no effective treatment if there is disease progression after initial response or if the patient does not respond to cytokine therapy [4]. Therefore, more active agents and effective treatment strategies are urgently needed against metastatic RCC.

An effective and novel approach for treating malignant tumors involves using conditionally replicative adenoviruses (CRAds), which can replicate in tumor cells. When the infected tumor cells lyse, the released CRAds infect the neighboring cells, rendering them highly advantageous over non-replicating adenoviruses (Ads) [5]. Two major approaches that can be used to target OAds against cancer cells specifically are: i) Alteration of viral gene products that attenuate replication in normal tissue only, e.g., E1B 55-kDa and E1A proteins that promote viral replication in cancer cells with defective p53 or pRb [6]; ii) Controlling early viral gene expression via promoters selective for cancer. Radiotherapy combined with Ad5-Δ24RGD, CV706 and Ad5-CD/TKrep has greater anti-tumor efficacy than either approach alone [7-9].

Traditional adenoviral vectors are commonly derived from adenovirus of serotype 5 (Ad5), which belongs to subgroup C [10]. Coxsackie

adenovirus receptor (CAR) is the Ad5 receptor on the cell membrane. Twelve fiber trimers of Ad5 are attached to the penton base proteins of the viral capsid. At the distal tip of each fiber monomer is a globular region which is referred to as the knob domain. The knob region associates with cellular adenoviral receptors. Replication of Ad5 in a multitude of primary tumor cells and tumors in vivo is often limited due to low or absent expression of CAR on tumor cells [11]. Cell transfection by certain adenovirus B mainly depends on the CD46 molecule, which is widely expressed on the surface of tumor cells, instead of CAR [12]. Type 35 in the adenovirus B2 subgroup (Ad35) also depends on the CD46 molecule, although Ad35 has more cell membrane receptors than Ad3, enabling closer integration with the CD46 molecule and more efficient infection of various tumor cells [13]. Based on the Ad5-ZD55 vector with the E1B-55K gene deleted, we constructed a 5/35 fiber chimeric adenovirus, F5/35-ZD55, in an attempt to evaluate the antitumor activity of F5/35-ZD55 in combination with radiotherapy in RCC.

Considering the findings of above mentioned recent studies, we hypothesized that a combination of gene therapy mediated by F5/35-ZD55 and radiotherapy would have an enhanced anti-tumor effect compared with either agent on its own. Our data suggest that radiotherapy combined with F5/35-ZD55 is a potential therapeutic strategy against RCC.

Materials and methods

Cell lines and culture conditions

We purchased the human RCC cell line Ketr-3 from Shanghai Cell Collection (Shanghai, China). We cultured the cells in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Grand Island, NY) that was supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO BRL), 4 mM glutamine, 50 U/mL penicillin, and 50 µg/mL streptomycin at 37°C in humidified 5% CO_2. We screened the cells routinely to ensure no mycoplasma contamination.

Construction and production of the virus

The recombinant adenoviruses used in this study have been previously described, which

included pZD55, an oncolytic Ad plasmid construct form which the E1B-55K gene had been deleted [14]. We generated the pF5/35-ZD55 plasmid by inserting F5/35 into pZD55, deleting the E1A promoter. We successfully replaced the E1A promoter with F5/35 in the pF5/35-ZD55 plasmid. We transfected the pF5/35-ZD55 plasmid into HEK-293 cells with the pBHGE3 plasmid to obtain the recombinant oncolytic Ads, designated F5/35-ZD55. Viral plaques that appeared 9-12 days after infection were recombinant adenoviruses, which we verified by PCR. We plaque-purified the viruses, propagated them in HEK-293 cells, and used plaque assay to determine functional plaque-forming unit (PFU) titers.

Radiation

We used an X-irradiator (X.S.S.205 FZ, Jiancheng Co. LTD., China). The dose rate was 0.287 Gy/min with 200 kV/10 mA and 0.5-mm thick Cu/Al filters; 56 cm spanned the distance between the X-ray source and target. Forty-eight hours after viral treatment, we irradiated the cells with 0.5-6 Gy ionizing radiation using a 137Cs g-radiation source. We treated tumor-bearing mice with the recombinant Ad; X-rays were delivered focally to the tumor tissue to ensure that the other body parts were shielded.

Detection of the expression of reporter gene in vitro

Ketr-3 cells were plated at a density of 10^5 cells/6 cm dish and were treated with F5/35-ZD55-EGFP (1 MOI and 10 MOI) for the indicated time. The expression of enhanced green fluorescent protein (EGFP) was detected under Olympus fluorescence microscope at 24, 48, 72 and 96 hours following treatment.

Cell viability assay

We plated Ketr-3 cells (10^5 cells/6-cm dish) and treated them with radiation alone (2 Gy, 4 Gy, 8 Gy), F5/35-ZD55 alone (0.1, 1, 10 MOI), or F5/35-ZD55 + radiation (0.1 MOI + 2 Gy, 1 MOI + 4 Gy, 10 MOI + 8 Gy). After 4-day treatment, we evaluated the rate of cell survival rate using a standard tetrazolium dye (MTT) assay (Sigma, St. Louis, MO) as per the manufacturer's protocol. We tested four replicate wells per assay; each experiment was repeated three times.
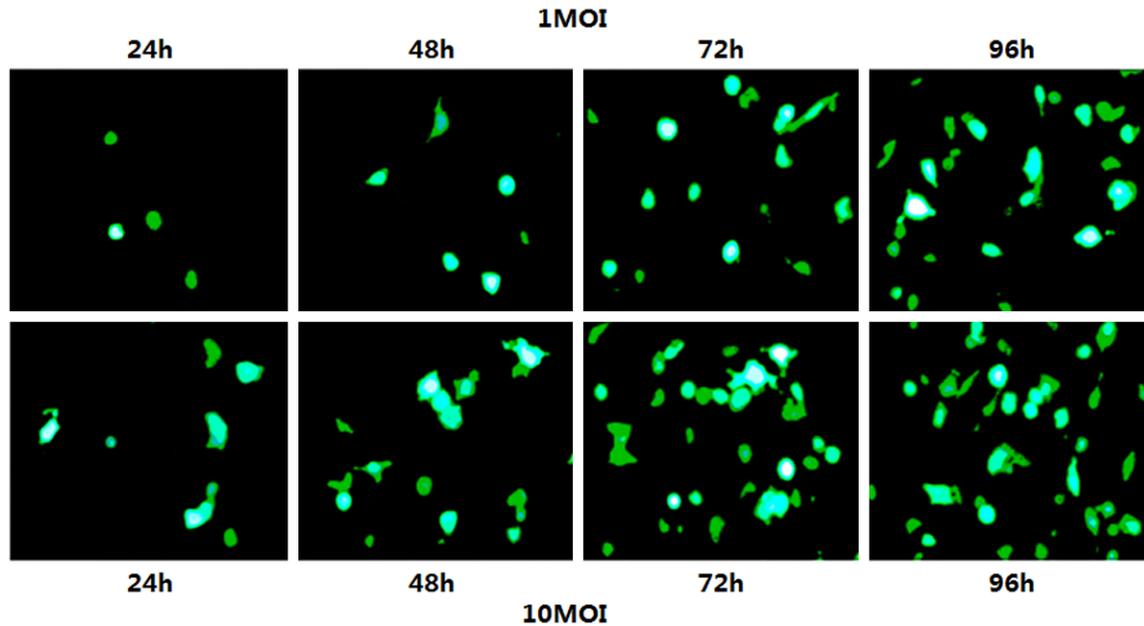
Figure 1. F5/35-ZD55 mediated reporter gene expression in Ketr-3 cells. Ketr-3 cells were treated with F5/35-ZD55-EGFP (1 MOI and 10 MOI). Expression of EGFP was detected under Olympus fluorescence microscopy at 24, 48, 72 and 96 h following treatment.

Western blot analysis

We separated cell extract aliquots from the harvested cells on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel. We transferred the proteins to nitrocellulose membranes and they were incubated overnight at 4°C with rabbit polyclonal antibodies against caspase-3, NF-κB, γH2AX, Bcl-2, Bax (Cell Signaling Technology, Beverly, MA), β-actin and E1A (Santa Cruz Biotechnology, Dallas, TX). The membranes were subsequently washed and incubated with secondary antibodies conjugated to alkaline phosphatase in Tris-buffered saline containing Tween (TBST) for 2 h and developed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Promega, Madison, WI). We scanned and analyzed the band density on the membrane using ImageJ analyzer (LabWorks Software, UVP, Upland, CA).

Apoptotic cell staining

Ketr-3 cells were seeded in flat-bottomed 6-well plates; F5/35-ZD55, radiation, or both agents combined were used to treat the cells. Untreated cells were used as the controls. After forty-eight hours, we incubated the cells with Hoechst 33258 (Nanjing Keygen Biotech, Nanjing, China) for 10 min, washed them twice with phosphate-buffered saline (PBS), and observed them using fluorescence microscopy.

Xenograft tumor nude mouse model

We obtained male BALB/c nude mice (4-5 weeks old) from Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China). The mice were quarantined for 1 week prior to xenografting. We carried the animal care and experimental procedures strictly in accordance with the Guide for the Care and Use of Laboratory Animal (National Research Council, 1996). We established the xenograft tumor model by subcutaneous injection of 2×10^6 Ketr-3 cells into the right flanks of the mice. When tumors grew to 100-150 mm^3, we divided the mice randomly into four groups (8 mice/group) and treated them with intratumoral injections of F5/35-ZD55 (5×10^8 PFU/day) for 3 successive days or with PBS (control). We treated mice in the combination group locally with a single radiation dose (10 Gy) using a clinical linear accelerator (Varian, Milpitas, CA). Tumors were monitored every week for 30 days; we measured the tumor size using a caliper. The tumor volume was calculated as follows: V (mm^3) = length × width^2 × 1/2. At the end of the experiment, we harvested the tumors for analyses. We tested
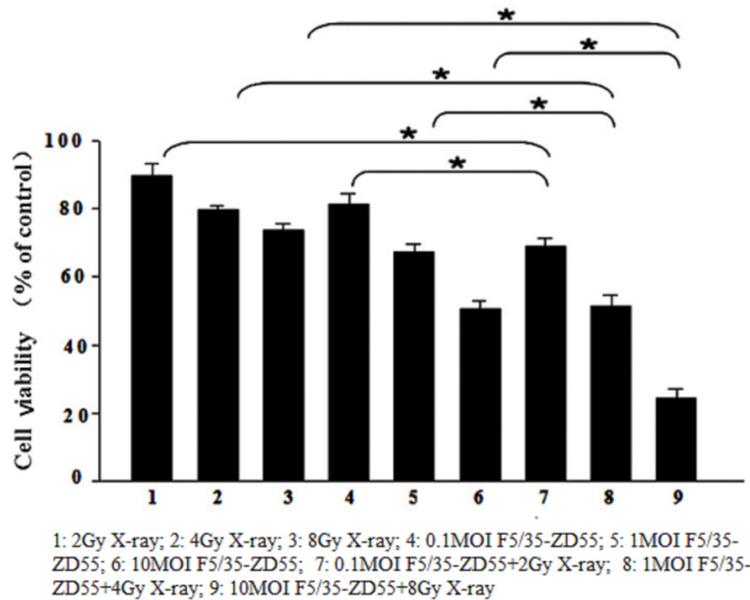
Figure 2. Effects of F5/35-ZD55 in combination with X-ray on viability of RCC cell line Ketr-3 cells. Ketr-3 cells were treated with varying doses of either F5/35-ZD55 or X-ray irradiation alone, and F5/35-ZD55 together with X-ray irradiation. The cells were subjected to MTT assay at the fourth day after treatment. Data are expressed as mean ± SD (error bars) of three independent experiments and the cell viability values corresponding to different treatment groups are displayed as a percentage of untreated control cells. The asterisks "*" denote p < 0.05 in comparison to F5/35-ZD55 or X-ray treated groups (n = 6).
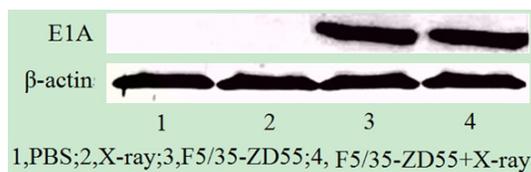

Figure 3. F5/35-ZD55 induced efficient delivery of E1A in renal cell carcinoma cell line Ketr-3 cells. Cells were treated with F5/35-ZD55 or X-ray alone and F5/35-ZD55 along with X-ray Phosphate-buffered saline (PBS) treatment served as a negative control. After 72 h treatment, E1A protein expression levels were analyzed by western blotting using anti-E1A antibody. β-actin served as a loading control.

differences in tumor growth for statistical significance.

Statistical analysis

The data are expressed as the mean ± SD. We analyzed the data using independent samples t-test and variance analysis as appropriate, using SPSS Base 13.0 for Windows (SPSS Inc., Chicago, IL). Findings were considered statistically significant at P < 0.05.

Results

Reporter gene assay

We examined EGFP expression levels in F5/35-ZD55-EGFP-treated Ketr-3 cells to determine the effect of adenovirus transduction. There were comparable numbers of EGFP-positive cells in the groups infected with F5/35-ZD55-EGFP (**Figure 1**). There was also much stronger green fluorescence in the F5/35-ZD55-EGFP-treated cells, presumably because of the viral replication. Altogether, the results indicate that higher and stable expression levels of the reporter gene are mediated by F5/35-ZD55.

F5/35-ZD55 in combination with radiotherapy enhances the loss of cell viability

To examine whether F5/35-ZD55 combined with radiotherapy has an inhibitory effect on RCC cell viability, we treated Ketr-3 cells with 2, 4, or 8 Gy radiation alone, F5/35-ZD55 alone (0.1, 1 or 10 MOI) or both F5/35-ZD55 and radiotherapy (0.1 MOI + 2 Gy, 1 MOI + 4 Gy, 10 MOI + 8 Gy). **Figure 2** shows that the combination treatments enhanced the inhibition of cell viability dose-dependently.

Following 4-day treatment of Ketr-3 cells with F5/35-ZD55 plus radiotherapy (10 MOI + 8 Gy), F5/35-ZD55 alone (10 MOI), and radiotherapy alone (8 Gy),the rate of Ketr-3 cell inhibition was 73.56 ± 2.58%, 32.3 ± 2.38%, and 15.75 ± 3.21%, respectively. The findings were considered statistically significant at P < 0.05. The combination of F5/35-ZD55 and radiotherapy (0.1 MOI + 2 Gy, 1 MOI + 4 Gy) was more effective than either treatment alone (P < 0.05) (**Figure 2**).

F5/35-ZD55 mediates efficient delivery of E1A in Ketr-3 cells

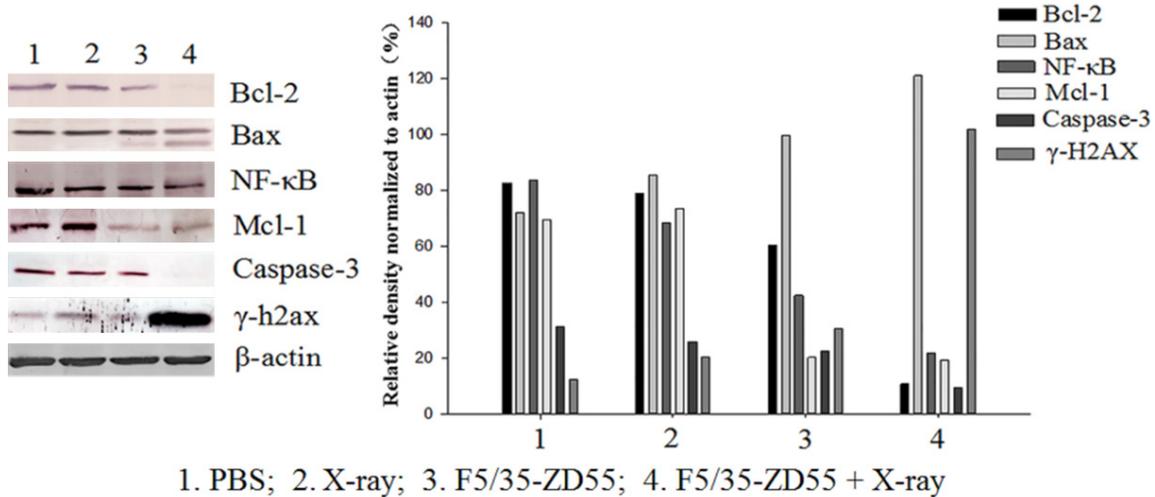Cells infected with F5/35-ZD55 and treated with or without radiotherapy had similar adeno-

Figure 4. F5/35-ZD55 sensitizes renal cell carcinoma cell line Ketr-3 cells to radiotherapy via mitochondrial apoptotic cell death. Ketr-3 cells were treated with F5/35-ZD55, F5/35-ZD55 + X-ray, or X-ray alone. Phosphate-buffered saline (PBS) treatment served as a negative control. After 72 h treatment, cell extracts were subjected to western blot analysis for caspase-3 expression levels. The anti-apoptotic proteins Bcl-2, Mcl-1, NF-κB and γ-H2AX, and the pro-apoptotic protein Bax expression levels were also examined. β-actin served as a loading control (n = 6).
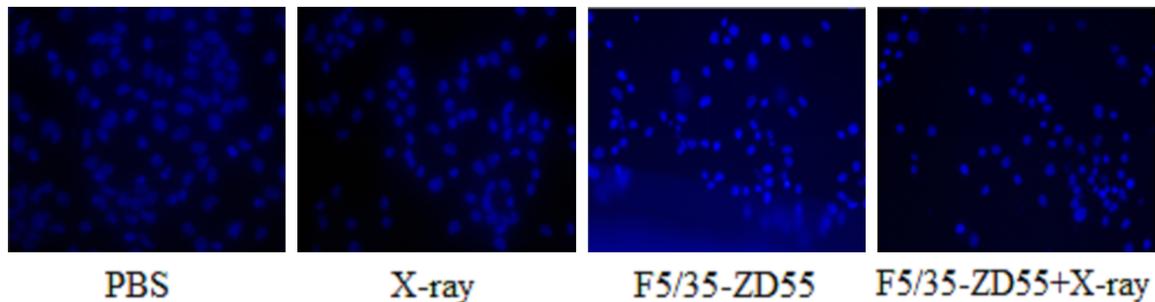

Figure 5. F5/35-ZD55 and X-ray induced morphological changes in the nuclei of Ketr-3 cells. Ketr-3 cells were treated with F5/35-ZD55 + X-ray (10 MOI + 8 Gy), F5/35-ZD55 (10 MOI) and X-ray (8 Gy), respectively. After 48 h, Ketr-3 cells were incubated with Hoechst 33258 for 2 h, and the nuclei condensation and fragmentation were observed under a fluorescence microscope. Arrows indicate apoptotic cells. Original magnification 400 ×.

viral E1A protein expression (**Figure 3**), indicating the productive replication of F5/35-ZD55 in the Ketr-3 cells and that the viral replication ability was not attenuated by radiotherapy.

Combination of F5/35-ZD55 with radiotherapy enhances mitochondria-mediated apoptosis in Ketr-3 cells

Ketr-3 cells treated with F5/35-ZD55 only had decreased Bcl-2 levels, which combination with radiotherapy reduced further (**Figure 4**). Bax expression was not increased significantly by PBS or radiotherapy alone, whereas it was increased significantly by F5/35-ZD55 or a combination of F5/35-ZD55 and radiotherapy.

Similarly, the combined treatment significantly decreased the expression of procaspase-3 (**Figure 4**). The results suggest that F5/35-ZD55 combined with radiotherapy enhances RCC cell apoptosis by skewing the Bcl-2 family protein balance towards the pro-apoptotic pathway efficiently.

Differences in cell apoptosis were detected following F5/35-ZD55 and radiotherapy treatment in RCC cell line Ketr-3 cells. Ketr-3 cells treated with the three treatments were stained with Hoechst 33258, and revealed markedly different cellular and nuclear morphology, e.g., there was DNA fragmentation as compared with the control (**Figure 5**).
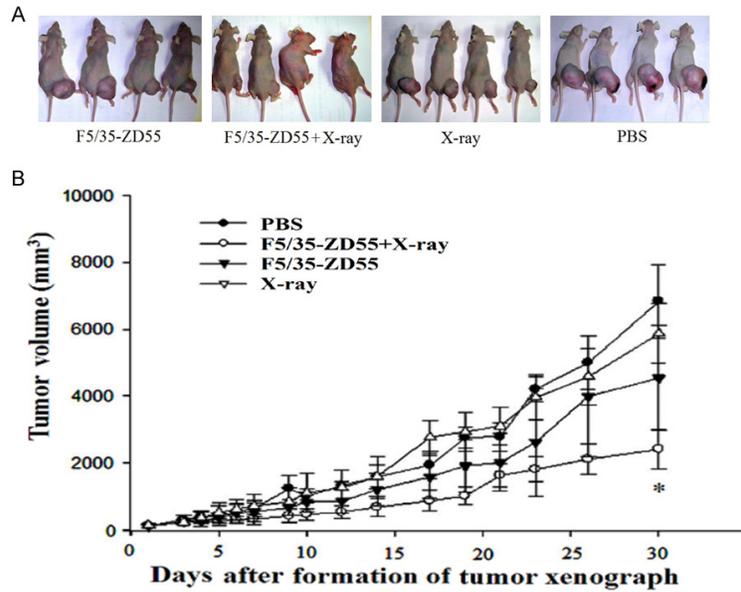
Figure 6. Anti-tumor activity of F5/35-ZD55 in the Ketr-3 xenograft model. Tumors were established by injecting Ketr-3 cells subcutaneously into the right flank of nude mice. When tumors reached a volume of 100-150 mm³, mice were divided randomly into four groups (8 mice per group) and were treated by three daily intratumoral injections of F5/35-ZD55 + X-ray (10 MOI + 8 Gy), F5/35-ZD55 (10 MOI) alone, X-ray (8 Gy) alone, or PBS as a control. (A) Representative tumors formed 30 days after injection. (B) The tumor size was measured and the tumor volume was calculated. Data are expressed as mean ± SD (error bars). The asterisk "*" indicates p < 0.05 in comparison to the F5/35-ZD55 or X-ray-treated groups (n = 6).

Anti-tumor efficacy of combination therapy using F5/35-ZD55 and radiotherapy in vivo

Based on the *in vitro* findings, we investigated the effects of the combined F5/35-ZD55 and radiotherapy treatment on tumor growth *in vivo*. Nude mice were subcutaneously injected with 2 × 10⁶ Ketr-3 cells. When the tumor grew to 100-150 mm³, we divided the mice randomly into four groups (8 mice/group), and treated them with a single radiation dose (10 Gy for 3 successive days), F5/35-ZD55 (5 × 10⁸ PFU/d for 3 consecutive days), or combined therapy (n = 8); the control group received Ketr-3 cells followed by PBS injection. The mice were sacrificed 30 days after xenografting and the tumors were harvested for analyses. Control group tumors had rapid and continued outgrowth during the experiment; the mean tumor size was 6835.14 ± 752.66 mm³; that of the radiotherapy group was 4384.76 ± 432.46 mm³ and that of the F5/35-ZD55 treatment group was 5770.25 ± 537.25 mm³. The mean tumor size of the combined treatment group was 2303.19 ± 130.22 mm³, much smaller

than that of the F5/35-ZD55 treatment group (p < 0.05) and the radiation treatment group (p < 0.05; **Figure 6**).

Discussion

The characteristics of RCC, the most common kidney malignancy, are suppressed apoptosis and marked invasiveness. Recent studies have focused on the possibility of combining modalities for improving the therapeutic value of existing standard therapies, e.g., chemotherapy and radiotherapy [15]. In radiotherapy, malignant tumor cells are destroyed by ionizing radiation. Although higher radiation doses exert greater anti-tumor effects, the potential toxicity associated with damage to normal tissue within the field of radiation limits the dosage.

Animal studies have demonstrated that radiation enhances OAd anti-tumor efficacy [16]. The radiation-enhanced efficacy of therapy mediated by OAds might also be due the ionizing radiation increasing viral replication [17]. Cross-resistance is theoretically unlikely because the mechanisms of oncolytic virus and ionizing radiation are independent of one another, consequently minimizing the possibility of treatment-resistant tumor cells developing.

Here, we examined the efficacy of treating RCC cells with gene therapy mediated by F5/35-ZD55 combined with radiotherapy. Western blotting confirmed higher E1A protein expression in cells treated with F5/35-ZD55 alone or a combination of F5/35-ZD55 and radiotherapy. Our results indicate that F5/35-ZD55 replicates efficiently in Ketr-3 RCC cells. Consistent with this finding, the MTT assay showed that the combined F5/35-ZD55 and radiotherapy induced cytopathic effects in Ketr-3 cells specifically. Furthermore, F5/35-ZD55 and radiotherapy significantly inhibited tumor growth when compared with F5/35-ZD55 treatment or radiotherapy alone.

Multiple model systems have established that the important determinants of cell survival or death are the relative levels of the anti-apoptotic proteins (e.g., Bcl-2 or Bcl-xL) and pro-apoptotic proteins (e.g., Bax) [18]. To describe the mechanism of the radiotherapy and F5/35-ZD55 induced cell death, we determined the effect of F5/35-ZD55, alone or combined with radiotherapy, on Bcl-2, Bax, NF-κB, γ-H2AX and caspase-3 protein levels in Ketr-3 cells. F5/35-ZD55 and radiotherapy combined greatly decreased the levels of Bcl-2, increased Bax significantly, and induced much stronger caspase-3 activation. The anti-tumor effect of combined treatment with F5/35-ZD55 and radiotherapy was correlated with γ-H2AX upregulation and NF-κB downregulation. Our results suggest that combining F5/35-ZD55 with radiotherapy alters pro-apoptotic and anti-apoptotic protein levels and ratio in RCC cells. These changes may contribute to the combined F5/35-ZD55 and radiotherapy-induced increase in apoptosis.

In summary, we demonstrate that F5/35-ZD-55-mediated gene therapy combined with radiotherapy might be a novel and effective approach for treating RCC.

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