RESEARCH ARTICLE

Replication-competent adenovirus expressing TRAIL synergistically potentiates the antitumor effect of gemcitabine in bladder cancer cells

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Abstract Replication-competent adenovirus armed with therapeutic tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene has been shown to sensitize cancer cells to chemotherapy and radiotherapy. However, the synergistic antitumor effect of replication-competent adenovirus expressing TRAIL and the cytotoxic chemotherapy in bladder cancer remains to be determined. Bladder cancer T24 cells or mouse tumor xenografts were infected with replication-competent adenovirus armed with human TRAIL (ZD55-TRAIL) alone or in combination with gemcitabine. The mRNA and protein levels of TRAIL were determined by "Reverse transcription polymerase chain reaction" and Western blotting, respectively. Cell viability was tested by CCK8 assay. Tumor growth in the mice was monitored every week by measuring tumor size. Cell apoptosis was detected by Annexin V-FITC staining and TUNEL assay. We found that adenovirus ZD55-TRAIL efficiently replicated both in cultured bladder cancer T24 cells and T24 mouse tumor xenograft as demonstrated by the overexpression of TRAIL and E1A. Gemcitabine did not affect the expression of TRAIL. In cultured T24 cells, ZD55-TRAIL enhanced the growth inhibitory effects of gemcitabine, accompanied by increased apoptosis. Similarly, ZD55-TRAIL synergistically enhanced the antitumor effect and induction of apoptosis following gemcitabine treatment in mouse T24 xenografts. In conclusion, replicative adenovirus armed with TRAIL synergistically potentiates the antitumor effect of gemcitabine in human bladder cancer. Our study provides the basis for the development of ZD55-TRAIL in combination with conventional chemotherapy for the treatment of bladder cancer.

Keywords Bladder cancer · TRAIL · Adenovirus · Gemcitabine · Apoptosis

Introduction

Following treatment, approximately 70 % of bladder cancers undergo recurrence and 10–20 % progress to invasive disease [1]. Bacillus Calmette-Guérin (BCG) is the reference standard for intravesical treatment of bladder cancer [2]. BCG reduces the risk of bladder cancer recurrence by 30–40 % and induces tumor regression by stimulating host immune response via tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) [3]. However, BCG frequently causes side effects that range from dysuria frequency to systemic tuberculosis [4]. Notably, recent studies have shown a correlation between increased urinary levels of TRAIL and BCG responsiveness, indicating that the release of TRAIL is important in the design of BCG-based bladder tumor immunotherapy protocols [5]. Therefore, there is an urgent need for the development of novel TRAIL-based strategies for the treatment of bladder cancer.

Gemcitabine is a nucleoside analog with a broad spectrum of antitumor activity in a variety of solid tumors. Gemcitabine inhibits DNA replication and thereby cell growth by direct incorporation of its diphosphate derivative into DNA [6]. Although clinical effectiveness of gemcitabine has been demonstrated in the systemic treatment of advanced urothelial

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carcinoma, gemcitabine monotherapy alone or in combination with other agents usually induces transient responses [7]. Furthermore, most responding diseases recur within the first year with a median survival of 12 to 14 months [8]. One of the underlying mechanisms may be the evasion of apoptosis of cancer cells. It is therefore intriguing to postulate that TRAIL may sensitize urothelial carcinoma of the bladder (UCB) cells to chemotherapeutic agents.

Oncolytic adenovirus has been used in cancer gene therapy largely due to its ability to selectively infect and replicate in tumor cells [9]. Not only do oncolytic adenovirus directly kills tumor cells at the end of its lytic cycle but also the progeny viruses spread throughout a tumor and infect other cancer cells. A recent study showed that oncolytic adenovirus encoding TRAIL could inhibit the growth and metastasis of triple-negative breast cancer [10]. In this study, we utilized replication-competent adenovirus to deliver TRAIL and examined its synergistic antitumor effect with the cytotoxic chemotherapeutic agent gemcitabine in bladder cancer.

Materials and methods

Cell lines and chemicals

Bladder cancer T24 cells were purchased from Shanghai Cell Collection (Shanghai, China) and maintained in RPMI supplemented with 10 % fetal bovine serum (GIBCO BRL, Grand Island, NY, USA), 100 U/mL penicillin, and 100 mg/L streptomycin (GIBCO BRL, Grand Island, NY, USA). Gemcitabine was obtained from Hanson Pharmaceutical (Jiangshu, China). An adenovirus overexpressing TRAIL (ZD55-TRAIL) was kindly provided by Dr. Xin-Yuan Liu (Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China).

CCK-8 assay

Cells were seeded in 96-well plates and treated with ZD55-TRAIL (10 MOI) plus gemcitabine (4.0 mg/mL), ZD55-TRAIL (10 MOI), gemcitabine (4.0 mg/mL), ZD55-EGFP (10 MOI), or PBS for 24 h. Ten microliters of CCK-8 solution (Cell counting kit-8, Dojindo Molecular Technologies, Gaithersburg, MD, USA) was added to each well. Absorbance was determined at 450 nm after 1, 2, 3, and 4 days of incubation.

Reverse transcription polymerase chain reaction

The expression of TRAIL gene was analyzed by reverse transcription polymerase chain reaction (RT-PCR) using Access RT-PCR System. Total RNA was isolated from cells using Total RNA Isolation kit. RNA integrity was assessed by checking the 28S and 18S ribosomal RNA in a glyoxal/dimethyl sulfoxide gel. RT-PCR was performed in a reaction volume of 25 μL, including 1 μL of following primers: 5′- TTCAAGCTTGATCATGGCTATGATGGAGGT-3′ and 5′-GCTCTAGATTAGCCAACTAAAAAGGCCCCGA-3′, respectively. The reaction conditions were 30 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, and 1 cycle at 72 °C for 5 min for final extension. β-Actin was used as an internal reference gene to normalize the expression of TRAIL. Quantification was performed with an image analyzer (Lab works Software 3.0; UVP).

Western blot analysis

After treatment with gemcitabine and/or ZD55-TRAIL for 48 h, cells were washed with cold PBS and lysed in lysis buffer (1 % Triton X-100, 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 10 mM PMSF, 1 mM benzamidine, 5 mg/mL aprotinin, 3 mg/mL pepstatin, 5 mg/mL leupeptin). Protein concentration was determined by Bradford assay. Fifty micrograms of total protein was mixed with equal volume of laemmli sample buffer, boiled for 5 min at 95 °C, loaded on a 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, and transferred onto a nitrocellulose membrane. Membranes were blocked for 4 h at room temperature in blocking buffer (3 % nonfat milk power in TBS-T: 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05 % Tween 20) and incubated for 4 h at room temperature in blocking buffer containing a rabbit anti-TRAIL polyclonal antibody (1:1,000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA). After washing in TBS-T buffer, membranes were incubated for 2 h at room temperature in blocking buffer containing a 1:10,000 dilution of peroxidase conjugated mouse anti-rabbit secondary antibody. After washing in TBS-T, membranes were developed using NBT/BCIP color substrate. The bands were scanned and analyzed with the image analyzer (Lab works Software).

Cellular apoptosis assay

Apoptosis was assessed with the Annexin V-FITC kit according to the manufacturer's protocol. After treatment with gemcitabine and/or ZD55-TRAIL for 48 h, cells were washed twice with cold PBS, resuspended in binding buffer, added with 5 mL of Annexin V-FITC and 10 mL of propidium iodide (PI) (BioVision, Mountain New, CA), and incubated at room temperature in the dark for 10 min. At the end of the incubation, 200 μL of binding buffer was added, and the cells were immediately analyzed with flow cytometry. Cell Quest software was used to analyze flow cytometry data (number of total apoptotic cells/100)×100 %.

Xenograft tumor model in nude mice

Male BALB/c nude mice (4–5 weeks old) were obtained from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China) and quarantined for a week before tumor implantation. Animal welfare and experimental procedures were carried out strictly in accordance with the "Guide for the Care and Use of Laboratory Animals" (National Research Council, 1996) and approved by Institutional Animal Care and Use Committee (IACUC) of Xuzhou Medical College. The bladder cancer xenograft model was established by subcutaneously injecting 2×10^6 T24 cells into the right flank of mice. When tumors reached 100–150 mm^3, mice were divided randomly into four groups (eight mice for each group) and received intratumoral injection of ZD55-TRAIL (10 MOI) plus gemcitabine (4.0 mg/mL), ZD55-TRAIL (10 MOI), and gemcitabine (4.0 mg/mL), which were dissolved in 200 μL saline or 200 μL PBS as the control, respectively, with three consecutive injections daily. At the seventh day after the first injection, three mice were randomly selected and killed to harvest tumors for additional analyses as described below. The remaining five mice were monitored every week by measuring tumor size using caliper for 9 weeks. The tumor volume was calculated by the following formula: $V(mm^3) = length \times width^2 / 2$. At the end of the experiment, all animals were killed.

Immunohistochemical staining

Tumors were harvested and fixed in 10 % formalin, embedded in paraffin, and cut into 4 μm sections. Deparaffinized tumor sections were treated with 3 % H_2O_2 for 10 min to block the endogenous peroxidase and incubated with blocking serum (goat serum) at room temperature for 30 min, then were incubated with anti-TRAIL or anti-E1A antibody (1:200). After incubation with goat anti-rabbit IgG (1:150), the expressions of TRAIL or E1A were detected with diaminobenzidine (DAB; Sigma, St. Louis, MO) by using an avidin–biotin reaction ABC kit (Vector Laboratories Burlingame, CA). Tissue sections stained without primary antibody served as negative control. The slides were counterstained with hematoxylin.

TUNEL assay

Apoptotic cells in tumor tissue sections were quantified using in situ apoptosis detection kit (Roche, Indianapolis, IN). In brief, formalin-fixed and paraffin-embedded sections were dewaxed, followed by permeabilization with proteinase K at room temperature for 15 min. Endogenous peroxidase was blocked with 3 % H_2O_2. Sections were incubated with equilibration buffer and terminal deoxynucleotidyl transferase (TdT) enzyme, and incubated with anti-digoxigenin–peroxidase conjugate. Peroxidase activity was determined by DAB.

Under microscopy, six fields were randomly selected from each sample and 100 cells were randomly examined in each field. The apoptotic rate = (number of total apoptotic cells / 100) × 100 %.

Statistical analysis

Values were expressed as mean±SD. Statistical analysis was carried out by one-way analysis of the variance (ANOVA) followed by the Duncan's new multiple range method or Newman–Keuls test. $p < 0.05$ was considered significant.

Results

Infection of adenovirus ZD55-TRAIL leads to efficient delivery of TRAIL in bladder cancer T24 cells

To determine the delivery efficiency of TRAIL in bladder cancer T24 cells by adenoviral vector ZD55-TRAIL, we checked the mRNA of TRAIL of T24 cells treated with gemcitabine and/or ZD55-TRAIL by RT-PCR. Compared to the cells treated with gemcitabine, control vector ZD55-EGFP or PBS, the cells treated with ZD55-TRAIL or ZD55-TRAIL plus gemcitabine displayed increased TRAIL mRNA. Furthermore, there was no apparent difference in TRAIL mRNA levels between ZD55-TRAIL plus gemcitabine and ZD55-TRAIL treatment (Fig. 1a). In consistence, TRAIL protein was overexpressed in ZD55-TRAIL plus gemcitabine- and ZD55-TRAIL-treated cells; there was no apparent difference of TRAIL protein levels between ZD55-TRAIL plus gemcitabine and ZD55-TRAIL treatment (Fig. 1b). These data demonstrated that the adenovirus ZD55-TRAIL is an efficient vector for the delivery of TRAIL and gemcitabine does not affect TRAIL expression of ZD55-TRAIL in bladder cancer T24 cells.

ZD55-TRAIL sensitizes bladder cancer T24 cells to gemcitabine

We next examined the cell proliferation following infection of T24 cells with ZD55-TRAIL with or without gemcitabine treatment by CCK8 assay. After 2 days, the survival rate of the ZD55-TRAIL (10 MOI) plus gemcitabine (4.0 mg/mL) group (18.44±2.38 %) was significantly lower than those of the ZD55-TRAIL group (40.75±4.12 %), gemcitabine group (53.6±1.41 %), and ZD55-EGFP (61.65±4.53 %) ($p < 0.01$) (Fig. 2a). Eight milligrams/milliliter of gemcitabine was required to reach the inhibitory effects of 4.0 mg/mL gemcitabine in combination with 10 MOI of ZD55-TRAIL in days 2 and 3 (Fig. 2b). Thus, ZD55-TRAIL significantly enhances the cytotoxic effect of gemcitabine in bladder cancer T24 cells.
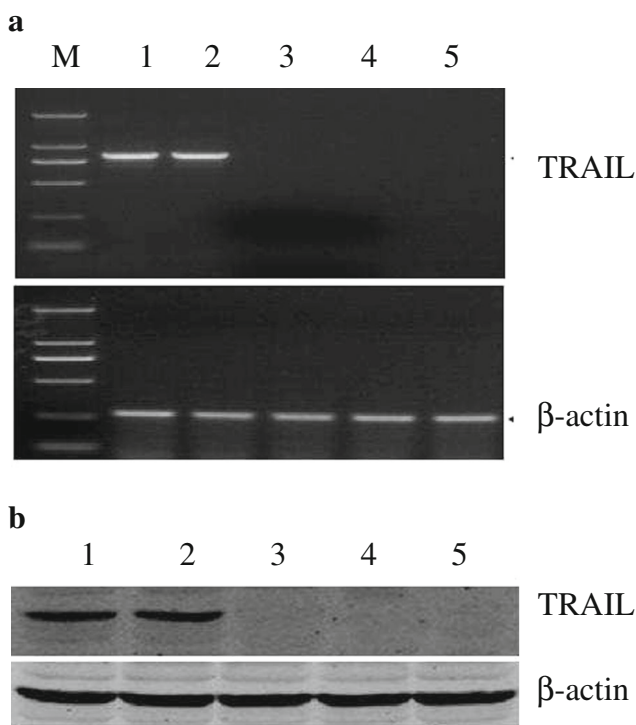
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Fig. 1 ZD55-TRAIL induced efficient expression of TRAIL in bladder cancer T24 cells. **a** Analysis of TRAIL mRNA level by RT-PCR. TRAIL mRNA level was much higher in ZD55-TRAIL+gemcitabine group and ZD55-TRAIL group. β-Actin was used as the internal control. **b** Analysis of TRAIL protein level by Western blot. TRAIL protein level was much higher in ZD55-TRAIL+gemcitabine group and ZD55-TRAIL group. β-Actin served as loading control. *M*, DL2000 RNA marker; *1*, ZD55-TRAIL+gemcitabine group; *2*, ZD55-TRAIL group; *3*, gemcitabine group; *4*, ZD55-EGFP group; *5*, PBS group. Shown were representative results from three independent experiments

ZD55-TRAIL increases the apoptosis of bladder cancer T24 cells treated with gemcitabine

To test whether the enhanced cytotoxic effect of gemcitabine in combination of ZD55-TRAIL is due to the induction of

apoptosis by TRAIL, we examined cell apoptosis by Annexin V-FITC staining. Compared with other groups, there was significantly increased apoptosis in the cells treated with gemcitabine and/or ZD55-TRAIL (Fig. 3a). Two days after treatment, Annexin V-FITC-positive cells in ZD55-TRAIL plus gemcitabine group was 2.01 times of that in ZD55-TRAIL group, 2.21 times of that in gemcitabine group, and 2.95 times of that in ZD55-EGFP group ($p < 0.01$), respectively (Fig. 3b).

ZD55-TRAIL synergistically potentiates the antitumor effect of gemcitabine in bladder cancer T24 mouse xenografts

To extend our in vitro observation, we investigated the effects of gemcitabine and/or ZD55-TRAIL treatment on tumor growth in mouse T24 tumor xenografts. As shown in Fig. 4, in the control group receiving PBS, tumors displayed rapid and continued outgrowth during the course of the experiment, with the mean tumor size of 2,501.0 mm^3. In sharp contrast, the mean tumor size of the ZD55-TRAIL (10 MOI) plus gemcitabine (4.0 mg/mL) group was 129.01 mm^3, significantly smaller than that of the ZD55-TRAIL group (1,760.6 mm^3; $p < 0.05$) and the gemcitabine group (1,129.3 mm^3; $p < 0.05$). At the end of experiments, tumors were excised from the mice and weighted. The results showed that tumor weight was significantly less in ZD55-TRAIL (10 MOI) plus gemcitabine group (44.6±7.24 mg) than in ZD55-TRAIL group (163.5± 18.6 mg, $p < 0.01$), gemcitabine group (127.3±26.4 mg, $p < 0.01$), and PBS group (235.6±23.6 mg, $p < 0.01$).

TUNEL staining showed that there was significantly increased apoptosis in the ZD55-TRAIL plus gemcitabine group (85.8±5.6 %) in comparison to that in the control groups with PBS (15.8±3.2 %), ZD55-TRAIL (61.4± 3.8 %), or gemcitabine (44.0±3.8 %) (Fig. 5a, b).

To verify that the enhanced therapeutic effect was due to TRAIL, we further determined the expression of TRAIL and E1A in the tumors by immunohistochemical analysis.
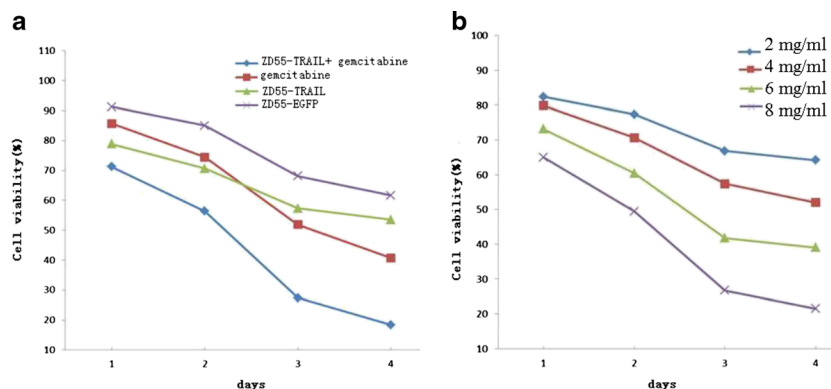
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Fig. 2 ZD55-TRAIL and gemcitabine synergistically inhibited bladder cancer T24 cell growth. **a** T24 cells were treated with the agents as indicated for up to 4 days. Cell viability was measured by MTT assay.

b T24 cells were treated with gemcitabine at the indicated concentration and time. Cell viability was measured by MTT assay. Shown were representative results from three independent experiments
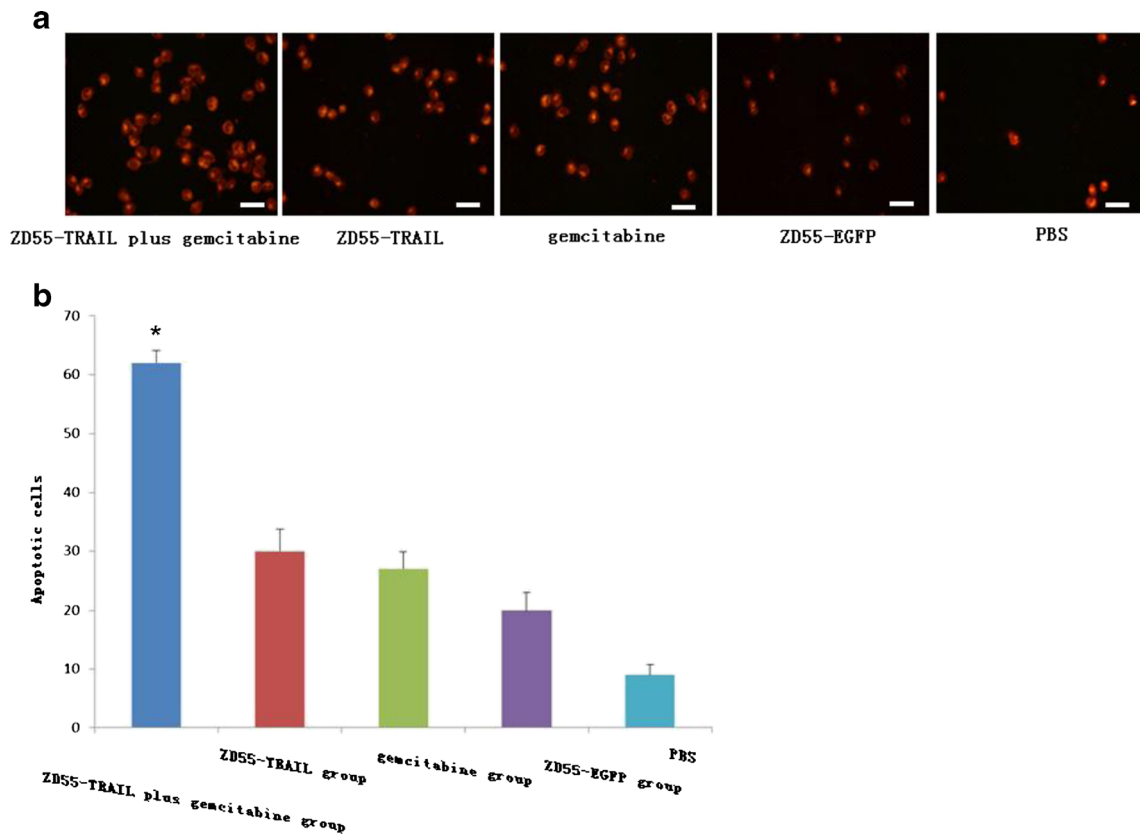
Fig. 3 ZD55-TRAIL and gemcitabine synergistically induced apoptosis of bladder cancer T24 cells. **a** At 48 h after transfection, apoptotic cells were detected as shown by the condensation of nuclear chromatin and its fragmentation. *Scale bar*, 20 μm. **b** Quantitative representation of apoptotic T24 cells ($n=3$). *$p<0.01$: ZD55-TRAIL+gemcitabine group vs. other groups

Compared to the gemcitabine- and PBS-treated groups, there was marked increase of TRAIL staining in the ZD55-TRAIL plus gemcitabine- and ZD55-TRAIL-treated groups (Fig. 6a). Moreover, E1A expression was detected only in ZD55-TRAIL plus gemcitabine- and ZD55-TRAIL-treated groups but not in the gemcitabine- and PBS-treated groups (Fig. 6b).

Discussion

TRAIL, a member of TNF family closely related to Fas ligand and TNF-α, induces apoptosis in a wide variety of cancer cells. Unlike Fas ligand and TNF-α, TRAIL triggers apoptosis in cancer cells while spares normal cells even though most cells express TRAIL receptors at significantly high levels,
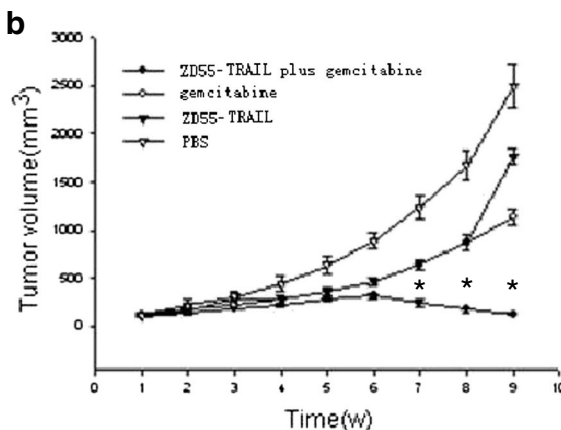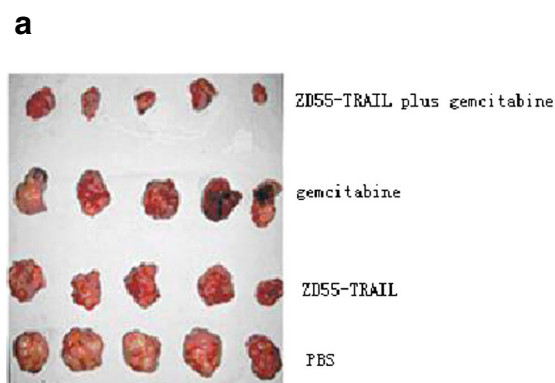

Fig. 4 ZD55-TRAIL and gemcitabine demonstrated synergistic antitumor effects in bladder cancer T24 xenografts. **a** Shown were representative bladder cancer T24 xenografts dissected from each group of nude mice. **b** Tumor growth curves to show quantitative analysis of tumor growth following treatment as indicated ($n=5$). *$p<0.01$: ZD55-TRAIL+gemcitabine group vs. other groups. *w*, weeks

Fig. 5 ZD55-TRAIL and gemcitabine exhibited synergistic effects in the induction of apoptosis in bladder cancer T24 xenografts. Tumor sections were excised from nude mice and analyzed for apoptosis by TUNEL staining. **a** Representative images of TUNEL staining. Apoptotic cells were stained as *brown. Scale bar*, 20 μm. **b** Quantitative analysis of apoptotic rate (%) in xenografts from each group based on TUNEL staining (*n*=5). *p<0.01: ZD55-TRAIL+gemcitabine group vs. other groups
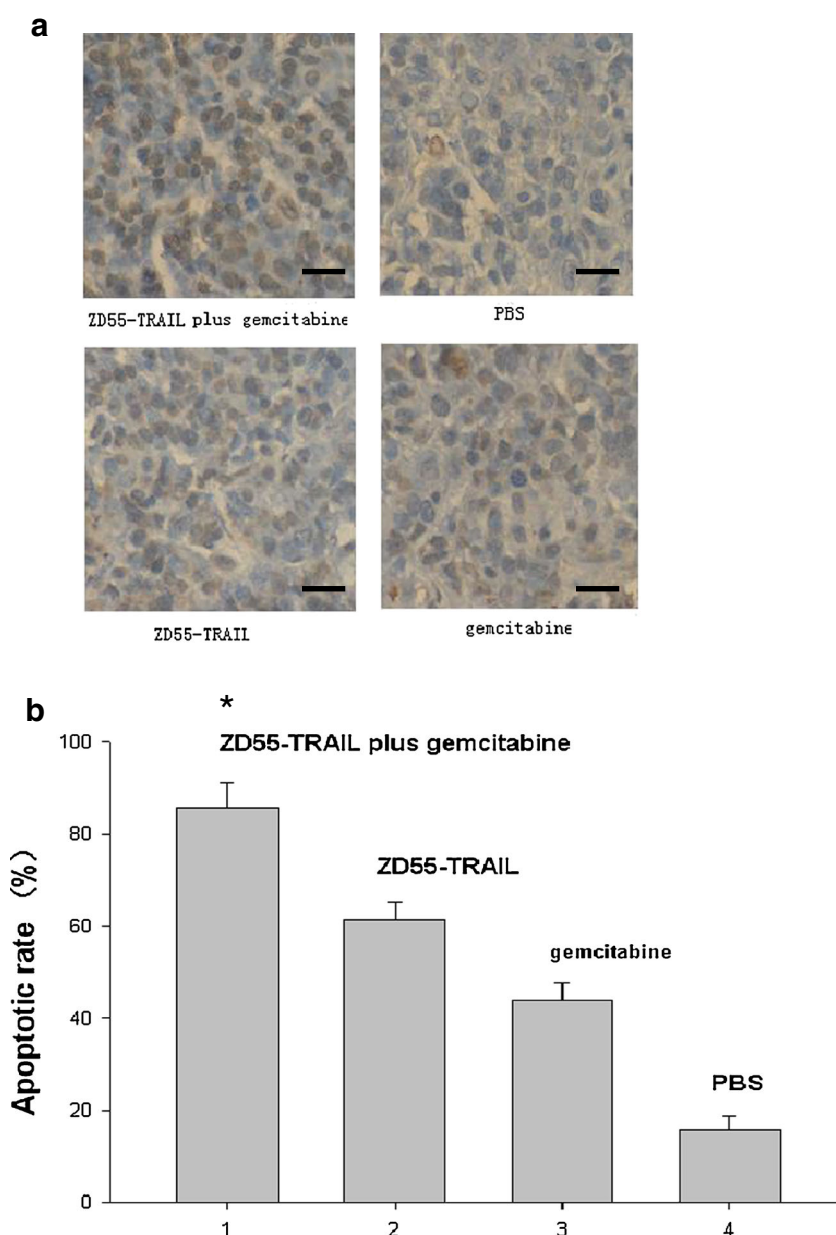
indicating that TRAIL is a promising agent for cancer therapy. Upon binding to its death receptors (DR4 and DR5), TRAIL not only stimulates apoptosis via the formation of the Death Inducing Signaling Complex (DISC) that contain procaspase-8 and Fas Associated Death Domain (FADD) but also activates NF-κB, which regulates the expression of survival factors such as members of the inhibitors of apoptosis and the Bcl-2 families [11]. ZD55-TRAIL is an E1B 55 kDa-deleted replication-competent adenovirus and armed with the therapeutic gene TRAIL. Due to the increased expression of TRAIL with the selective replication of the oncolytic adenovirus (ZD55), ZD55-TRAIL has displayed more significant activity than the replication defective adenovirus Ad-TRAIL in vitro and in vivo [12]. Although ZD55-TRAIL has obvious

antitumor activity, tumors could not be completely eradicated with ZD55-TRAIL alone. In agreement with our results, ZD55-TRAIL has been reported to have synergistic antitumor activity with various treatment modalities, including targeted agents, chemotherapy drugs, and even radiation therapy [7–10].

Gemcitabine is the drug of choice in the context of a multidisciplinary clinical strategy aimed at sparing the bladder in patients with transitional infiltrating cancer [13, 14]. In addition, the combination of gemcitabine chemotherapy with biotherapy has been effective in treating pancreatic cancer [15, 16]. However, the synergistic antitumor effect of ZD55-TRAIL biotherapy and gemcitabine chemotherapy in bladder cancer is unknown. In this study, we analyzed the synergistic

Fig. 6 ZD55-TRAIL was efficiently replicated in bladder cancer T24 xenografts. Tumor sections were excised from nude mice and subjected to immunohistochemical analysis. **a** Immunohistochemical staining of TRAIL. *Upper*, representative images of TRAIL staining. TRAIL-positive cells were stained as *brown*. *Lower*, quantitative analysis of TRAIL staining in xenografts from each group (*n*=5). **p*<0.01, vs. gemcitabine or PBS group. **b** Immunohistochemical staining of E1A. *Upper*, representative images of E1A staining. E1A-positive cells were stained as *brown*. *Lower*, quantitative analysis of E1A staining in xenografts from each group (*n*= 5). **p*<0.01, vs. gemcitabine or PBS group
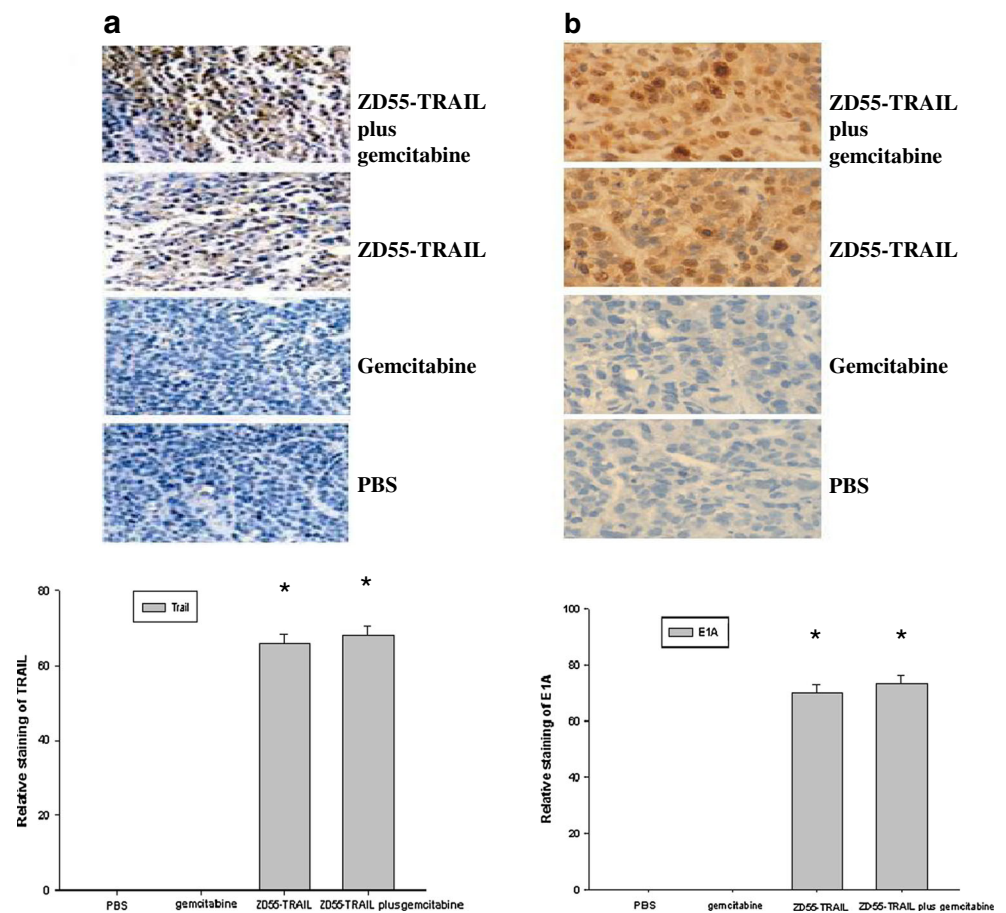
antitumor effect of ZD55-TRAIL and gemcitabine. Western blot analysis confirmed higher expression of E1A protein in cells treated with either ZD55-TRAIL alone or ZD55-TRAIL plus gemcitabine. We observed elevated expression of TRAIL only in ZD55-hTRAIL plus gemcitabine- or ZD55-TRAIL-treated T24 cells. These results indicated that ZD55-TRAIL could efficiently replicate in T24 cells, and that gemcitabine did not affect the expression of TRAIL. Consistent with the ability to replicate in tumor cells, CCK8 assay showed that ZD55-TRAIL plus gemcitabine could specifically induce cytopathologic effects on T24 cells. Significant inhibition of tumor growth was also demonstrated in the ZD55-TRAIL plus gemcitabine groups when compared with the ZD55-TRAIL or gemcitabine.

Furthermore, by using cytometry and TUNEL assays, we provided both in vitro and in vivo evidence that the synergistic antitumor effect of ZD55-TRAIL and gemcitabine in bladder cancer is at least in part due to the induction of apoptosis. This is consistent with previous reports that TRAIL potently induces apoptosis in a variety of cancer cells including bladder cancer [17–19]. However, several limitations of this study should be noted. First, we used xenograft nude mouse model, which could not truly reflect the in vivo situation of bladder cancer development. Next, we need employ orthotopic bladder cancer models to evaluate the potential of ZD55-TRAIL in combination with gemcitabine for bladder cancer therapy [20]. Second, in this study, we used intratumoral TRAIL gene delivery which has limitations that restrict its full potential [21]. Further studies to develop targeted delivery of TRAIL gene into bladder cancer will be essential for translating preclinical TRAIL studies into the clinic.

In summary, we demonstrated the synergistic antitumor effect of ZD55-TRAIL and gemcitabine on bladder cancer in vitro and in vivo. These findings provide a rationale for the development of ZD55-hTRAIL-based combination therapy regimen with conventional chemotherapy agents such as gemcitabine for bladder cancer treatment.

Conflicts of interest None.

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