Autophagy promotes oncolysis of an adenovirus expressing apoptin in human bladder cancer models

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Summary

As a potential cancer therapy, we developed a recombinant adenovirus named Ad-VT, which was designed to express the apoptosis-inducing gene (apoptin) and selectively replicate in cancer cells via E1a manipulation. However, how it performs in bladder cancer remains unclear. We examined the antitumor efficacy of Ad-VT in bladder cancers using CCK-8 assays and xenograft models. Autophagy levels were evaluated by western blotting, MDC staining, and RFP-GFP-LC3 aggregates' analyses. Here, we report the selective replication and antitumor efficacy (viability inhibition and apoptosis induction) of Ad-VT in bladder cancer cells. Using xenograft tumor models, we demonstrate that its effects are tumor specific resulting in the inhibition of tumor growth and improvement of the survival of mice models. Most Importantly, Ad-VT induced a complete autophagy flux leading to autophagic cancer cell death through a signaling pathway involving AMPK, raptor and mTOR. Finally, we suggest that treatment combination of Ad-VT and rapamycin results in a synergistic improvement of tumor control and survival compared to monotherapy. This study suggests that Ad-VT can induce selective autophagic antitumor activities in bladder cancer through the AMPK-Raptor-mTOR pathway, which can be further improved by rapamycin.

Keywords Bladder cancer · Adenovirus · Autophagy · AMPK · Rapamycin

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Introduction

In 2014, an estimated 78,000 new cases and 32,000 deaths of urinary bladder cancer occurred in China [1]. More than 90 % of bladder cancer cases are transitional cell carcinoma (TCC) and transurethral resection, along with intravesical chemotherapy, has been its first line of therapy [2]. However, patients with multilocular bladder tumors are at high risk of tumor recurrence and development of muscle-invasive or metastatic cancer. For the treatment of metastatic cancer, chemotherapy remains the most widely used approach; however, this regime is highly toxic to patients and may lead to chemoresistance [3]. Thus, these limitations highlight an unmet need for the development of safer modalities to treat bladder cancer.

Oncolytic viruses can be genetically engineered to selectively infect and lyse cancer cells with little harmful effects on normal cells, making oncolytic virotherapy a promising antitumor approach. In our previous study, we constructed a novel adenovirus-based oncolytic agent, which we designated as Ad-hTERT-E1a-apoptin (Ad-VT). Ad-VT contains a hTERT-driven E1a cassette in which the expression of the adenovirus early viral 1A (E1a) gene is controlled by the

promoter of the human telomerase reverse transcriptase (hTERT) that is highly active in over 85 % of human cancers and without activity in most normal human somatic cells [4]. Furthermore, Ad-VT expresses apoptin, which is the product of the chicken anemia virus VP3 gene and selectively induces apoptosis in a variety of tumors with no toxic or transforming activity in normal, non-transformed human cells [5]. Previous studies showed selective antitumor activities of Ad-VT in cell lines derived from melanoma, colorectal carcinomas and lung, gastric, prostate and breast cancers. Moreover, it showed a favorable safety profile in mammal animals, including mice, rats, guinea pigs, and dogs [6–9]. However, it is not known how Ad-VT acts on bladder cancer.

In the current study, we assessed tumor-selective replication and oncolytic actions of Ad-VT on a variety of human bladder cancer cell lines and an xenograft mice model. Furthermore, we reported that autophagy-related cancer cell death is triggered by Ad-VT infection possibly through the AMPK-Raptor-mTOR pathway. In addition, the antitumor synergistic effects of Ad-VT were evaluated in combination with the well-accepted mTOR inhibitor and autophagy agonist, rapamycin, in a bladder xenograft tumor model.

Materials and methods

Viruses, cell lines and animals

The oncolytic Ad-VT (Ad-hTERT-E1a-Apoptin) was constructed in our laboratory and as previously described. [6] Briefly, the transgene cassettes containing the hTERT promoter (the 5' flanking region of the hTERT gene between positions −283 to -78) that drives E1a expression and the CMV promoter that drives apoptin expression were subcloned into the BD Adeno-X Viral DNA (the adenoviral genome) via the shuttle vector pShuttle2. The HEK-293 human embryonic kidney cells were used to package and produce the recombinant adenoviruses. The HEK-293 cell line, the human bladder cancer cell lines (UM-UC-3, T24, 5637 and EJ-1) and the immortalized human bladder urothelium cell line (SV-HUC-1) were obtained from the Committee on Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). UM-UC-3 and HEK-293 cells were cultured in DMEM with 10 % FBS. SV-HUC-1 was cultured in Ham's F-12 medium with 10 % FBS, and T24, 5637 and EJ-1 were cultured in 1640 medium with 10 % FBS. DNA Fingerprinting (monitoring the mutation of human cell lines), isoenzyme analysis (verify the species of origin), Scharfe Casy TT system (evaluating cell proliferation) and Hoechst staining (mycoplasma detection) were used by the supplier to characterize the cell lines. Female BALB/c nude mice (6 weeks, 22 ± 2 g) were purchased from the Beijing SPF Laboratory Animal Technology Company and used for constructing the xenograft tumor model. All animals were maintained in groups of 8 mice per cage and under standard conditions of controlled temperature (23 ± 1°C), humidity (45 %), and lighting (12-hour light/dark). All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

SiRNAs, plasmids, and reagents

SiRNAs of Control (sc-37,007) and Atg5 (sc-41,445) were purchased from Santa Cruz Biotechnology. The pmCherry-EGFP-LC3 plasmid, which encodes for a fusion protein of mCherry, enhanced green fluorescent protein (EGFP) and LC3, was purchased from Public Protein/Plasmid Library (PPL70001). The 3-MA (M9281), CQ (C6628), LY294002 (L9908), Q-VD (SML0063) and rapamycin (553,210) were purchased from Sigma-Aldrich. Taxol (HY-B0015) were purchased from MedChemExpress. Cisplatin (S1166) and gemcitabine (S1714) were purchased from Selleck.

Measurement of viral titers

UM-UC-3, T24, 5637 and SV-HUC-1 cells were plated onto 6-well plates at a density of 2×10^5 cells per well. After 24 h incubation, the cells were infected with Ad-VT at a MOI of 10, and re-incubated at 37 for 24, 48 or 72 h. The supernatants of infected cultures were harvested and serially diluted in serum-free medium. HEK-293 cells were cultured in a 96-well plate and infected with tenfold concentration of the viruses for 96 h. The virus titers were calculated with the Reed-Muench method and expressed as TCID50 per milliliter of supernatant. In the 3-MA or rapamycin treatment groups, the cells were pretreated with 3-MA or rapamycin 2 h before Ad-VT infection.

Cell viability assay

Cell viability was determined by the CCK-8 assay following the manufacturer's instructions. Briefly, the cells were first counted and seeded at 5×10^3 cells/well in a 96-well plate. After 24 h incubation, treatments with viruses or drugs were applied. After another incubation for an indicated time (24 h, 48 h or 72 h), 10 μL of the CCK-8 reagent (Dojindo Molecular Technologies) was added into each well for 4 h. OD at 450 nm was measured using a multifunction microplate reader. In the drug treatment groups, cisplatin (10 uM), taxol (50 nM), gemcitabine (25 nM), 3-MA (2 mM), CQ (10 μM), LY294002 (5 μM), rapamycin (100 nM), or Q-VD (40 μM) was added 2 h before viral administration. In the siRNA knockdown groups, the cells were first seeded on the 6-well plates and transfected with siRNA. After 48 h, these cells were re-seeded in 96-well plates (5×10^3 per well) and treated as previously described.

Western blotting analysis

Cells were collected for western blotting analysis as previously described [10]. Equal amounts (30 µg) of proteins were electrophoresed, transferred into a membrane and then incubated with primary rabbit antibodies. The primary antibodies against LC3 (3868), P62 (16,177), Atg5 (2630), AMPK (5831), p-AMPK (2535), raptor (2280), p-raptor (2083), mTOR (2983), p-mTOR (5536), S6 (2317), p-S6 (4858) and GAPDH (5174) were purchased from Cell Signaling Technology. The antibody against E1a (sc-58,658) was purchased from Santa Cruz Biotechnology. The antibodies against apoptin (ab193612) and β-actin (ab8226) were purchased from Abcam. After incubation with secondary antibodies (anti-rabbit or anti-mouse from Abcam), the specific bands were detected using the Pierce ECL Western Blotting Substrate.

MDC labeling

Autophagic vacuoles were detected as previously described [11]. The cells (2×10^5 per well) were first seeded on coverslips in 6-well plates, and then infected with Ad-VT at a dose of 100 MOI or treated with an equal amount of vehicle after 24 h incubation. After an additional 24 h, the culture medium was removed, and each well was washed with 0.1 mol/L PBS (pH 7.4) 3 times. Then 100 nM monodansylcadaverine (MDC, Sigma-Aldrich) was dissolved in serum-free medium, added into each well and incubated for 30 min at 37. After incubation, the cells were washed 3 times with PBS and immediately analyzed with a fluorescence microscope (Olympus BX43). A total of 30 cells were observed in 40x fields within each well to calculate the percentage of positive cells, for three wells per experiment. A cell containing 20 or more MDC-labelled dots was defined as a positive cell.

Analysis of mCherry-EGFP-LC3 aggregates

The cells (70–90 % confluent) were grown in 6-well plates and transfected with plasmid encoding mCherry-EGFP-LC3. After 48 h incubation, the cells were trypsinized and re-seeded in new 6-well plates (2×10^5 per well). On the next day, the transfected cells were infected with vehicle or Ad-VT at a MOI of 100 for 12 or 24 h. Images were captured by a fluorescence microscopy (Olympus BX43) and the average number of EGFP or mCherry-containing dots in 30 cells were counted in 40x fields.

Animal experiments

T24 cells (3×10^6) were subcutaneously inoculated into the right flank of female BALB/c nude mice (6 weeks, 22 ± 2 g) and 7 days later, the mice were randomly assigned to different groups (n = 8 per group) and subjected to indicated treatments. Tumors' size was measured every three days using calipers and calculated with the following formula: [$(W^2 \times L)/2$; W, width; L, length; in cubic millimeters]. Mice were sacrificed by cervical dislocation when tumor volume exceeded 3000 mm^3 with ulceration. In the Ad-VT-treated groups, $9 \times 10^{\wedge 7}$ plague-forming units of Ad-VT were given intratumorally to mice in a volume of 100 ul in PBS. The control PBS group received 100ul of PBS alone. The viruses were given every three days for a total of six treatments. In the drug-treated groups, 3-MA (15 mg/kg) and rapamycin (2 mg/kg or 4 mg/kg) were given intraperitoneally in a volume of 100ul PBS (3-MA) or corn oil (rapamycin). The drugs were given every three days for a total of six treatments. In the siRNA knockdown groups, Atg5 (15 ug) were dissolved in 15 ul distilled water, and then mixed with 7.5 µL of 10 % glucose solution and 7.5ul of Entranster-in vivo reagent (Engreen, China), according to the manufacturer's instructions. The siRNAs were given intratumorally a day prior to virus treatment to reduce interference with virus treatment and every three days for a total of six treatments. Tumor, liver, spleen or kidney tissues (n = 3) were harvested 21 days after the initial viral injection, fixed in 4 % paraformaldehyde.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

Tumor sections were incubated with 50 µL TUNEL reaction mixture (5 µL enzyme solution and 45 µL label solution) for 1 h as recommended by manufacturer (In Situ Cell Death Detection Kit, Roche). Then, these tumor sections were incubated with DAB for 15 min and counterstained with hematoxylin. At least three random fields from each section were examined at ×400 magnification.

Histopathological analysis

Tissue sections were blocked with normal horse serum for 1 h, and then incubated at 4 overnight with antibodies against Ki-67 (Cell Signaling Technology) or adenovirus hexon protein (1 in 50 dilution: Abcam). After washing, the sections were incubated with secondary antibodies (1:100) for 1 h, washed with PBS and the proteins were visualized by incubating sections with DAB for 15 min, followed by counterstaining with hematoxylin. Images were captured at ×200 or ×400 magnification.

Statistical analysis

All data are presented as means ± SEM and analyzed with GraphPad Prism, Version 6.0. The Data were analyzed by t-test or analysis of variance (ANOVA) followed by a two-tailed t-test; except for the survival data that were subjected

to a Kaplan-Meier survival analysis. For all tests, differences with a $p < 0.05$ were considered significant.

Results

Ad-VT selectively replicates in bladder cancer cells

Ad-hTERT-E1a-apoptin (Ad-VT) contains an hTERT-driven E1a cassette and a CMV-driven apoptin cassette (Fig. 1a). Previous studies reported that apoptin induced apoptosis in tumors but had no toxic or transforming activity in normal and non-transformed human cells [5]. Following infection with Ad-VT, bladder tumor cells (UM-UC-3, T24 and 5637) and immortalized normal bladder cells (SV-HUC-1) expressed apoptin (Fig. 1a). Consistent with the specific expression of E1a in tumor cells, the viral titers of Ad-VT significantly increased in UM-UC-3, T24 and 5636 cells but not in SV-HUC-1 cell, suggesting a tumor-specific replication of Ad-VT (Fig. 1a, b).

Ad-VT induces tumor-specific oncolytic activities in bladder cancer cells

The CCK-8 assay was used to investigate the oncolytic activity of Ad-VT and the results showed that Ad-VT infection significantly decreased the viability of a variety of bladder tumor cells (UM-UC-3, T24, 5637 and EJ-1) in a time-dependent fashion. This effect was not observed in the SV-HUC-1 normal bladder cells, suggesting a tumor-specific antitumor efficacy of Ad-VT on bladder cancer cells (Fig. 2a). Correspondingly, Ad-VT infection (100 MOI) significantly

increased the number of apoptotic cells and cleaved caspase 3 levels in a time-dependent manner, suggesting an Ad-VT-induced apoptosis of tumor cells (Fig. 2b).

Finally, we made a comparative study between the effects of cell treatments with Ad-VT and chemotherapy. A significant decrease in cell viability was observed in Ad-VT and that was similar to that observed following induction with by chemotherapeutic agents (cisplatin, taxol and gemcitabine) in bladder cancer cells (UM-UC-3, T24, 5637 and EJ-1) (Fig. 2c). In addition, in contrast to that these drugs significantly reduced the viability of SV-HUC-1 cells, Ad-VT did not affect its cell viability. The results indicated the safe and potent oncolytic characteristics of Ad-VT.

Ad-VT induces an antitumor effect in T24 bladder cancer xenograft models

To evaluate the potential application of Ad-VT antitumor efficacy as a monotherapy in vivo, T24 xenograft bladder transitional cell carcinoma tumor models were used. In the T24 tumor model, a significant inhibition of tumor growth was observed following six intratumoral treatments with Ad-VT (9×10^7 plague-forming units per time) when compared with the PBS-treated control group (Fig. 3a). In addition, the survival rate was also substantially improved in the Ad-VT treated group (Fig. 3b). No significant difference in body weight gain was observed between the Ad-VT and PBS groups (data not shown). These results demonstrate the antitumor efficacy of Ad-VT in vivo.

Following the intratumoral injection of Ad-VT, the alterations in cell apoptosis and proliferation in the T24 tumors
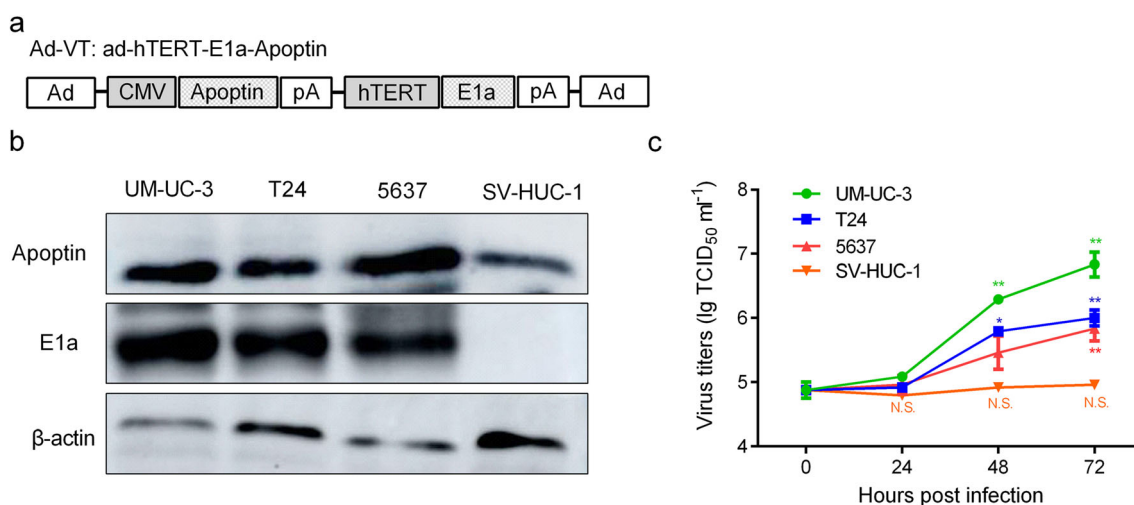

Fig. 1 Ad-VT selectively replicates in bladder cancer cells. **a** Schematic diagram showing the E1a and apoptin expressing cassettes of Ad-VT. **b** Western blotting showing the expression of apoptin and E1a in the bladder cancer cell lines UM-UC-3, T24 and 5637, and in the normal bladder epithelium immortalized cell line SV-HUC-1 24 h after Ad-VT infection (MOI = 100). (c) The viral titers in the indicated cell lines at 0, 24, 48 and 72 h after Ad-VT infection (MOI = 10) $^*p < 0.05$, $^{**}p < 0.01$; N.S., not significant; n = 3 in each group
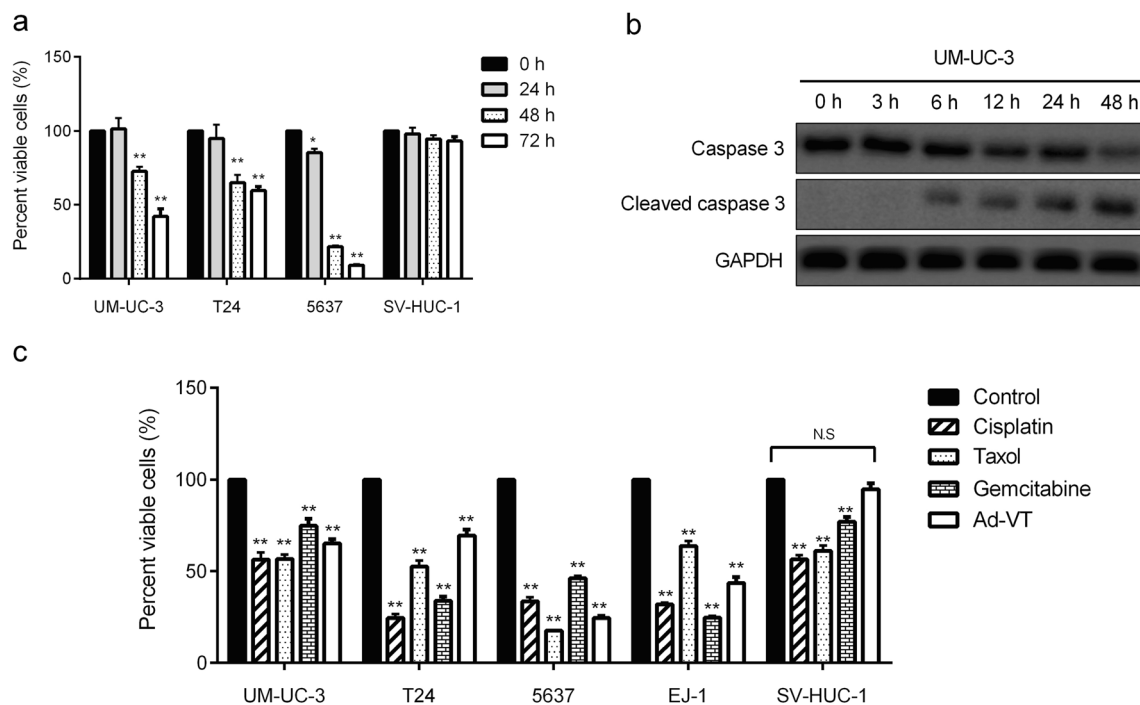
Fig. 2 Ad-VT induces tumor-specific oncolytic activities in bladder cancer cells. **a** CCK-8 assays showing the viability of the indicated cell lines at different time points (0, 24, 48,72 h) after treatment with 100 MOI Ad-VT. **b** Representative western blotting of the expression of caspase 3 and cleaved caspase 3 in UM-UC-3 cells at 0, 3, 6, 12, 24 and 48 h after Ad-VT infection (MOI = 100). **c** CCK-8 assays showing the viability of indicated cell lines at 48 h after Ad-VT infection (MOI = 100) and cisplatin (10 uM), taxol (50 nM) or gemcitabine (25 nM) treatments. $^{*}p < 0.05$, $^{**}p < 0.01$; N.S., not significant; n = 8 in each group

were monitored by TUNEL assay and Ki-67 staining, respectively (Fig. 3c, d). The tumors were collected on day 21 (6 days after Ad-VT or PBS last administration) and serial sections of the paraffin-embedded tumors were used for staining. As expected, The Ad-VT-infected tumors displayed a significantly higher number of apoptotic cells and a lower level of cell proliferation compared with the tumors from the PBS group. These results are consistent with the in vitro study and demonstrate the apoptosis-inducing and proliferation-inhibiting efficacies of Ad-VT in bladder cancer cells.

Furthermore, we examined the virus replication in T24 tumors and extra-tumoral organs following Ad-VT intratumoral injection for 21 days (Fig. 3e, f). A staining for the adenovirus hexon protein was observed in Ad-VT infected tumors tissue, which was absent in the tumors tissue from the PBS group. In addition, no viral replication was observed in extra-tumoral organs, including the liver, spleen and kidney. These results demonstrate a tumor-specific replication of Ad-VT in vivo.

AD-VT induces complete autophagy flux in bladder cancer cells

To determine whether Ad-VT induces autophagy in bladder cancer cell lines, we examined the expression of biomarkers that are involved in autophagy. As shown in Fig. 4a, b), a

decrease in the relative ratio of protein abundance between P62 and GAPDH and an increase in that of the microtubule-associated protein 1 light chain 3-II (LC3-II) were observed over time in the Ad-VT-infected UM-UC-3 and T24 cells. These results suggest that Ad-VT enhances autophagy in bladder cancer cells. In addition, we also assessed the accumulation of autophagosomes, another hallmark of autophagy, in Ad-VT-infected cells. MDC labelling showed that after 24 h infection with Ad-VT (100 MOI), the number of autophgosome-positive UM-UC-3 or T24 cells, was significantly increased. These results demonstrate that Ad-VT induced autophagy in bladder cancer cells (Fig. 4c, d).

LC3-I is generated by the cleavage of newly synthesized LC3 by Atg4B. Upon induction of autophagy, cytosolic LC3-I is converted to lapidated LC3 (LC3-II) that adheres to autophagosome membranes. Autophagosomes fuse with lysosome and form autophagolysosomes, resulting in the degradation of autophagosome contents [12, 13]. We used a mCherry-EGFP-LC3 reporter to observe the stepwise progression of autophagy. The EGFP signal in the mCherry-EGFP-LC3 fusion protein is quenched under the acidic pH of autophagolysosomes, which eases the distinction between autophagosomes and autophagolysosomes [14]. As shown in Fig. 4e, f, red spots were predominantly observed compared to the green ones in the UM-UC-3 and T24 cells infected with
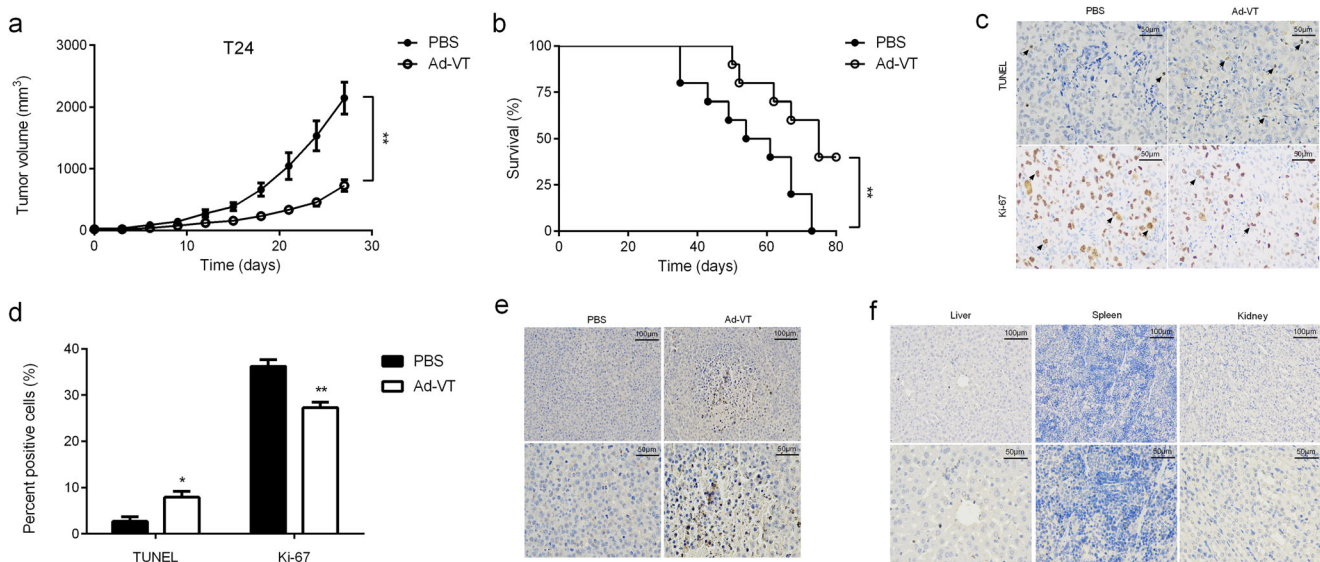
Fig. 3 Ad-VT induces an antitumor effect in T24 bladder cancer xenograft models. BALB/c nude mice bearing T24 cells were intratumorally injected with PBS or with 9×10^7 plague-forming units of Ad-VT on day 0, 3, 6, 9, 12 and 15. (a-b) The tumor growth curves (a) and survival curves (b) of mice are shown. (c-d) T24 tumors were collected on day 21 following intratumoral administration and serial sections of the paraffin-embedded tumors were used for staining. Representative images (c) and analysis (d) of cell apoptosis, as determined by the TUNEL assay, and cell proliferation, as determined by Ki-67 staining, in the PBS or Ad-VT treated tumors. Scale bar = 50 or 100 μm. (e) Virus replication in PBS or Ad-VT treated tumors, as determined by staining for the adenovirus hexon protein. Scale bar = 50 or 100 μm. (f) Virus replication in the liver, spleen and kidney of mice intratumorally treated with Ad-VT, as determined by staining for the adenovirus hexon protein. Scale bar = 50 or 100 μm. $^{*}p < 0.05$, $^{**}p < 0.01$; n = 8 mice in each group (a-b), n = 3 from each group for histological staining (d)

Ad-VT for 12 or 24 h. These results suggest that autophagosomes and autophagolysosomes accumulated and were unimpaired under the above conditions. Moreover, compared with the mock infection, Ad-VT treatment for 24 h significantly increased the number of red spots in bladder cancer cells. These results suggest a complete autophagic flux, induced by Ad-VT infection.

Autophagy promotes oncolytic effects of Ad-VT in bladder cancer cells

Although autophagy can cause cell death, it is also thought to be a survival mechanism for cells under certain conditions [15]. To determine whether autophagy was directly involved in Ad-VT-induced cancer cell death, we used autophagy inhibitors, including 3-methyladenine (3-MA) and LY294007 that suppress autophagy from an early stage, and chloroquine (CQ) that inhibits the fusion of autophagosomes and lysosomes. As shown in Fig. 5a, the Ad-VT-induced decrease in cell viability was partially reversed by the autophagy inhibitors after 48 h treatments, suggesting a cell death-promoting role of autophagy. In addition, Q-VD, an inhibitor of apoptosis, substantially increased cell viability in the Ad-VT-infected bladder cancer cells (UM-UC-3, 5637), demonstrating a vital role of apoptosis in the antitumor activities of Ad-VT. Furthermore, we used siRNA to knockdown Atg5, a key

component for autophagic membrane, in UM-UC-3 and 5637 cells. Western blotting analysis showed that Atg5 expression was successfully downregulated (Fig. 5b) and CCK-8 assay showed that Atg5 knockdown partially reversed cell death caused by Ad-VT infection (Fig. 5c). These results indicate that autophagy promotes Ad-VT-induced cell death.

Finally, we explored whether autophagy causes an increase in Ad-VT replication which could strengthen its oncolytic efficacy. Ad-VT viral titers did not change after treated with 3-MA or rapamycin, an autophagy activator, for 48 h, suggesting that Ad-VT-induced autophagy does not affect virus replication in bladder cancer cells (Fig. 5d).

Autophagy promotes the antitumor activity of Ad-VT in bladder T24 xenograft models

The role of autophagy in Ad-VT induced antitumor activity was investigated in T24 xenograft models. As shown in Fig. 6a and b, Ad-VT (9×10^7 plague-forming units per time) markedly decreased tumors' volume and promoted the percentage of surviving mice, while 3-ma (15 mg/kg) partially inhibited this therapeutic effect. In line with this result, Atg5 knockdown reduced the percentage of surviving mice that were subjected to Ad-VT treatment, further supporting our
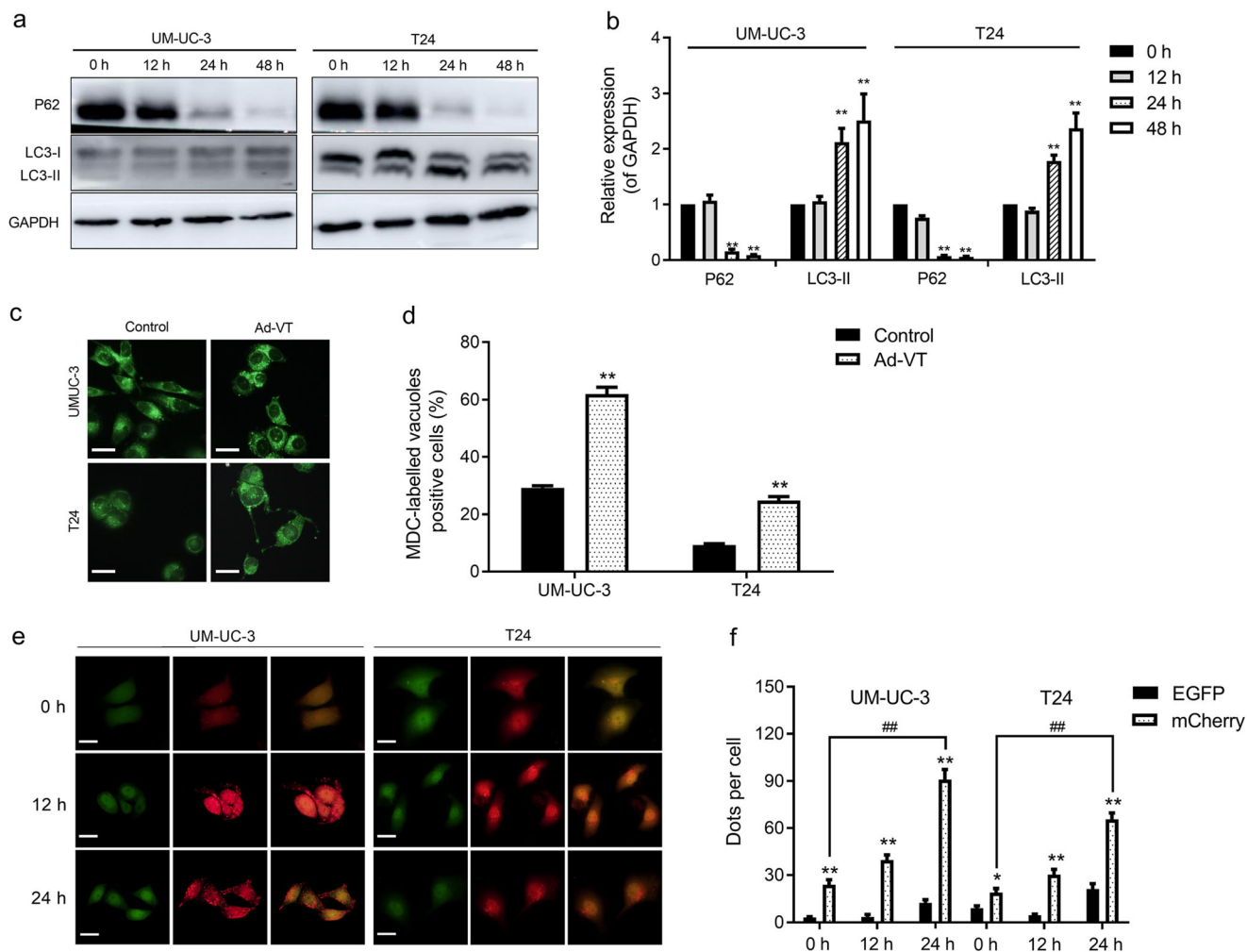
Fig. 4 AD-VT induces complete autophagy flux in bladder cancer cells. **a-b** Representative western blotting (**a**) and their analysis (**b**) for the expression of the autophagy-related proteins P62 and LC3-II in UM-UC-3 and T24 cells at 0, 12, 24 and 48 h after Ad-VT infection. The bar graph shows the relative abundance of these proteins (normalized to that of GAPDH). **c, d** Representative microscopic images from the MDC staining (**c**) and their analysis (**d**) of autophagosomes in UM-UC-3 and

T24 cells at 24 h after Ad-VT infection. Scale bar = 25 µm. **e, f** Microscopic images (**e**) and their analysis (**f**) of mCherry-GFP-LC3 integration into autophagosomes and autophagolysosomes in UM-UC-3 and T24 cells at 0, 12 and 24 h after Ad-VT infection. The average number of dots were calculated in 30 cells. Scale bar = 25 µm. $^*p < 0.05$, $^{**}p < 0.01$; n = 3 for each group. Ad-VT were used at a MOI of 100

results on the involvement of autophagy in Ad-VT induced antitumor effect (Fig. 6c).

Ad-VT activates AMPK-Raptor-mTOR pathway in bladder cancer cells

Autophagy can be induced by inhibiting the mammalian target of rapamycin complex 1 (mTORC1) pathway [16]. To explore the signaling pathway involved in Ad-VT-induced autophagy, we examined the expression of mTOR-related proteins. As shown in Fig. 7b, the expression of phosphorylated mTOR was substantially decreased 24 h and 48 h after Ad-VT infection (100 MOI). In addition, a decrease in phosphorylated S6, a major downstream target of mTOR, was also

observed, suggesting an Ad-VT-induced inhibition of mTOR signaling [17].

To elucidate the mechanisms by which mTOR was inhibited, we examined the AMPK-Raptor pathways [18]. It was reported that the activation of the AMPK-Raptor pathway was involved in the inhibition of mTOR signaling. As shown in Fig. 7d, phosphorylated AMPK and phosphorylated raptor were markedly increased in UM-UC-3 cells 24 and 48 h after Ad-VT infection, suggesting the activation of the AMPK-Raptor pathway by Ad-VT infection.

Taken together, our results indicate that Ad-VT may induce autophagy by targeting the AMPK-Raptor-mTOR signaling pathway.
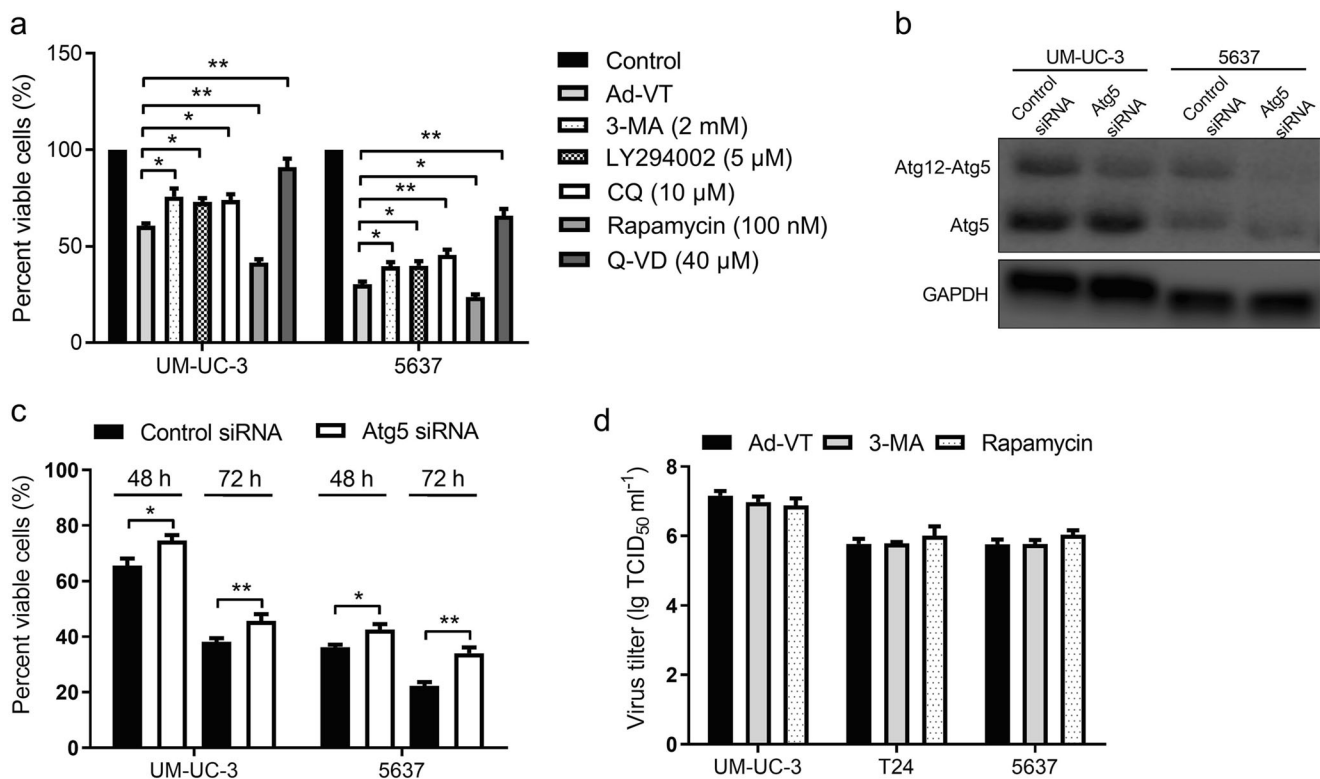
Fig. 5 Autophagy promotes oncolytic effects of Ad-VT in bladder cancer cells. **a** CCK-8 assay showing the cell viability of uninfected control bladder cancer cells UM-UC-3 and 5637 and in the cells infected with Ad-VT, following treatment with 3-MA (2 mM), LY294002 (5 μM), CQ (10 μM) or Q-VD (40 μM) for 48 h. **b** Western blotting analysis of Atg5 knockdown effectiveness in UM-UC-3 and 5637 cells. **c** CCK-8 assays showing the effect of Atg5 knockdown on the viability of UM-UC-3 and 5637 cells, infected with Ad-VT for 48 and 72 h. (j) Virus replication in the bladder cancer cells UM-UC-3, T24 and 5637, infected with Ad-VT for 48 h, in the absence or presence of 3-MA (2 mM) or rapamycin (100 nM). $^*p < 0.05$, $^{**}p < 0.01$; n = 8 for each group (a, c), n = 3 for each group (d). Ad-VT were used at a MOI of 100

Ad-VT induces an enhanced antitumor activity in combination with rapamycin

We hypothesize that Ad-VT therapeutic activity could be further improved when combined with other autophagy-stimulating agents. Rapamycin is known to inhibit mTOR signaling and induces a strong autophagy [19]. In accordance with our hypothesis, rapamycin (100 nM) substantially enhanced the inhibitory activity of Ad-VT (100 MOI) in bladder cancer cells (Fig. 8a). Similarly, the combination of rapamycin (2 mg/kg or 4 mg/kg) and Ad-VT (9×10^7 plague-forming units per time) resulted in a synergistic improvement of tumor control and survival compared to monotherapy (Fig. 8b, c).

Discussion

The present study reported that Ad-VT was able to induce autophagic cell death that promoted antitumor activity in bladder cancer cells in vitro and in vivo. Moreover, the AMPK-Raptor-mTOR pathway was involved in the autophagy induced by Ad-VT. Finally, we demonstrated that the

combination of Ad-VT with rapamycin, a mTOR inhibitor known to stimulate autophagy, resulted in an enhanced antitumor efficacy in bladder cancer models compared to monotherapy.

A specific antitumor efficacy determines the safety and clinical application of an oncolytic therapy [20]. Ad-VT, which contains the hTERT promoter, that controls E1a expression, is a conditionally replicating oncolytic Ad5 adenovirus designed to preferentially replicate in and kill cancer cells [6]. The present study showed that Ad-VT selectively replicated and induced apoptosis in the bladder cancer cell lines UM-UC-3, T24, 5637 and EJ-1, but not in the normal bladder cell line (SV-HUC-1). Consistent with our previous studies showing that Ad-VT specifically expressed E1a protein in melanoma cells lines (A375 and B16) but not in normal human epidermal melanocyte cells [6]. Furthermore, a more favorable safety profile of Ad-VT was found in the present study compared to the typical first-line chemotherapy agents, including cisplatin, taxol and gemcitabine.

In line with the results of the in vitro studies, the intratumoral injection of Ad-VT in T24 xenografted mice models produced significant antitumor efficacy, as demonstrated by decreased tumor volumes and improved survival
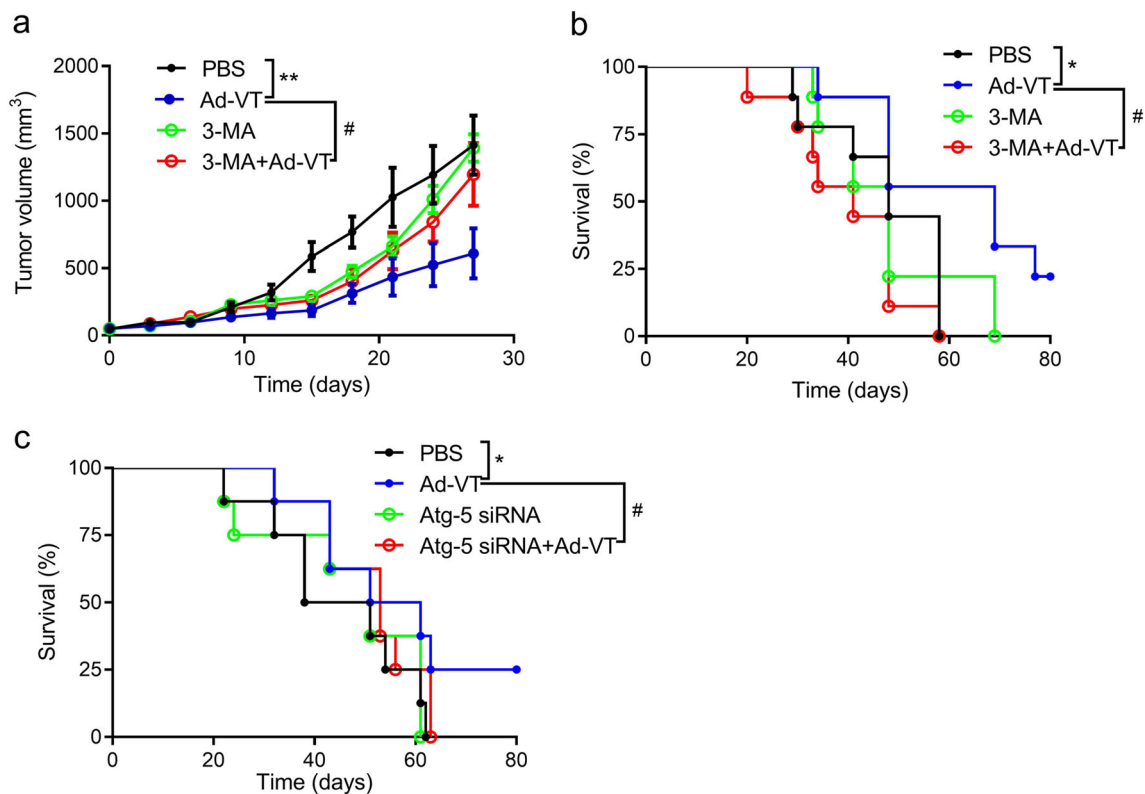
Fig. 6 Autophagy promotes antitumor activity of Ad-VT in bladder T24 xenograft models. BALB/c nude mice bearing T24 cells were intratumorally injected with PBS or Ad-VT. 3-MA (15 mg/kg) was intraperitoneally injected and Atg5 (15ug) was intratumorally injected every three day for a total of six times. The siRNAs were given one day prior to virus treatment. (**a**-**b**) Effects of 3-MA on tumor volume (**a**) and survival rate (**b**) of mice treated with PBS or Ad-VT. (**c**) Effects of Atg5 siRNA on survival rate of mice treated with PBS or Ad-VT. $P^* < 0.05$, $^{**}p < 0.01$. $^{\#}p < 0.05$, N.S., not significant. n = 8 for each group

rate. Moreover, Ad-VT was barely detected in extra-tumoral organs of the following intratumoral virus injection. However, human adenoviruses replicate less efficiently in mouse cells and the ability of our study to recapitulate the actual effect of an oncolytic adenoviral therapy in human patients required further investigation. To partially compensate for this, we intratumorally injected the virus multiple times and over 3-day intervals to mimic the previously reported viral infection-cell lysis-re-infection cycle [21]. Our study demonstrates that Ad-VT may be useful as a precision oncolytic virotherapeutic agent for bladder cancer.

The levels of the autophagic membranes' marker, LC3-II, were increased indicating an increased number of autophagosomes or autophagolysosomes. At the late stage of autophagy, autophagosomes fuse with lysosomes to form autophagolysosomes, which results in the degradation and removal of the enveloped contents, such as p62 which decreased levels indicated a normal function of autopaglysosomes. The present study showed an enhanced and unimpaired autophagic flux caused by Ad-VT, as demonstrated by increased LC3-II levels, decreased P62 expression and an accumulation of autophagosomes and autophagolysosomes. The results were in accordance with a previous study that showed an induction of autophagy in

bladder cancer models by replication competent adenoviruses [22].

However, autophagy has opposing and context-dependent roles in cancer. It is thought that once cancer is established, an increased autophagic flux often enables tumor cell survival and growth. Conversely, excessive autophagy could induce cell death [15]. To elucidate the exact role of autophagy in Ad-VT-induced cancer cell death, we used inhibitors and siRNAs to impede autophagy at multiple stages, including the formation (3-ma and ly294002) and elongation (Atg5 siRNA) of the phagophore, and the fusion of autophagosome and lysosome (chloroquine). Our results showed that the above interventions markedly inhibited bladder cancer cell death induced by Ad-VT. Correspondingly, in the T24 xenograft models, attenuated tumor control and survival by Ad-VT were observed in the 3-ma or Atg5 siRNA treated mice, demonstrating a promoting role of autophagy in Ad-VT antitumor activity. Previous studies showed that autophagy is beneficial for adenoviruses' replication in A549 cells [23]. Thus, we investigated whether the improved oncolytic actions can partially be attributed to an enhanced Ad-VT replication, caused by autophagy. However, neither 3-ma nor rapamycin affected Ad-VT replication in human bladder cancer cells. This discrepancy may be explained by some differences between
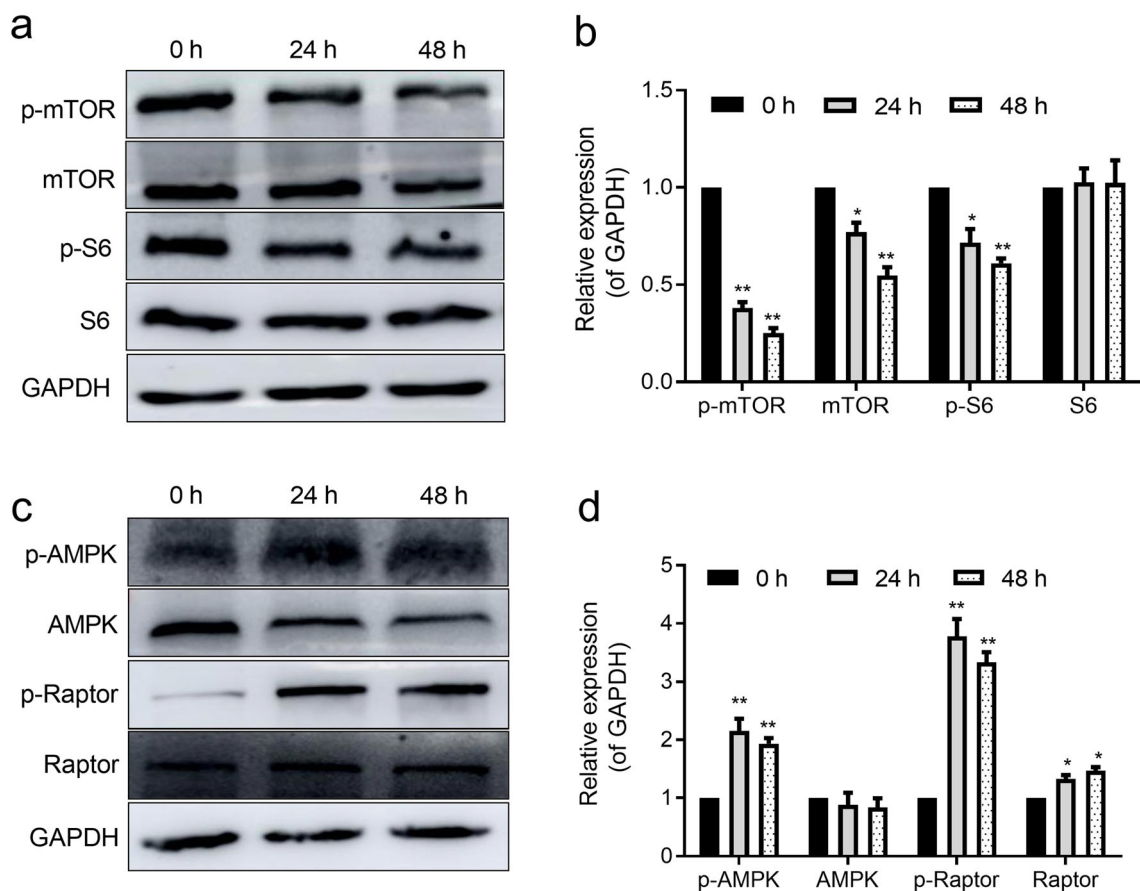
Fig. 7 Ad-VT activates AMPK-Raptor-mTOR pathway in bladder cancer cells. **a**, **b** Representative western blotting (**a**) and their analysis (**b**) of the expression of p-mTOR, mTOR, p-S6 and S6 in UM-UC-3 cells at 0, 24 and 48 h after Ad-VT infection. **c**, **d** Representative western blotting (**c**) and their analysis (**d**) of the expression of p-AMPK, AMPK, p-raptor

and raptor in UM-UC-3 cells at 0, 24 and 48 h after Ad-VT infection. $^*p < 0.05$, $^{**}p < 0.01$. n = 3 for each group. A bar graph for western blotting analysis that shows the relative abundance of proteins (normalized to that of GAPDH). Ad-VT was infected at a MOI of 100
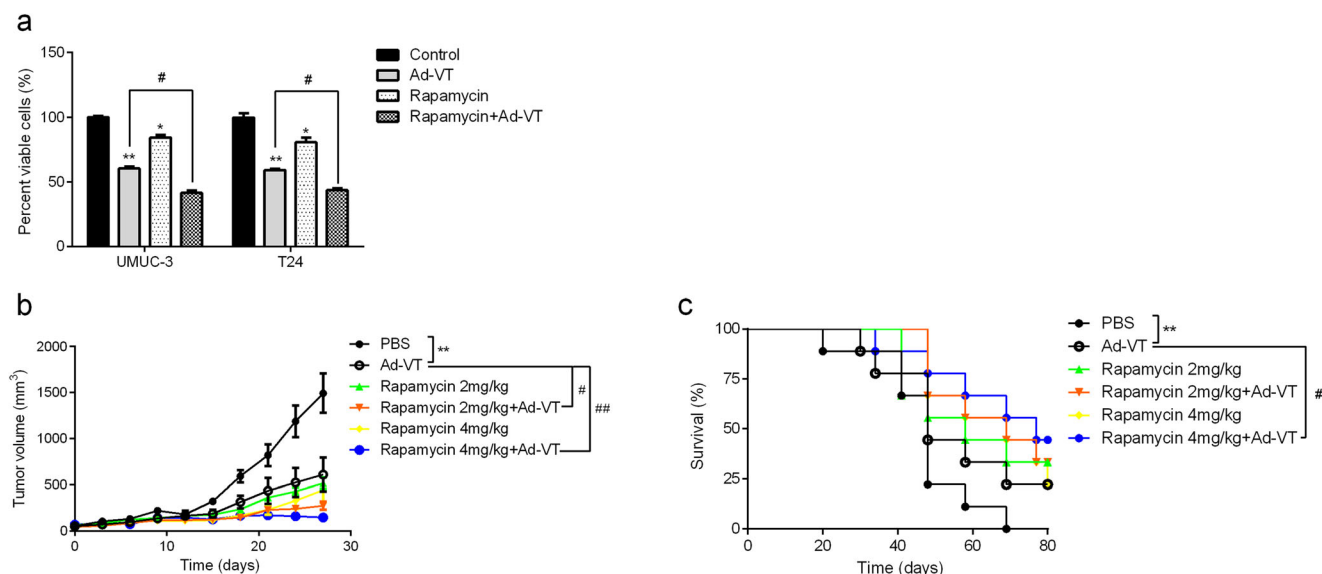

Fig. 8 Ad-VT induces an enhanced antitumor activity in combination with rapamycin. (**a**) A CCK-8 assay showing the viability of Ad-VT (100 MOI)-infected UMUC-3 and T24 cells, with or without rapamycin treatment (100nM). (**b-c**) Tumor volume (**b**) and percentage of surviving

mice (**c**). BALB/c nude mice bearing T24 cells were intratumorally injected with PBS or Ad-VT on day 0, 3, 6, 9, 12 and 15, along with an intraperitoneal injection of rapamycin (2 mg/kg or 4 mg/kg). $P < 0.05$, $^{**}p < 0.01$. $^#p < 0.05$, $^{##}p < 0.01$. n = 8 for each group

A549 and bladder cancer cell lines in the adenoviral replication process after infection.

Autophagy is monitored by a multi-layered control system. The best recognized inhibitor of autophagy is the mammalian target of rapamycin complex 1 (mTORC1) [24]. This complex can phosphorylate the 40S ribosomal protein S6 and depress the formation of autophagy-related proteins, including LC3-II [25]. In addition, AMPK can inhibit mTORC1 by increasing the phosphorylation of raptor when ATP levels dwindle and AMP accumulates [13]. Accordingly, we found that Ad-VT significantly raised AMPK and raptor phosphorylation and consequently decreased p-mTOR and p-S6 levels, suggesting that the AMPK-Raptor-mTOR pathway is involved in the Ad-VT-induced autophagy. These results demonstrate that the autophagic antitumor activity of Ad-VT may involve the AMPK-Raptor-mTOR pathway.

We further inhibited mTOR signaling and hypothesize that increasing autophagic cell death by combining adenovirus-derived oncolytic therapy and autophagy stimulators may be an appealing approach to improve antitumor activity. Rapamycin, a specific and preferential mTOR inhibitor, can trigger autophagy and suppress tumor growth. In the present study, we reported a synergistic improvement in tumor control and survival outcomes when combining Ad-VT and rapamycin (2 mg/kg, 4 mg/kg) treatments and when compared to monotherapy.

In summary, Ad-VT exerts significant antitumor efficacy in bladder cancer, that is partially attributed to an enhanced autophagic process. More importantly, the AMPK-Raptor-mTOR pathway plays a critical role in the autophagic cancer cell death induced by Ad-VT. Finally, combining oncolytic adenovirus with autophagy stimulators is a promising approach to promote therapeutic efficacy.

Conclusions

We identified Ad-VT as a highly selective oncolytic therapeutic tool in bladder cancer. We also showed that the enhanced autophagy through AMPK-Raptor-mTOR pathway was associated with Ad-VT-induced cell death. Treatment combination of Ad-VT and rapamycin resulted in a synergistic improvement in antitumor efficacy compared to monotherapy. Our results provide mechanistic insights into the autophagic cell death induced by adenovirus-based oncolytic virotherapy in bladder cancer, and prompt its therapeutic use in combination with autophagy stimulators.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethics approval and consent to participate The animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the Academy of Military Medical Science (AMMS) and all efforts were made to minimize animal suffering and reduce the number of animals used for the experiments.

Consent for publication Not applicable.

Research involving human participants and/or animals The animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the Academy of Military Medical Science (AMMS). The manuscript does not contain clinical studies or patient data.

Informed consent For this type of study, informed consent is not required.

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