**Original Article**

**The antitumor effect of the novel cancer-specific adenovirus Ad-VEGFR on bladder cancer in vitro and in vivo**

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**Abstract:** Bladder cancer is one of the most common cancers. Approaches that block tumor angiogenesis are a new therapeutic strategy for locally advanced or metastatic BC. VEGF/VEGFR signaling has been obviously and negatively correlated with the progression and invasion of cancer. In this study, we constructed the recombinant adenovirus vAd-VEGFR-3 to investigate its antitumor effect in vitro/vivo. First, we used the recombinant adenovirus vAd-VEGFR-3 to infect bladder cancer cells and then collected the cell culture supernatant to treat human umbilical vein endothelial cells (HUVECs). The proliferation, migration and apoptosis of HUVECs were respectively detected by MTT, transwell and Annexin V-FITC/PI double staining. In addition, mouse bladder mucosa was injured by trypsin, and the orthotopic transplantation model of human bladder cancer was successfully constructed to clarify the anti-tumor effect of Ad-VEGFR in vivo. The results showed that Ad-VEGFR could inhibit the cancer’s proliferation and migration, while promoting the apoptosis of HUVECs in vitro. Moreover, Ad-VEGFR could significantly promote the apoptosis of bladder cancer cells and then prevent tumor growth in vivo. In addition, it also down-regulated the expression levels of CD31, an endothelial cell marker which is closely related to the angiogenesis. Taken together, it suggests that the infection of adenovirus-carrying VEGFR in bladder cancer cells may inhibit blood vessel formation and prevent tumor progression.

**Keywords:** Bladder cancer, VEGFR, adenovirus, anti-tumor effect, bladder mucosa injury

**Introduction**

Bladder cancer (BC), a common urogenital system malignant tumor, ranks as the 7th most frequently diagnosed cancer in men [1] and is the most expensive malignancy to treat on a per-patient basis due to the requirement for lifelong routine monitoring and treatment from diagnosis to mortality [2, 3]. Histologically, BC is categorized into non-muscle invasive BC (NMIBC, ∼70%) and muscle invasive BC (MIBC, ∼30%) [4]. Current therapeutic strategies for treating BC usually combine surgery with multiple adjuvant therapies, such as chemotherapy [5]. However, it still has a low survival rate for patients with locally advanced or metastatic BC [5]. Additionally, drug resistance is a major obstacle for successful systemic therapeutics [6].

Expansion of vasculature is critical for tumor growth, which is closely related to angiogenic support provided by vascular endothelial growth factor-A (VEGF-A or VEGF) and other soluble factors [7]. VEGF is a key angiogenic factor that exerts multiple functions including the stimulation of angiogenesis, inflammation, vascular permeability and vasculogenesis [8]. Meanwhile, some reports showed that VEGF produced by tumor cells was able to act on neighboring VEGFR-expressing ECs to promote neovascularization for continued tumor growth [9]. A mountain of evidence has accumulated to support the hypothesis that anti-VEGF therapies may have a dual effect on both paracrine and autocrine VEGF loops within tumors [10, 11]. It has been suggested that VEGFR were abundantly expressed by a large percentage of solid tumors, and VEGF/VEGFR signaling was
significantly and negatively correlated to the progression and invasion of cancer [12, 13]. Hence, it is a useful strategy for BC treatment by interfering with the expression VEGFR.

Gene therapy has the advantage of selectively correcting or eradicating defective tissue and targeting defects in malignant cells [5]. Hence, it offers a novel and effective strategy for patients who are resistant to traditional therapies [5]. Several recent studies pay attention to transfection efficiency and expression reliability in cancer gene therapy [14]. Due to the advantage of preferential replication in tumor cells, adenovirus vector was more and more attractive in gene therapy [15]. Oncolytic adenoviruses had adapted to a number of clinical trials in recent decades [15]. However, no replicating adenoviruses have had any effect on eradicating tumor cells [16]. Therefore, it is important to develop a bladder cancer gene therapy using a novel adenoviral vector system.

In this study, we mainly investigated the biological processes between VEGFR and BC in vitro/vivo and aimed to illustrate whether the infection of adenovirus carrying VEGFR in bladder cancer cells could decrease the progression and invasion of BC. Our study may provide an effective and promising therapeutic strategy for BC.

Materials and methods

Cell lines and culture

The human EJ cell line and human HUVECs were purchased from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and 100 U/ml penicillin/streptomycin (Beyotime Biotechnology, Nanjing, China). All cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

The preparation and transfection of recombinant adenovirus vector

To construct an adenovirus vector carrying VEGFR, the human VEGFR-3 extracellular domain gene was amplified by RT-PCR according to the primer as: forward, 5'-GGA TCC GAC GGC CTG GTG AGT GAC TA-3'; reverse, 3'-GAA TTC CTT TGA GCC ACT CGA CGC TGA TGA-5'. The amplified DNA products were double digested by restriction endonucleases EcoR1 and BamH1, and then cloned to the adenovial shuttle plasmid pAdTrack-CMV to obtain the resultant pAdTrack-CMV-VEGFR-3 (Ad-VEGFR group). The empty adenovirus vector was used as a negative control (Ad-NC group). All constructed adenovirus vector were co-transfected into BJ5183 bacteria with the plasmid pAdeasy-1. The adenoviral plasmid carrying VEGFR-3 was generated with a homologous recombinant in the bacteria. The adenoviruses were transfected in 293 cells. The recombinant adenovirus vAd-VEGFR-3, which has infective stability, was packed in 293 cells. The protein expression level of VEGFR-3 was validated by western blot with an anti-VEGFR antibody (Abcam, Cambridge, Massachusetts, USA).

Validation for the expression of target gene by qRT-PCR: EJ cell lines were infected with recombinant adenoviruses vAd-VEGFR-3 or an empty adenovirus vector at a multiplicity of infection (MOI) of 10 pfu/cell for 12 h. The expression of the target gene was tested by qRT-PCR. In brief, cells were harvested and total RNA samples were isolated after cell lysis using the NucleoSpin RNA II Kit (Macherey-Nagel, Duren, Germany). The amount of isolated RNA was quantified by measuring its absorbance at 260 nm. RNA samples were reverse-transcripted into cDNA with a Reverse Transcription Kit (Bio-Rad laboratories, CA, USA). qRT-PCR was then carried out on a MyiQ single-color RT-PCR detection system with SYBR Green Supermix (ComWin Biotech Co., Ltd, Jiangsu, China). Primer sequences for each gene were as follows: VEGFR, reverse: 5'-CAG GTT ATT CGC TTC CCA TCA-3'; forward: 5'-TGA AGG AAG GCA GAT CGT CAT-3'; GAPDH, forward: 5'-CATGGGTGGAA-TCAATTGGGA-3', reverse: 5'-GAAGGTGAGGT CGGAGT-3'. The 2ΔΔCt method was used for data analysis. All samples were normalized to the expression of GAPDH.

Cell viability analysis for HUVECs proliferation: A cell viability assay was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA), as described previously [17]. Briefly, the cell culture supernatant from EJ cells infected with recombinant adenoviruses vAd-VEG-
FR-3 for 12 h was collected for the following experiments. HUVECs were seeded into a 96-well plate at density of $5 \times 10^4$ cells/mL and treated with cell culture supernatant collected above for 24 hours. Aspirate off supernatant, 20 μl MTT (5 mg/ml) was added to each well and incubation was continued at 37°C for 4 h. The culture medium was aspirated and 150 μL dimethylsulfoxide was added to dissolve the insoluble purple formazan product into a colored solution. Absorbance was subsequently measured at 490 nm.

Flow cytometry analysis for HUVECs apoptosis: HUVECs were inoculated in a 12-well plate at density of $5 \times 10^5$ cells/mL and treated with cell culture supernatant collected above for 24 hours or 48 hours. The cells were washed with PBS and suspended in a 100 μL binding buffer, and then apoptosis was determined using an Annexin V/pyridine iodide (PI) apoptosis detection kit (Beyotime Biotechnology, Nanjing, China).

Transwell detection for HUVECs migration: HUVECs was re-suspended in serum-free RPMI1640 at a density of $5 \times 10^5$ cells/mL, and then added to a transwell chamber (100 μL/well) with a lower layer xx 600 μL RPMI 1640 containing 10% FBS. Three duplicates were set for one group. The transwell chambers were removed after 24 hours or 48 hours incubation. Non-migrated cells on the insert membrane were wiped with a cotton swab, and migrated cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The cells were counted under a light microscopy (BX51, Olympus, USA).

Bladder mucosa injury and orthotopic BC model in mouse

Male nude mice (BALB/c-foxn1<sup>−/−</sup>, 4-6 weeks old) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, Jiangsu, China). The animals were provided with continuous access to standard rodent chow, water and housing. The mice were randomly divided into two groups with each group containing ten mice. The mice were treated as follows: 1) control group, normal saline stimulation; 2) model group, trypsin stimulation. In brief, the animals were anesthetized by 1% pentobarbital sodium and fixed on a plate. After urination by squeezing the lower abdomen of mice, each bladder was washed with PBS by inserting a catheter through the urethra. Then, each bladder was injected with 100 μL trypsin or normal saline, and the urethras were ligated by a thin wire for 30 min. Twenty-four hours later, the bladders were removed from the sacrificed mice, followed by hematoxylin and eosin (H&E) staining. After validating the mucosal injury induced by trypsin, we further clarified the relationship between mucosal injury and orthotopic BC. Male nude mice were randomly divided into two groups (n=10 for each group) as following: 1) normal saline+EJ cells implantation; 2) trypsin+EJ cells implantation, which were anesthetized, urinated and then injected with trypsin or normal saline (100 μL) in the bladders as described above. After 30 min of urethral ligation, trypsin or normal saline was released and the bladders, with or without mucosal injury, were washed with PBS. Then, bladder cancer cells-EJ cell lines were injected into bladder and the urethras maintained ligation for one hour. To evaluate the effect of mucosal injury on the rate of bladder tumor implantation, each bladder was palpated to identify the lump, sclerosis and hematuria symptoms at 1, 2, 3 and 4 weeks after surgery. Meanwhile, at the fourth week, all animals were sacrificed via cervical dislocation and their bladders were subjected to routine H&E staining.

The effect of Ad-VEGFR on tumor growth: The mice orthotopic BC model was established as described above. That is, all animals were anesthetized, urinated and then injected with trypsin (100 μL) in their bladders. After 30 min of urethral ligation, trypsin was released and the bladder with mucosal injury was washed with PBS. Then, the bladder was subjected to tumor implantation with EJ cells, following urethral ligation for one hour. The criteria of the orthotopic BC model were intended to be the formation of a lump, hematuria symptoms and bladder sclerosis. These model mice were randomly divided into a control group, an Ad-NC group and an Ad-VEGFR group, n=5 for each group. The mice were respectively treated by injecting normal saline, recombinant adenoviruses vAd-VEGFR-3, or empty adenovirus vector into their bladders. All groups were treated two times per week. After 3 weeks, all animals were weighed and sacrificed via cervical dislocation. The
Tumors were immediately weighted and then fixed with 4% paraformaldehyde.

**TUNEL assay for bladder cancer cells apoptosis in tumor tissue:** Immunoperoxidase detection using the indirect TUNEL method was performed to analyze the apoptosis (Chemicon International, Temecula, CA, USA). The tumor tissue specimens fixed in 4% paraformaldehyde were embedded in paraffin and cut into 4 μm thick sections. Slices were deparaffinized, re-hydrated and then incubated with 20 μg/mL proteinase-K at room temperature for 15 min. After treatment with hydrogen peroxide to block endogenous peroxidase activity, slices were washed with PBS 3 times and then immersed in TdT reaction enzyme for 20 min. After we added a stop/wash buffer to quench the reaction, the slices were incubated with anti-digoxigenin-peroxidase conjugate in a humidified chamber for 30 min at room temperature. Then, the slices were washed in PBS and visualized using a metal enhanced DAB substrate kit (Thermo Fisher Scientific, Waltham, MA, USA). Finally, the slices were counterstained with hematoxylin, dehydrated with ethanol and Citrisol V (Thermo Fisher Scientific, Waltham, MA, USA) and mounted.

**Immunofluorescence for CD31 expression in tumor tissue:** To detect the expression of CD31, the bladder cancer mouse model was established as described above. Meanwhile, all the animals were subcutaneously injected with 0.5...
ml Matrigel™ (BD Biosciences, Bedford, MA) containing recombinant mouse (rm) VEGF (0.4 μg/mL) to induce angiogenesis. One day after the Matrigel™ injection, the mice were treated with adenovirus as described above. All groups were treated two times per week. The stimulation of Matrigel™ lasted for 7 days, and the tumors were collected and stored frozen at -80°C after 3 weeks treatment with the adenovirus. The frozen specimens were fixed with cold acetone, permeabilized in 0.1% Triton, blocked in 8% goat serum, incubated overnight at 4°C with anti-CD31 (Abcam, Cambridge, Massachusetts, USA), and visualized under a fluorescence microscope (BX51, Olympus, USA).

**Statistical analysis**

The statistical significance of differences was assessed using a one-way analysis of variance, where P<0.05 was considered to indicate a statistically significant difference. All statistical tests were performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). All data are presented as mean ± SD.

**Results**

**Infection of recombinant adenovirus vAd-VEGFR-3 could stable over-expressing VEGFR gene in EJ cell lines**

After infecting with recombinant adenovirus vAd-VEGFR-3, we detected the expression levels of the VEGFR gene by qRT-PCR and found that adenovirus could significantly up-regulate VEGF mRNA expression with an increased production rate of nearly more than five times when compared with empty adenovirus vector treated EJ cells (P<0.001, vs Ad-NC group), as shown in Figure 1A, suggesting that recombinant adenoviruses carrying VEGFR-3 could stably replicate in bladder cancer cells.

**Ad-VEGFR inhibited the proliferation of HUVECs:**

Previous studies have reported that VEGF-A/VEGFR-2 signaling pathways could stimulate the secretion and activation of matrix metalloproteinases (MMPs) in endothelial cells which result in cell proliferation, migration and survival [18, 19]. To investigate the regulatory role of Ad-VEGFR on cell motility we generated the ad-VEGFR plasmid and transfected it into bladder cancer cells. The MTT assay showed that the proliferation of EJ cells was inhibited with the prolongation of the processing time, especially after 48 hours treatment (P<0.01 or P<0.001, vs Ad-NC group), as shown in Figure 1B. In addition, we analyzed the apoptosis ratio of HUVECs using flow cytometry. As shown in Figure 2, Ad-VEGFR suppresses the transmigration of EJ cells detected by the transwell method. A. The cells were stained with 0.1% crystal violet. Representative results are shown. B. The number of cell in the upper, lower, left, right and middle visual fields were all counted under light microscopy. The values presented are the mean ± SD of three independent experiments. Ctrl: control group; Ad-NC: EJ cells infected with empty adenoviruses vector; Ad-VEGFR: EJ cells infected with recombinant adenoviruses vAd-VEGFR-3. ***P<0.001, vs Ad-NC group.

**Ad-VEGFR promoted the apoptosis of HUVECs:**

In addition, we analyzed the apoptosis rate of HUVECs using flow cytometry. The results showed that Ad-VEGFR could enhance HUVECs apoptosis at 24 h or 48 h (Figure 1C, 1D). The apoptosis ratio respectively enhanced from 5.79% and 6.38% to 17.29% and 24.25% at 24 h and 48 h.

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Figure 1C and 1D, the cell culture supernatant treatment obviously augmented the apoptosis rate of HUVECs (P<0.001, vs Ad-NC group). At 24 hours, the apoptosis rate enhanced from 5.79% to 17.29%; while at 48 hours, the apoptosis rate enhanced from 6.38% to 24.25%.

Ad-VEGFR suppressed the transmigration of HUVECs: Endothelial cell migration is known to be driven by VEGF gradient [20]. Here, we detected the transmigration of HUVECs by the transwell method and found that HUVECs treated with the recombinant adenoviruses vAd-VEGFR-3 had a significantly lower transmigration compared with the Ad-NC group (P<0.001), especially at 48 hours, with a 65% decrease (140 cells in the Ad-NC group vs 48 cells in the Ad-VEGFR group), as shown in Figure 2.

Trypsin could induce mouse bladder mucosa injury

To construct the bladder mucosa injury model, we injected normal saline or trypsin into mouse bladders with emptied of urine. The gross anatomy showed that, in the normal saline group, the bladder mucosae of the nude mice were smooth, the bladder walls were thin and uniform in thickness, the blood vessel texture was clear, and no lumps were found; however, in the trypsin group, the bladder mucosae of the nude mice showed diffuse hyperemia, edema and

Figure 3. Mouse bladder mucosa injury was closely related to the high incidence of bladder cancer. A. H&E staining (×200) of mice bladder mucosa. In the normal saline group, mouse bladder mucous was composed of 3~4 layers of transitional epithelial cells, which were flat in shape, uniform in size, with a round nucleus and an ordered arrangement; while trypsin-treatment led to bladder mucosa edema and mucosal shedding, capillary expansion, and leukocyte infiltration. B. H&E staining (×200) of mice bladder mucosa. In the trypsin-treated group, the disordered structure and irregular shape of some transitional epithelial cells and the infiltrated cells in tunica muscularis vesicae urinariae was observed. Three parallel samples for each group.
were dark red. Meanwhile, the H&E staining indicated that the normal mouse bladder mucous was composed of 3~4 layers of transitional epithelial cells, which were flat in shape, uniform in size, with a round nucleus and ordered arrangement; while the trypsin treatment led to bladder mucosa edema and mucosal shedding, capillary expansion, and leukocyte infiltration (Figure 3A), suggesting bladder mucosa injury.

Mouse bladder mucosa injury was closely related to the high incidence of bladder cancer

After validating the mucosal injury induced by trypsin, we further clarified the relationship between mucosal injury and the rate of bladder tumor implantation. Similarly, in the normal saline+EJ cells implantation group, the gross anatomy and H&E staining of mouse bladder mucous exhibited no abnormal changes compared with the normal mouse bladder mucous. This indicates that bladder cancer cell implantation without bladder mucosa injury could not induce bladder cancer. Interestingly, after the bladder mucosa injury stimulated by trypsin, the implantation of bladder cancer cells easily led to a high incidence of bladder cancer, showing as the disordered structure and irregular shape of some transitional epithelial cells and the infiltrated cells in tunica muscularis vesicae urinariae (Figure 3B). All of these data demonstrated that the orthotopic transplantation model of human bladder cancer was successfully constructed.

Ad-VEGFR could apparently reduce the growth of bladder cancer in vivo: We have confirmed that Ad-VEGFR could obviously inhibit the proliferation of HUVECs in vitro. Hence, we further utilized the orthotopic transplantation model of human bladder cancer to evaluate the anti-

Figure 4. Ad-VEGFR could obviously suppress tumor growth. Tumor tissue photos (A), tumor volume (B) and tumor weight (C) are shown. (D) The apoptosis of bladder cancer cells in tumor tissue was analyzed using the TUNEL assay, with three parallel samples for each group. Ctrl: control group; Ad-NC: EJ cells infected with empty adenoviruses vector; Ad-VEGFR: EJ cells infected with recombinant adenoviruses vAd-VEGFR-3. *P<0.05, vs Ad-NC group.
tumor effect of Ad-VEGFR in vivo. The bladder cancer bearing mice were treated respectively with normal saline, recombinant adenoviruses vAd-VEGFR-3 or an empty adenovirus vector. The results showed that the tumor growth could be significantly suppressed by Ad-VEGFR compared with the control group and the Ad-NC group with the volume and weight of the tumor decreased by a factor of two (Figure 4A-C).

Ad-VEGFR promoted the apoptosis of bladder cancer cells in tumor tissue: To investigate whether Ad-VEGFR could induce the apoptosis of bladder cancer cells, the bladder cancer bearing mice were treated as described above. The results of the TUNEL assay also demonstrated that Ad-VEGFR could enhance the apoptosis of bladder cancer cells in vivo, suggesting that Ad-VEGFR could inhibit tumor growth by promoting bladder cancer cell apoptosis (Figure 4D).

Ad-VEGFR obviously inhibited the expression of CD31 in vivo: CD31 is expressed on endothelial cells [21] and acts as an endothelial cell marker. CD31 signaling participates in the regulation of angiogenesis [22]. Therefore, we also detected the expression of CD31 in tumor tissue by

Figure 5. Ad-VEGFR down-regulated CD31 expression and was detected by immunofluorescence assay (×400). Red represents CD31 protein; Blue represents the nucleus stained by DAPI. Ctrl: control group; Ad-NC: EJ cells infected with empty adenoviruses vector; Ad-VEGFR: EJ cells infected with recombinant adenoviruses vAd-VEGFR-3.
immunofluorescence analysis. The results show that CD31 expression was obviously reduced after the Ad-VEGFR treatment (Figure 5). These data suggest that Ad-VEGFR may inhibit tumor blood vessel formation and prevent tumor progression.

Discussion

Bladder cancer is the most common malignant urologic neoplasm in the world, especially in elderly men [23, 24]. In the last decades, only minor changes have been reported in the 5-year survival of patients with bladder cancer [24]. The reason for this dismal prognosis may be partially explained by the small number of successful clinical trials and effective therapeutic targets lack [25]. Hence, it is novel therapeutic approaches that reproduce and validate biomarkers [25, 26]. The VEGF family consists of several members that play an important role in tumor angiogenesis and progression [27]. VEGF produced by tumor cells acts on neighboring VEGFR-expressing ECs to promote neovascularization for continued tumor growth [27, 28]. Some reports showed that VEGF expression level had a strong association with disease recurrence in superficial and in muscle invasion bladder tumors [29]. In addition, VEGF and VEGFR mRNA levels are reported to be higher in superficial bladder cancer patients compared with normal patients' bladder samples [30].

In this study, we constructed recombinant adenovirus vAd-VEGFR-3 to evaluate its antitumor effector in vitro/vivo. In our study, recombinant adenoviruses carrying VEGFR-3 could stably replicate in bladder cancer cells. The angiogenesis in tumor tissue is a complicated process involved in the activation, proliferation and migration of vascular endothelial cells. Our results showed that Ad-VEGFR could inhibit the proliferation and migration of HUVECs and promote the apoptosis of HUVECs in vitro, suggesting that the infection of adenovirus-carrying VEGFR in bladder cancer cells may inhibit angiogenesis in tumor tissue. Further, to clarify the anti-tumor effects of Ad-VEGFR in vivo on bladder cancer, the orthotopic transplantation model of human bladder cancer was established. Here, the bladder mucosa injury model was induced by trypsin, because we found that mouse bladder mucosa injury was closely relat-ed to the high incidence of bladder cancer, while bladder cancer cell implantation without bladder mucosa injury could not induce the incidence of bladder cancer. The data from an in vivo study demonstrate that Ad-VEGFR could significantly promote the apoptosis of bladder cancer cells and prevent the tumor growth in vivo. CD31 often acts as an endothelial cell marker because it mainly expresses on endothelial cells [21]. In addition, CD31 signaling participates in the regulation of angiogenesis [22]. Interestingly, our study indicated that Ad-VEGFR also suppresses the expression levels of CD31, which is closely related to angiogenesis in cancer. Taken together, it suggests that the infection of adenovirus carrying VEGFR in bladder cancer cells may inhibit blood vessel formation, and prevent tumor progression. Therefore, it may be a better and novel strategy for bladder cancer treatment.

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Disclosure of conflict of interest

None.

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