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Original article

Prostate stem cell antigen enhancer and uroplakin II promoter based bladder cancer targeted tissue-specific vector☆

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Abstract

Purpose: To construct a dual specific vector which contains prostate stem cell antigen enhancer (PSCAE) and uroplakin II (UPII) promoter targeted bladder cancer.

Methods: UPII promoter and PSCAE were amplified by polymerase chain reaction (PCR). Luciferase gene (LUC) was obtained from plasmid pBK-CMV-LUC. PSCAE, UPII promoter and LUC were inserted into shuttle plasmid to create Rp-UPII-LUC and Rp-PSCAE-UPII-LUC. Rp-UPII-LUC and Rp-PSCAE-UPII-LUC were cotransfected with pCMV-β-gal into various cell lines at the presence or absence of androgen receptor agonist R1881 and androgen receptor antagonist flutamide. Luminescence was detected with luciferase assay kit and counted on liquid scintillation counter.

Results: Bladder cancer cells showed higher LUC activity than non-bladder cancer cells after transfected with plasmids Rp-UPII-LUC and Rp-PSCAE-UPII-LUC. PSCAE could improve the LUC activity in both AR positive and AR negative bladder cancer cells but not in non-bladder cancer cells and normal human urothelial (NHU) cells. R1881 could increase the LUC activity in AR positive bladder cancer cells but not in AR negative bladder cancer cells and non-bladder cancer cells. Flutamide could not inactivate PSCAE in bladder cancer cells.

Conclusions: PSCAE can improve target gene expression in bladder cancer cells but not in non-bladder cancer cells and NHU cells. PSCAE maintains a certain level of androgen independent activity in bladder cancer cells. PSCAE is active in both AR positive and AR negative bladder cancer cells. The results suggest that combination of PSCAE with UPII promoter is feasible in constructing bladder cancer-specific vectors.

Keywords: Prostate stem cell antigen enhancer; Uroplakin II promoter; Gene therapy; Bladder cancer

1. Introduction

Bladder cancer is a commonly occurring cancer. Existing local therapies for transitional cell carcinoma (TCC) of the bladder include local resection for nonmuscle-invasive disease and cystectomy for muscle-invasive disease. These strategies are effective but far from satisfactory. Nearly 50% to 70% of patients treated for superficial disease develop recurrence, and as many as 20% progress to more aggressive disease [1]. Thus, novel treatments need to be developed.

Cancer is a disease arising from aberrant gene regulation. In principle, therefore, gene therapy should be able to reverse this process. Recent advances in genetics and molecular biology have allowed the development of gene therapy as a novel approach for augmenting current cancer treatment. Present gene therapy treatments have their limitations because they are inefficient and prone to cause side effects as a result of the lack of tissue or cancer specificity. Tissue- or tumor-specific vector represents one of the most promising gene therapy strategies for a variety of malignancies. The differential expression of the desired gene in the target tissue is essential for optimal effect [2]. One approach for

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targeting gene therapy is using tissue-specific promoter to drive therapeutic gene expression. Since tissue-specific promoter has activity only in target cells, toxicity from treatment can be limited [1]. To find and to identify ideal tissue- or cancer-specific promoters are essential for targeting gene therapy. In our previous studies, we placed a tissue-specific promoter prostate-specific antigen (PSA) in control of the replication genes of adenovirus [3–6]. The results demonstrated that adenoviral replication is limited to only those cells that are capable of expressing PSA (prostate epithelium).

With its anatomic location, bladder is an easily accessible structure and appears to be an ideal site for gene transfer. Hence, most efforts involving bladder cancer gene therapy are being directed towards intravesical delivery. Direct intravesical treatment has low toxicity and thus can be readily combined with existing treatment. On the gene therapy of bladder cancer, Zhang and colleagues [7] reported a conditionally replicative adenovirus targeted to bladder cancer through the use of the urothelial-specific UPII promoter. Uroplakins are a group of integral membrane proteins synthesized as the major differentiation products of mammalian urothelium [8], are highly conserved, and may be employed as tumor markers for TCCs [9]. UPII promoter results in preferential expression in bladder carcinoma cells, with negligible expression in nonurothelium cells [7]. The utility of the UPII promoter in the generation of urothelium-specific adenoviral vectors provided a potential foundation for the development of bladder tumor-specific oncolytic viral therapies. This promoter is particularly attractive, as it is active even in the most poorly differentiated urothelial cancers. However, UPII is also expressed in normal bladder. We try to further functionalize UPII promoter in bladder cancer cells and limit its activity in nonbladder cancer cells by inserting a cancer-specific enhancer at the upstream of UPII promoter.

Prostate stem cell antigen (PSCA), a homologue of the Ly-6/Thy-1 family of cell surface antigens, is expressed by a majority of human prostate cancers and is a promising target for prostate cancer immunotherapy [10,11]. In addition to its expression in normal and malignant prostate, PSCA is also overexpressed in a large percentage of bladder and pancreatic cancers [4,12,13]. Amara et al. [12] reported that PSCA is over-expressed in human transitional cell carcinoma (TCC). PSCA might also be a valuable target for bladder cancer diagnosis and therapy. PSCA enhancer (PSCAE) is active in LNCaP cells (prostate cancer) and HT1376 (bladder cancer), but inactive in 293T cells and fAR-HeLa cells, indicating that the enhancer maintains tissue selectivity [14].

PSCAE contains binding site for androgen receptor (AR) as androgen response elements (AREs). When androgen combines with AR and binds to AREs of PSCAE, the activity of PSCAE can be increased. Administrate of R1881 can increase the function of PSCAE in AR positive prostate cancer cells [14], but it is still unknown whether it can increase the function of PSCAE in bladder cancer cells or not. In this study, we constructed a novel dual specific vector targeted to bladder cancers that contain PSCAE and UPII promoter. We investigated the specificity of the vector and effects of AR agonist R1881 and AR antagonist flutamide on PSCAE function.

2. Materials and methods

2.1. Reagents and cells

RPMI 1640 and fetal bovine serum were obtained from GIBCO (Grand Island, NY). Luciferase assay system and β-galactosidase enzyme assay system with lyses buffer were purchased from Promega (Madison, WI). T24, UMUC-3, 5637 (bladder TCC) HepG2 (liver carcinoma), PC3 and LNCAP (prostate cancer), BGC823 (gastric cancer) cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum in 5% CO_2 incubator. Normal human urothelial (NHU) cells were derived from redundant ureteral segments from transplanted kidneys. The NHU cells were established and maintained in keratinocyte serum-free medium (GIBCO). R1881, a synthetic androgen receptor agonist, was purchased from DuPont (Boston, MA), flutamide, an androgen receptor antagonist, was obtained from SIGMA (St. Louis, MO).

2.2. Clone of PSCAE and UPII promoter

PSCAE and UPII promoter were cloned by method of polymerase chain reaction (PCR): Primers used for PSCAE was 5-GCTGACCGGTAGAGGCCAGCAGCACCCCTG-3 and 5-CGGTGCTAGCAACTGCTTCCGTGTGTGGCTGACAG-3, and T-24 Genomic DNA was used as templates. UPII promoter was amplified by PCR using CP1131 plasmid (a kind gift from D. C. Yu, Cell Genesys, Inc.) containing UPII as template, and the primers were as follow: 5-CGTAGCTAGCCAGGCTTCACCCCAGACCCA-3 and 5-CCGCGGATCCGGGATGCTGGGCTGGGAGGT-3. PCR was carried out in a 50-μl reaction mixture composed of 5 μL 10 X Taq buffer, 1.5 mM $MgCl_2$, 0.2 mM concentrations of each deoxynucleoside triphosphate, 10 pmol of each prime, 1 unit of *Taq* DNA polymerase (Roche, Indianapolis, IN). The reaction was performed in a thermal cycler GeneAmp 2400 PCR system; Perkin Elmer (Waltham, MA). The PCR conditions for PSCAE were 30 cycles of 95°C, 60°C, and 72°C for 30 s each after 95°C for 15 min. A final extension step of 10 min at 72°C was performed to ensure complete amplification of all DNA fragments. The PCR for UPII promoter was conducted with an initial activation step at 95°C for 15 min, followed by 30 cycles each consisting of a denaturation step at 95°C for 1 min, an annealing step at 60°C for 30 s, and an extension step at 72°C for 1 min. A final extension step of 10 min at 72°C was performed to ensure complete amplification of all DNA fragments. PCR products were analyzed by electrophoresis in agarose gels containing ethidium bromide (1 μg/ml). The expected PCR products were 324 bp for PSCAE and 2,204 bp for UPII.

2.3. Vector construction

To generate our recombinant constructs containing PSCAE and UPII promoter to drive the expression of luciferase gene (LUC), the original shuttle vector from AdEasy System (Stratagene, Inc., La Jolla, CA) was modified as follows: for insertion of our favorite genes combined with other restriction enzymes, an internal BamH I site was ablated and re-introduced into multiple cloning sites (MCS) that contain Age1, Nhe1, BamH1, and Not1 sites. UPII promoter was inserted into the plasmid at the sites of Nhe1 and BamH1, and then LUC was inserted at BamH1 and Not1 sites, this created Rp-UPII-LUC. PSCAE was inserted into Rp-UPII-LUC at Age1 and Nhe1 to create Rp-PSCAE-UPII-LUC. The plasmids were confirmed by enzyme digest.

2.4. Vectors transfection and luciferase assay

Rp-UPII-LUC and Rp-PSCAE-UPII-LUC were co-transfected with pCMV-β-gal (Promega) to 8 cell lines respectively (T24, UMUC-3, 5637, HepG2, PC3, LNCAP, BGC823, and NHU cells. pCMV-β-gal was used as an internal control (contains SV40 early promoter and enhancer drive transcription of the bacterial lac Z gene). Transfections were carried out in 24-well plates by lipofectamine 2000 (GIBCO) according to the manufacturer's instructions. Briefly, cells were trypsinized from plates, resuspended in RPMI-1640 with 10% FBS to 4×10^5 cells/ml and plated into a 24-well plate at a concentration of 2×10^5 cells per well and grown overnight. Cells were washed twice with 500 ul RPMI 1640 medium. For each well of plates, 0.8 ug luciferase recombinant plasmid DNA, 0.2 ug β-galactosidase plasmid, and 2 ul lipofection were mixed into 50 ul RPMI 1640 medium. Those mixtures were added into plates. Cells were incubated at 37°C for 5 h, followed by adding 600 ul medium with/without 10 nM R1881 or 10 nM flutamide into plates and incubated at 37°C for 24 h. 200 ul reporter lyses buffer (Promega) was added to cover the cells and a single freeze thaw was performed to ensure complete lysis. β-galactosidase levels in 100 ul lysate were measured by Bioelisa Reader (Winooski, VT) at 420 um. Luminescence was measured with luciferase assay kit (Promega) and counted on a Wallac 1450 Microβ liquid scintillation counter (Wallac, Gaithersburg, MD). L/G values for transfection efficiency in all cells were acquired by dividing the luciferase values by the β-galactosidase expression from the transfected pCMV-β-gal plasmid. All samples were in triplicate.

2.5. Detection of the AR and PSCA by Western blotting

T24, UMUC-3, 5637, NHU, PC3, and LNCAP cells were homogenized with the Dounce homogenizer and resuspended in M-PER (Mammalian Protein Extraction Reagent) (Pierce, Rockford, IL). The BCA protein assay kit (BioRad, Hercules, CA) was used to determine total protein concentration. Proteins were separated on a 12% Tris-HCl polyacrylamide gel (Bio-Rad) and transferred to PVDF Membrane (BioRad, Hercules, CA). The membrane was blocked for 1 h in blocking buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) with 5% nonfat dry milk, then incubated overnight with a 1:1000 dilution of rabbit anti-androgen receptor antibody, rabbit anti-PSCA antibody and rabbit anti-β-actin antibody (Cell Signaling Technology, Danvers, MA), respectively, followed by anti-rabbit IgG peroxidase conjugate (1:20,000) (Boster, Wuhan, China) for 1.5 h at room temperature. Immunoreactive bands were detected using the BeyoECL Plus Western Blotting detection System (Beyotime, Jiangsu, China) according to the manufacturer's instructions.

3. Results

3.1. Digest of plasmids with restriction endonucleases

Restrictive enzymes digestion assay confirmed the correct construction of the vectors. Rp-UPII-LUC and Rp-PSCAE-UPII-LUC were digested with multi-endonucleases and electrophoretically analyzed on 1% agarose gel. The restriction endonucleases and expecting bandings for Rp-PSCAE-UPII-LUC were as follows: EagI: 7,059, 5,073; HincII: 9,277, 2,334, 521; HindIII: 7,469, 1,686, 1,597, 1,379; SacII: 10,478, 1654; XhoI: 9,396, 2,736. The banding patterns were correct (Fig. 1).

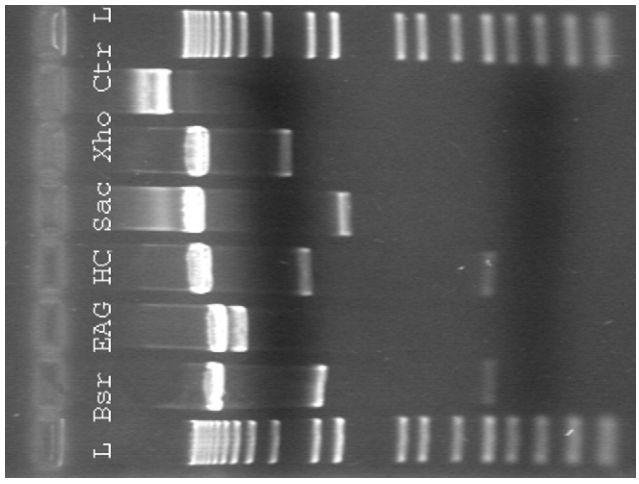

Fig. 1. The restriction endonucleases digest analysis of Rp-PSCAE-UPII-LUC. The plasmid of Rp-PSCAE-UPII-LUC was digested with 5 different enzymes and the banding patterns are correct (1 kb plus marker). The enzymes and expecting bandings are as follows: EagI: 7,059, 5,773. HincII: 9577, 2,334, 521. HindIII: 7,770, 1,686, 1,597, 1,379, SacII: 10,477, 1,654. XhoI: 9,395, 2,736.

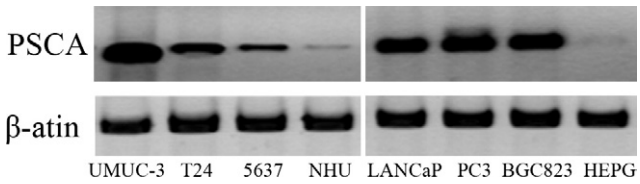

Fig. 2. Western blot analysis of PSCA in TCC cell lines and control cells. Bladder cancer cell lines (UMUC-3, T24, 5637), prostate cancer cell lines (LNCaP, PC3), and BGC823 (gastric carcinoma) showed higher PSCA than NHU and HEPG cells.

3.2. Western blot analysis of PSCA in TCC cell lines and control cells

PSCA expression in the cell lines was shown in Fig 2. Bladder cancer cell lines (UMUC-3, T24, 5637), prostate cancer cell lines (LNCaP, PC3) and gastric carcinoma cell line BGC823 showed higher PSCA expression while NHU and HEPG showed lower PSCA expression.

3.3. Tissue-specificity of UPII promoter and PSCAE in bladder cancer cells

To test tissue specificity of the vectors, a variety of cell lines were transfected with Rp-UPII-LUC and Rp-PSCAE-UPII-LUC respectively. The cell lines used represent several tissues including human liver carcinoma (HepG2), human gastric carcinoma (BGC823), human prostate cancer (PC3 and LNCAP), human bladder transitional carcinoma (T24, UMUC-3, 5637), and NHU cells. pCMV-β-gal was used as an internal control. Summary of the results is shown in Fig. 3. Both Rp-UPII-LUC and Rp-PSCAE-UPII-LUC produced higher LUC activity in T24, UMUC-3, and 5637 cells than in HepG2, BGC823, PC3, and LNCAP cells ($P <$

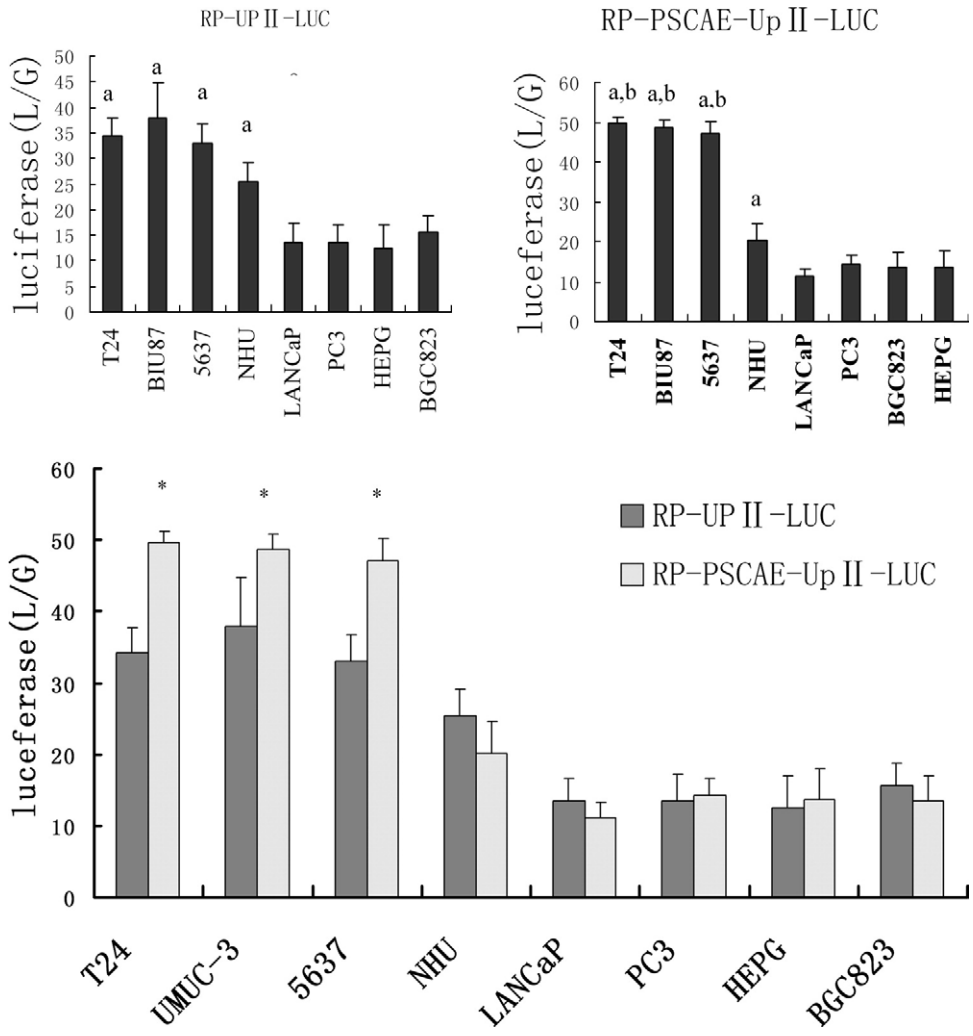

Fig. 3. Tissue specificity of the UPII promoter and PSCAE in vitro. Eight cell lines were transfected with plasmids Rp-UPII-LUC and Rp-PSCAE-UPII-LUC, and pSV-β-galactosidase vector was used as internal control. For each cell lines and plasmids, the ratio of luciferase activity to β-galactosidase activity (L/G) was calculated. L/G values showed a higher luciferase activity in bladder cancer cells than in non-bladder cancer cells. Inserting PSCAE into the plasmid could improve LUC activity in T24, UMUC-3, 5637 calls, but not in NHU cells and other nonbladder cancer cell lines (A) $P < 0.01$ vs. LNCaP, PC3, BGC823, and HEPG cells. (B) $P < 0.01$ vs. NHU cells. *$P < 0.05$ vs. Rp-UPII-LUC).

0.01). NHU cells transfected with Rp-UPII-LUC also showed higher luciferase activity than nonbladder cells. T24, UMUC-3, 5637 cells showed higher luciferase activity than NHU cells after transfected with Rp-PSCAE-UPII-LUC ($P < 0.01$). The bladder cancer cells transfected with Rp-PSCAE-UPII-LUC exhibit higher luciferase activity than the cells transfected with Rp-UPII-LUC, but this was not so in NHU cells and nonbladder cancer cells. LANCaP, PC3, and BGC823 are PSCA positive cells but PSCAE can not increased luciferase activity in those cells after transfected with the UPII based vectors. The results indicated that the UPII promoter maintained bladder selectivity and the PSCAE maintained cancer selectivity in bladder tissues.

3.4. Western blot analysis of AR in cells concerned and effects of R1881 and flutamide on vector activity in transfected cells

AR expression in the cell lines was shown in Fig. 4. Bladder cancer cell lines T24, 5637 and prostate cancer cell line LNCaP were AR positive while UMUC-3, NHU and PC3 were AR negative. The plasmid Rp-PSCAE-UPII-LUC was active and androgen inducible in AR-positive bladder cancer cells (5637 and T24) but not inducible in AR-negative bladder cancer cells (UMUC-3) and prostate cancer cells (both AR-positive LNCaP and AR-negative PC3). When androgen receptors were blocked by flutamide, Rp-PSCAE-UPII-LUC could still induce higher luciferase activity in bladder cancer cells than in nonbladder cancer cells and NHU cells (Fig. 4). The results indicated that PSCAE maintained a certain level of androgen independent activity in bladder cancer cells. R1881 and flutamide did not affect luciferase activity in cells transfected with Rp-UPII-LUC (Fig. 5).

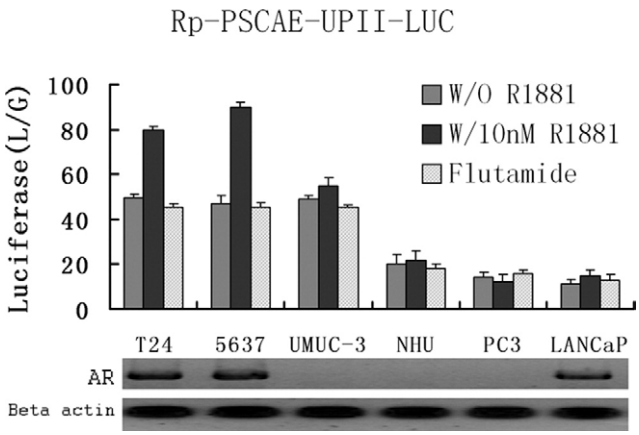

Fig. 4. Detection of AR expression and effects of R1881 and flutamide on Rp-PSCAE-UPII-LUC activity in bladder cancer cells. Bladder cancer cell lines T24, 5637, and prostate cancer cell line LNCaP were AR positive while UMUC-3, NHU and PC3 were AR negative. The plasmid Rp-PSCAE-UPII-LUC was active and androgen inducible in AR-positive bladder cancer cells (5637 and T24) but not androgen inducible in AR-negative bladder cancer cells (UMUC-3) and prostate cancer cells (both AR-positive LNCaP and AR-negative PC3). When androgen receptors were blocked by flutamide, Rp-PSCAE-UPII-LUC could still induce higher luciferase activity in bladder cancer cells than in nonbladder cancer cells. The results indicate that PSCAE maintains a certain level of androgen independent activity in bladder cancer cells. Combine PSCAE and UPII promoter can induce higher luciferase activity in both AR-positive and AR-negative bladder cancer cells.

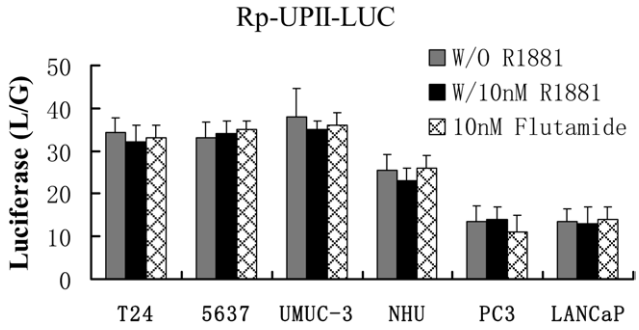

Fig. 5. Effects of R1881 and flutamide on Rp-UPII-LUC activity in cells. A panel of cells was transfected with Rp-UPII-LUC and incubated in medium containing R1881 or flutamide. R1881 and flutamide did not affect luciferase activity in cells transfected by Rp-UPII-LUC.

4. Discussion

Gene therapy approaches to cancer are attractive because they hold the possibility of selectively targeting therapy to affected cancer cells, thereby avoiding toxicities associated with treatments such as cytotoxic chemotherapy. The development of cancer-specific and tissue-specific systems for delivering therapeutic genes to cancer cells is essential for the success of gene therapy. An important approach is using tissue-specific promoter to direct therapeutic genes into cancer cells, such as using the prostate antigen (PSA) promoter [5,15] to construct prostate-specific vectors. In this study, we designed a novel dual specific vector by combining PSCAE and UPII promoter.

Urothelial plaques cover a large portion of the apical surface of mammalian urothelium and prevent the urothelial luminal surface from rupturing during bladder distention. Furthermore, it has been shown that mammalian urothelial plaques contain 4 major integral membrane proteins: UP-Ia (27 kDa), UP-Ib (28 kDa), UPII (15 kDa), and UPIII. Immunohistochemical survey of various tissues found UPs to be urothelium-specific. Antibodies to UPs stained 88% of papillary noninvasive TCCs, 53% of invasive TCCs, and 66% of TCC metastases, without significant binding to any of the nonurothelial carcinomas [8]. These findings suggest that UPs may serve as a unique marker for the positive identification of urothelial-derived TCCs [8,13]. Therefore, the promoters that direct the expression of the uroplakins may be useful in constructing tissue-specific vectors for bladder cancer gene therapy. Cloning and analysis of human UPII and its application for gene therapy in bladder cancer was reported [7]. The UPII promoter was used in the genera-

tion of urothelium-specific adenovirus vectors (CG8840), and this urothelium-specific adenovirus variant showed significant synergy with chemotherapeutic agents in achieving complete tumor regression. Zhu et al. [16] cloned mouse UPII promoter to construct tissue-specific replicated adenovirus. The data demonstrated that the mouse UPII promoter had a high activity in human bladder cancer cells. Our study also indicates that the human UPII promoter gene is able to drive the expression of target genes in bladder cancer cells, and this is in accordance with the results of Zhang et al. [7] (Fig. 3). However, UPII is also expressed in normal urothelium, both normal and cancer cells will be targeted. The published literatures did not address the activity of UPII promoter based vectors in normal urothelium. Our data indicated that UPII promoter based plasmid Rp-UPII-LUC can induce higher luciferase activity in NHU cells. New approaches for improve the specificity of vectors to cancer cells are needed. In an attempt to increase the gene expression and specify the expression to bladder cancer cells, a cancer-specific enhancer PSCAE was introduced upstream of the UPII promoter in this study.

PSCA is a cell surface antigen that has potential utility in the diagnosis and treatment of prostate cancer. PSCA expression is up-regulated in prostate cancers [10]. Overexpression of PSCA correlates with increasing tumor stage, grade, and metastasis to bone. In situ hybridization analyses demonstrated PSCA expression in more than 80% of local and 100% of bone-metastatic prostate cancer specimens [17]. PSCA is also overexpressed in a large percentage of bladder and pancreatic cancers. PSCA might serve as a novel marker in the diagnosis of bladder cancer and pancreatic cancer [18]. PSCA expression level could be a valuable prognostic marker for tumor recurrence in superficial TCC of the bladder [19]. These results show a potential therapeutic role of PSCA in bladder cancer. In this study, we demonstrated that tissue-specific enhancer PSCAE could function in bladder cancer when positioned upstream UPII promoter. Combination of PSCAE and UPII promoter together in the specific vector could improve the expression of luciferase gene in TCCs but not in nonbladder cancer cells and NHU cells. PSCAE contains binding site for AR as AREs. When androgen combines with AR and binds to AREs of PSCAE, the activity of PSCAE can be increased [14]. Androgen could increase the function of PSCAE in AR positive bladder cancer cells (Fig. 4). But blocking androgen receptor by flutamide could not inactivate PSCAE. The results indicated that PSCAE maintained a certain level of androgen independent activity in bladder cancer cells. PSCAE was active in both AR positive and AR negative bladder cancer cells. PSCAE could not increase vector activity in NHU cells. Based on the above results, we concluded that PSCAE and UPII promoter could drive the expression of therapeutic target gene expression in bladder cancer cells. Our work on constructing oncolytic viruses by using PSCAE and UPII promoter to control the replication of virus is at work at present.

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