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Original Article

Development of targeted therapy for bladder cancer mediated by a double promoter plasmid expressing diphtheria toxin under the control of IGF2-P3 and IGF2-P4 regulatory sequences

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Abstract: Background**:** The human IGF2-P3 and IGF2-P4 promoters are highly active in bladder carcinoma, while existing at a nearly undetectable level in the surrounding normal tissue. A double promoter DTA-expressing vector was created, carrying on a single construct two separate genes expressing diphtheria toxin A-fragment (DTA), from two different regulatory sequences, selected from the cancer-specific promoters IGF2-P3 and IGF2-P4. Methods: IGF2-P3 and IGF2-P4 expression was tested in samples of urothelial carcinoma (UC) of the bladder (n=67) by RT-PCR or by ISH. The therapeutic potential of single promoter expression vectors (P3-DTA and P4-DTA) was tested and compared to the double promoter toxin vector P4-DTA-P3-DTA in UC cell lines and in heterotopic and orthotopic animal models for bladder cancer. Results: Nearly 86% of UC patients highly expressed IGF2-P4 and IGF2-P3, as determined by ISH. The double promoter vector (P4-DTA-P3-DTA) exhibited superior ability to inhibit tumor development by 68% (P=0.004) compared to the single promoter expression vectors, in heterotopic bladder tumors. The average size of the P4-DTA-P3-DTA bladder tumors (in orthotopically treated mice) was 83% smaller (P<0.001) than that of the control group. Conclusions: Overall, the double promoter vector exhibited enhanced anti-cancer activity relative to single promoter expression vectors carrying either gene alone. Our findings show that bladder tumors may be successfully treated by intravesical instillation of the double promoter vector P4-DTA-P3-DTA.

Keywords: IGF2, bladder cancer cells, cancer, targeted cancer therapy

Introduction

Non-muscle invasive bladder carcinoma can be removed by transurethral resection, but tend to recur in 50-70% of the patients. Measures to decrease this high recurrence rate include intravesical chemotherapy and immunotherapy (BCG). These treatments decrease the recurrence rate, but are associated with side effects and frequent failures [1].

Based on early studies of our group and others, the transcriptional regulatory sequences of the IGF2 gene emerged as candidates for cancer targeted therapy. IGF2 (the human P3 and P4 promoters) are onco-fetal genes and are oncogenes [2-4], expressed in the fetus and in a broad spectrum of tumors, but rarely in normal adult tissues [5-7]. The 67-aa IGF2 is a member of the insulin like growth factor family that is involved in cell proliferation and differentiation [8]. The human IGF2 gene contains 9 exons (E1 –9) and 8 introns [8, 9], and is transcribed from 4 different promoters (P1–P4) producing 4 different transcripts [9-11]. All four transcripts share a common coding region and a common 3.9kb 3-UTR, but variable 5-UTRs [9]. IGF2 is an imprinted gene that is almost exclusively expressed from the paternal allele [12-14]. The P3 and P4 promoters are the major IGF2 promoters during embryogenesis and tumor development [8]. Increased expression of IGF2 as a result of the loss of its imprinting is frequently seen in a variety of human tumors [14-16]. In addition, abnormal signal transduction and/or promoter activation was reported as a major mechanism

for the IGF2 overexpression in a variety of tumors including bladder carcinoma [17-20].

Our group has previously reported the construction of single promoter vectors expressing diphtheria toxin A-chain gene, under the control of IGF2-P4, IGF2-P3 (P4-DTA and P3-DTA), or H19 regulatory sequences [7, 21]. We showed that these constructs were able to selectively kill tumor cell lines and inhibit tumor growth in-vitro and in-vivo (P3-DTA) in accordance to the transcriptional activity of the above-mentioned regulatory sequences. However, there are UC cells that do not express IGF2-P3 and as a result, there are patients that could not match this treatment.

Thus, a double promoter DTA-expressing vector was created, carrying on a single construct two separate genes expressing DTA from two different regulatory sequences, IGF2-P4 and IGF2-P3 ('P4-DTA-P3-DTA').

This vector was then used to transfect and to eradicate tumor cells in culture or to inhibit tumor growth in heterotopic and orthotopic bladder tumor models.

The activity of P4-DTA-P3-DTA was tested and compared to the activity of the single promoter vectors.

The results showed enhanced activity of the double promoter vector, P4-DTA-P3-DTA, relative to the single promoter expression vectors carrying either DTA sequence alone.

Materials and methods

Cell culture

The human bladder carcinoma cell line T24P was obtained from the American Type Culture Collection (ATCC; Rockville, MD). The human bladder carcinoma cell line HT-1376 was kindly provided by Prof W. Schulz, Heinrich-Heine University of Dusseldorf, Germany. Cells were grown to confluency in a humidified incubator with 5% CO2 in polystyrene culture flasks and were maintained in DMEM-F12 (1:1) medium containing 10% fetal calf serum.

RNA Isolation, cDNA synthesis and PCR

RNA was extracted from cell lines or frozen tissue blocks, using the RNA STAT-60TM Total RNA/mRNA isolation reagent, according to the manufacture's instructions. The RNA was treated by RNAse-free DNAse I to eliminate any contaminating DNA.

Total cDNA was synthesized from 2μg total RNA in 20μl reaction volume with 10ng/μl of the oligo-(dT)15 primer and 10 units/μl M-MLV Reverse Transcriptase according to the manufacturer instructions. 2μl of cDNA samples were taken for the amplification of the different transcripts using the different primers. The amplification conditions were 95°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 59°C for 45 sec and 72°C for 60 sec, and finally 72°C for 5 min. The PCR reactions were carried out in 25μl volumes in the presence of 6ng/μl of each of the forward and the reverse primers using 0.05 units/μl of Taq polymerase according to the kit instructions (Takara). The primers designed for the detection of IGF2-P3 or IGF2-P4 RNA were designed to bind at exon 5 or exon 6 respectively (5'-CGTCGCACATTCGGCCCCCGCGACT and 5'-TCCTCCTCCTCCTGCCCCAGCG), for the P3 and P4 transcripts in the forward direction and the common reverse primer (5'- CAGCAATGCAGCACGAGGCGAAGCC) was designed to bind the 3' end of exon 7 and the 5' end of exon 8 without the introns in between. The integrity of the cDNA was assayed by PCR analysis of the ubiquitous, cell cycle independent, histone variant, H3.3 [7]. The PCR products were separated by electrophoresis on 2% gel agarose, and detected by ethidium bromide dye.

DIG-labeled probe synthesis

A PCR strategy was used to generate template DNA for synthesis of labeled RNA probes. Forward primers for the human IGF2-P3 and IGF2-P4 genes were designed. Each primer contain Sp6 promoter sequence in its 5'-end. Accordingly, a reverse primer was also designed with T7 promoter sequence incorporated in its 5'-end. The PCR products obtained for the IGF2-P3 and IGF2-P4 transcript were purified from the gel by the DNA and Gel Band Purification Kit (Amersham), and used as templates for the PCR -based incorporation of T7 and Sp6 RNA polymerase promoter. The PCR conditions used to generate the T7/Sp6 templates were the same as described earlier for the synthesis of IGF2 specific transcripts. The PCR products (containing T7 and Sp6 promoters) were purified from the gel, sequenced and found to be identical to the relevant published sequences in

the gene bank.

100 to 200ng from the purified products were used as templates for the T7 and Sp6 polymerase (2 units/μl), according to the manufacturer instructions in the presence of 2 units/μl RNase inhibitor. T7 and Sp6 promoters were respectively used to drive the synthesis of the antisense and the control sense Digoxigenin-labeled UTP probes. The resulting probes were treated by 2 units of RNase free DNase I, pelleted and resuspended in appropriate volume of DEPC-treated double distilled water. The sizes of the synthesized probes were analyzed by running on 4% denaturing agarose minigel, and their labeling efficiency was determined by dot blot analysis.

In situ hybridization (ISH)

The non radioactive ISH washing and treatments were as described in [7]. Each section was rehydrated by 30μl of the hybridization solution containing about 30ng of DIG labeled RNA probe at 52°C.

The ISH was performed on successive slides of TCC tissue for IGF2-P3 and IGF2-P4 transcripts. The intensity of hybridization signal was indicated as (0) for no staining, (+1) for weak, (+2) for moderate and (+3) for strong signals. The distribution of the hybridization signal was referred to as up to one third of the cells, + (1), one to two thirds, ++ (2), and more than two thirds, +++ (3). Therefore the total scoring (intensity + quantity) for each sample varied from 0 (no expression) to 6 (very high expression). Low expression was set as total scoring of $0 < X < 3$ and high expression was set as total scoring of $3 \leq X \leq 6$. Positive expression was defined as the overall IGF2 P3 or P4 expression (low and high expression).

Plasmid construction

The DTA (P3-DTA) and P4-DTA or luciferase single promoter vectors were designed as described [7]. We constructed double promoter expression plasmids, carrying on a single construct two separate genes expressing DTA from two different regulatory sequences: IGF2-P3 + IGF2-P4 (hereinafter: "P4-DTA-P3-DTA") (cloned by GENEART™ (Germany)).

A double promoter control construct was created, using the same strategy, expressing the luciferase reporter gene ('P4-Luc-P3-Luc').

Cationic polymer (jetPEI) transient transfection

The *in vitro* jetPEI™ transfection reagent compact the DNA into positively charged particles capable of interacting with anionic proteoglycans at the cell surface and entering cells by endocytosis. The transfection procedure was done as recommended by the manufacturer (Polyplus-transfection, France). A total of 0.1×10^6 cells/well were grown overnight in a twelve-well Nunc multidish (75mm). For each well, 2μg DNA and 4μl of the jetPEI (N/P =5) were diluted separately with 50μl of 150mM NaCl each, and vortex-mixed gently. The jetPEI solution was added at once to the DNA solution, the mixture was vortex-mixed and the mixture was incubated for 15 minuets at room temperature. The 100μl jetPEI/DNA mixture was then applied drop-wise onto the serum containing medium of each well. The transfection experiment was stopped after 48 hours.

Luciferase activity

The cells were harvested and the luciferase activity was determined using the luciferase Assay System kit (Promega). The light output was measured using a Lumac Biocounter apparatus. The total protein content of the lysates was determined by the Bio-Rad protein assay reagent and the results were normalized to the total protein and expressed as Light units/μg protein. LucSV40 (Luc-4) was used as a positive control for the efficiency of transfection as it contains the SV40 promoter and enhancer, while Luc-1 that lacks any regulatory sequences was used as a negative control to determine the basal nonspecific luciferase expression, which was found to be negligible in all of the cell lines. All experiments were done in triplicates and the results expressed as mean and standard error.

In vitro gene therapy

The cells were cotransfected with 2μg of the LucSV40 control vector and with the indicated amounts of the DTA expressing vector (P3-DTA, P4-DTA or the DTA double promoter expressing vector P4-DTA-P3-DTA). The same cells were additionally transfected with 2μg LucSV40 alone in the same experiment. The P3-DTA, P4-DTA and P4-DTA-P3-DTA cytotoxic activity was

determined by calculating the % of decrease in the cotransfected LucSV40 activity compared to that of LucSV40 transfected alone in the same cell type. The total protein content of the lysates was determined by the Bio-Rad protein assay reagent and the results were normalized to the total protein and expressed as Light units/μg protein. Therefore the reduction in luciferase activity, reflect the inhibition of protein synthesis activity by the DTA.

In vivo gene *therapy animal models*

All surgical procedures and the care given to the animals were approved by the local committee for animal welfare. Animals were kept in the Hebrew University's animal facility with water and food *ad librum*. The histopathological examinations of the different tumors were performed in consultation with a trained pathologist.

Heterotopic nude mice model

Confluent T24P and HT-1376 human bladder carcinoma cells were trypsinized to a single cell suspension and resuspended in PBS. $2x10^6$ T24P cells or HT-1376 cells (in 150 μl volume) were subcutaneously injected into the back of female CD1 nude mice, 6-8 weeks old. 10 days after cell inoculation the developing tumors were measured in two dimensions and randomized to different treatments. Animals were separated to different groups of the same size (n=6). The ability to inhibit tumor growth by the single promoter DTA expression vectors (P4-DTA, P3-DTA) and by the double promoter DTA expression vector (P4-DTA-P3-DTA), was tested.

Intratumoral injections of 25 mg of either DTA expressing constructs (treatment groups) or Luc expressing constructs (control groups) were given 10, 12 and 14 days after cells inoculation. *In vivo* Jet-PEI a 22 kDa linear form of polyethylenimine (PEI) was used as a transfection enhancer reagent. PEI/DNA complexes with a ratio of PEI nitrogen to DNA phosphate of 6 were prepared in a solution of 5% w/v glucose according to the manufacture's instructions. Tumor dimensions were measured, and the tumor volume was calculated according to the formula $width^2$ x length x 0.5. The animals were sacrificed 3 days after the last treatment, the tumors were excised and their ex-vivo weight and volume were measured. Samples of the tumors were fixed in 4% buffered formaldehyde and processed for histological examination for evidence of necrosis and persistent tumor. Computerized measurements of tumor surface area and of the necrotic surface area were made using the Image Pro Plus software (Media cybernetics, Silver Springs, USA).

Orthotopic model for bladder cancer

Female CD1 nude mice, 6-8 weeks old were used to develop orthotopic superficial bladder tumors. Mice were anesthetized with intraperitoneal injection of ketamine (85 mg/kg) and xylazine (3 mg/kg). The bladder was catheterized with a 24 gauge catheter, than drained and its mucosa was mildly disrupted with 0.1 ml HCl 0.1N for 15-sec. (The bladder is rather resistant to implantation of cells, and therefore it is necessary to create abrasions in the bladder mucosa of the anesthetized rodent either by acid, in order to increase "tumor take" [22]). The acid was immediately neutralized with 0.1 ml NaOH 0.1N, and the bladder was washed three times with 0.1ml PBS. Subsequently, a 0.1 ml suspension of PBS containing $10x10^6$ T24P human bladder carcinoma cells was instilled into the bladder. The urethra was ligated with 6/0 silk suture to assure that cells were retained in the bladder. After 2 hours the sutures were removed and the bladders were evacuated by spontaneous voiding. Four healthy mice were left without T24P cells instillation. Seven days after cell instillation, the animals were anesthetized and the bladders were catheterized the same way. The bladders were washed three times with 0.1ml of PBS. Animals were separated to different groups of the same size (n=6). Mice of the DTA treatment groups received 20 μg of the toxin vector P4-DTA-P3-DTA. The control group received 20 μg of the reporter vector P4-Luc-P3-Luc. A group of 4 mice were kept with no treatment. The same treatments were repeated after 3 days. The *in vivo*-jetPEITM was used as a transfection enhancer agent. For preparation of the solution, 2.4 μl of the jetPEI (N/P ratio = 6) in 50μl glucose 5 % (w/v) were mixed with 20 mg of treatment plasmids respectively, in 50μl of 5% glucose solution. The resulting mixture was vortex-mixed and left for 10-15 minutes at room temperature and subsequently instilled into the mice bladder transurethrally using the catheter as described above. The animals were sacrificed 4 days after the last plasmid instillation, their bladders were

Table 1. The endogenous IGF2-P3 and IGF2-P4 expression levels in UC tissue samples determined by ISH

	IGF2-P3	IGF2-P4	IGF2-P3 + IGF2-P4
Positive expression	27 /28	24 /28	27 /28
High expression	21 /28	14 /28	24 /28

Human UC samples (n=28) were examined by ISH and the expression levels of IGF2-P3 and IGF2-P4 transcripts were determined by the intensity of the hybridization signal and by the quantity of the stained cells. The table shows the level of IGF2-P4 and IGF2-P3 transcripts, defined as 'positive' (overall) expression and 'High' expression. The ISH was performed on successive slides of UC tissue for IGF2-P3 and IGF2-P4 transcripts. A semi quantitative scoring system was established to define the levels of IGF2 expression after ISH. The intensity of hybridization signal was indicated as (0) for no staining, (+1) for weak, (+2) for moderate and (+3) for strong signals. The distribution of the hybridization signal was referred to as up to one third of the cells, + (1), one to two thirds, ++ (2), and more than two thirds, +++ (3). Therefore the total scoring (intensity + quantity) for each sample varied from 0 (no expression) to 6 (very high expression). Low expression was set as total scoring of $0 < X < 3$ and high expression was set as total scoring of $3 \leq X \leq 6$. Positive expression was defined as the overall IGF2 P3 or P4 expression (low and high expression).

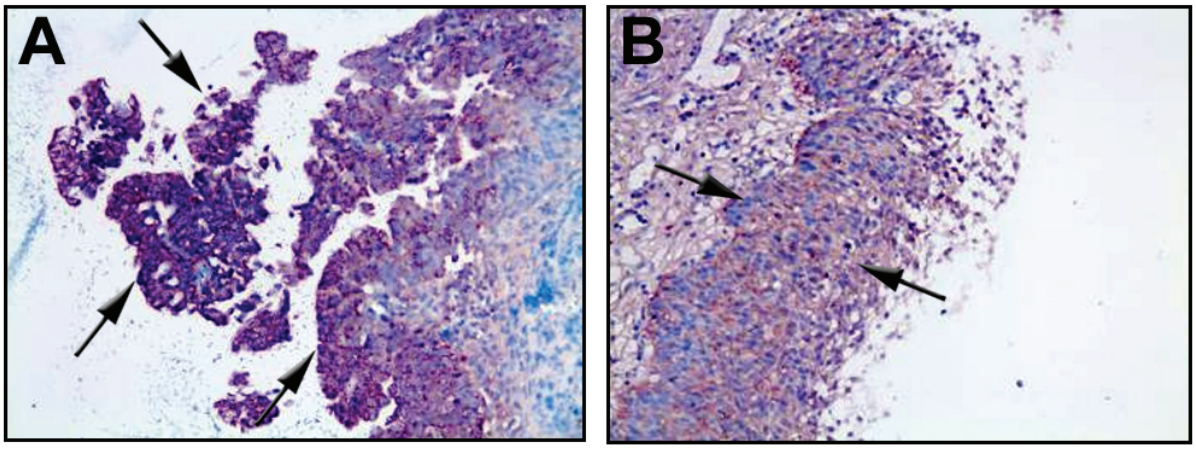

Figure 1. ISH detection of the expression of IGF2-P3 and IGF2-P4 transcripts in human TCC tissue samples: IGF2-P3 (**A**) and IGF2-P4 (**B**) specific transcripts, detected by ISH. The positive stained cells are marked by black arrows (Magnification are X20).

removed and the serosal surface and the adjacent sex glands were dissected carefully. Samples of the tumors were fixed in 4% buffered formaldehyde and processed for histological examination for evidence of necrosis and persistent tumor. Computerized measurements of tumor surface area and of the necrotic surface area were made using Image Pro Plus software (Media cybernetics, Silver Springs, USA). Other samples were frozen by liquid nitrogen and stored at -80°C to be analyzed by RT-PCR for evidence of IGF2, luciferase and DTA mRNA expression.

Results

Expression of IGF2-P3 and IGF2-P4 transcripts in UC tissues determined by RT-PCR or by ISH

To evaluate the possible use of IGF2-P3 and IGF2 -P4 regulatory sequences for targeted therapy of bladder cancer, we determined the expression of IGF2-P3 and IGF2-P4 transcripts by RT-PCR and by ISH.

The IGF2-P3 and IGF2-P4 overall expression in UC tissue samples determined by RT-PCR (n=39). Human UC samples were obtained from patients undergoing TUR or radical cystectomy

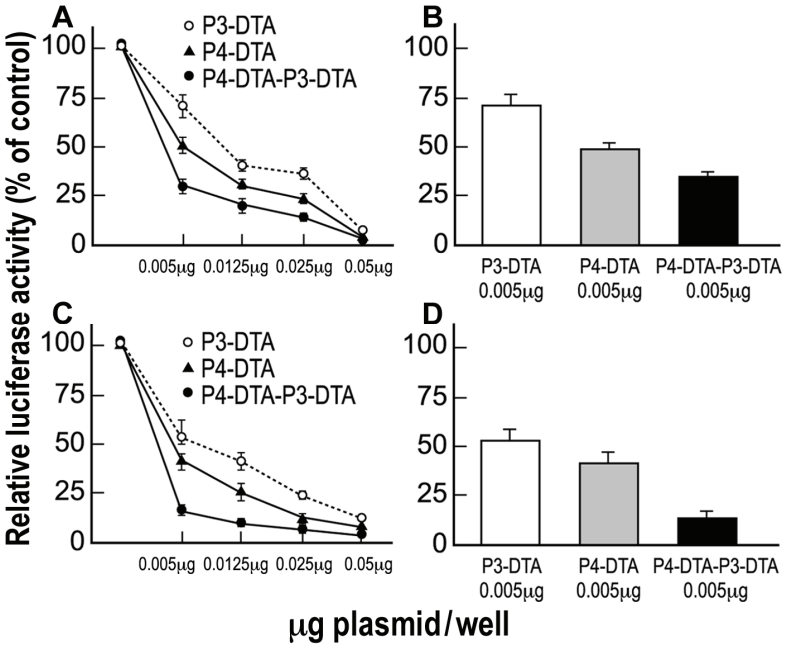

Figure 2. In vitro enhanced protein synthesis inhibition activity of P4-DTA-P3-DTA in UC cell lines: Tumor growth inhibition activity of the double promoter construct P4-DTA-P3-DTA was first tested *in vitro* by determining its ability to lyse two different human bladder carcinoma cell lines, relative to the single promoter constructs (P3-DTA, P4-DTA). Anti-tumor therapeutic activity was determined by measuring the inhibition of luciferase activity following co-transfection with LucSV40. T24P (**A-B**) and HT-1376 (**C-D**) UC cells were co-transfected with the indicated vectors (P4-DTA, P3-DTA, or P4-DTA-P3-DTA) in a dose-response manner at the indicated concentrations and with 2μg of LucSV40. Luciferase activity as an indicator of survival of the transfected cells was determined and compared to that of cells transfected with LucSV40 alone. Transfection experiments were stopped after 48 hours and luciferase activity was assessed. The decrease in LucSV40 activity was determined by comparison to the same cell type transfected with LucSV40 alone as a measure for cytotoxicity. The diverse effect of each vector at the lowest plasmid transfected concentration is indicated (**B**, **D**).

at Hadassah Hospital, following permission of the local IRB. Samples (n=39) were first tested for IGF2-P3 and IGF2-P4 overall expression by RT-PCR. 38 out of 39 UC samples (97.5%) examined by RT-PCR showed positive IGF2-P3 transcripts expression and 38 out of 39 (97.5%) UC samples showed positive IGF2-P4 expression.

Out of 28 UC samples, 24 and 27 out of 28 samples positively expressed IGF2-P4 and IGF2-P3, respectively (**Table 1**).

High expression levels of IGF2-P3 and IGF2-P4 (**Figure 1**), were found in 75% (21/28) and 50% (14/28) of the UC samples, respectively (**Table 1**).

However when the overall combined expression of the intensity and quantity of both transcripts IGF2-P3 + IGF2-P4 was determined, then nearly 100% (27/28) of the samples showed positive expression and 86% of UC samples (24/28) showed high expression levels.

In-vitro DTA expression by a single construct containing DTA genes separately expressed from IGF2-P4 and IGF2-P3 regulatory sequences

P4-DTA or P3-DTA vectors were able to drive the expression of the DTA gene and thus reduce luciferase activity in a dose-response manner. P4-DTA-P3-DTA, however, exhibited far enhanced efficiency in lysing cancer cell lines, relative to each of the single promoter constructs, in T24P (**Figure 2A-B**) and HT-1376 cells (**Figure 2C-D**). P4-DTA-P3-DTA was able to reduce the LucSV40 activity to more than 70% at concentrations as low as (0.005 μg/well) in T24P (figure 2B) and HT-1376 (**Figure 2D**) cells, respectively. Less significant inhibition was obtained by P4-DTA or P3-DTA at same concentrations (0.005 μg/well) in T24P (**Figure 2B**) and HT-1376 (**Figure 2D**) cells.

Tumor growth inhibition by P4-DTA-P3-DTA in heterotopic bladder carcinoma model

High expression of IGF2-P4 and IGF2-P3 RNA was found in heterotopic subcutaneous tumors induced by T24P or HT-1376 UC cells (**Figure 3**).

The therapeutic potency of the vectors was tested by direct intratumoral injection of 25mg of the DTA-expressing vectors (P4-DTA, P3-DTA, or P4-DTA-P3-DTA), or of the Luc-control vectors

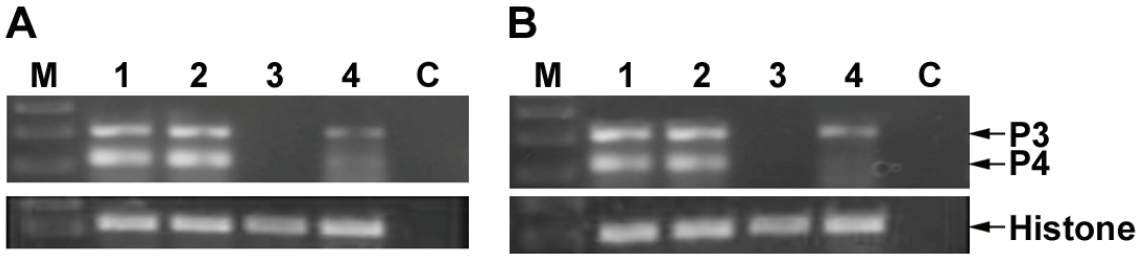

Figure 3. The expression of IGF2-P3 and IGF2-P4 in heterotopic subcutaneous tumors determined by RT-PCR: High expression of IGF2-P3 and IGF2-P4 transcripts in heterotopic subcutaneous tumors after injection of T24P (**A**) or HT-1376 cells (**B**) was determined by RT-PCR. "M": 100-bp molecular weight marker, lanes 1-2: heterotopic subcutaneous tumors from different mice induced by injection of T24P (**A**) or HT-1376 (**B**) cells, lane 3: subcutaneous tissue of normal mouse, lanes 4: T24P (**A**) or HT-1376 (**B**) cell lines, "C": negative control for PCR. The sizes of the PCR products are 211bp for human IGF2-P3, 119bp for IGF2-P4 and 213bp for Histone 3.3 internal control, respectively. There was no IGF2 expression in normal control mice (lane 3). Interestingly, the expression of IGF2-P 4and IGF2-P3 RNA in the heterotopic tumors was higher compared to the *in vitro* expression of T24P cells (lane A4) or HT-1376 cells (lane B4) used for inoculation.

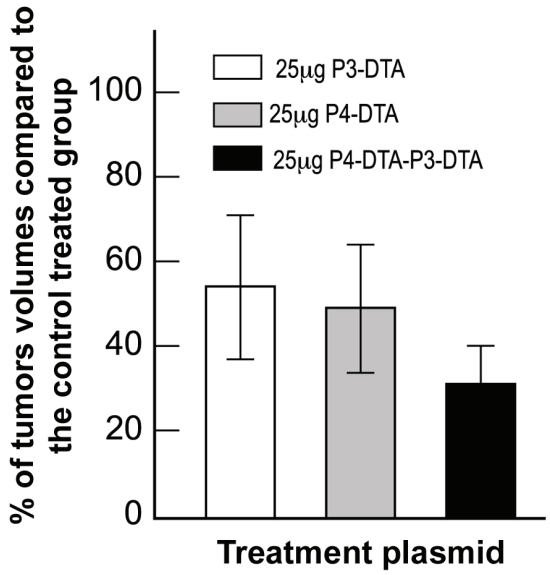

Figure 4. In vivo inhibition of heterotopic tumors in response to P4-DTA-P3-DTA treatments. Inhibition of tumor growth in response to P3-DTA, P4-DTA, or P4-DTA-P3-DTA treatments is shown. The tumor sizes of tumors treated with the DTA expressing vectors (n=6), or with control luciferase expressing vectors (n=6) were measured and the *in vivo* fold increase of the tumor size was calculated prior to each treatment and before sacrifice. The fold increase in tumor volume was calculated relative to the initial volume at the day of the first treatment.

into each heterotopic tumor. Three injections of P4-DTA or P3-DTA (Figure 4) at two-day intervals were able to inhibit tumor development by 50.5% (P=0.003) and 55.5% (P=0.005), respectively compared to P4-Luc and P3-Luc treatments. However, three injections of the double promoter plasmid P4-DTA-P3-DTA at two-day intervals inhibited tumor development by 68% (P=0.004) compared to P4-Luc-P3-Luc treatment (**Figure 4**). The P4-DTA-P3-DTA thus exhibited enhanced ability to inhibit tumor development *in-vivo*, compared to each of the single-promoter constructs (P4-DTA, or P3-DTA).

To confirm the difference between the P4-DTA-P3-DTA and P4-Luc-P3-Luc groups, tumors were excised and their *ex vivo* volume and weight were determined as well. Mice treated with P4-DTA-P3-DTA exhibited a 67% (P=0.01) reduction of the *ex-vivo* tumor volume (**Figure 5A**) and a 69% (P=0.01) reduction of the *ex-vivo* tumor weight (**Figure 5B**) compared to P4-Luc-P3-Luc treated mice.

Treatment of the orthotopic tumors bladder tumors by P4-DTA-P3-DTA

Treatment group (n=6) was intravesically treated with 20μg of P4-DTA-P3-DTA and the control group (n=6) received 20μg of P4-Luc-P3-Luc.

Two treatments of 20μg of P4-DTA-P3-DTA in three day intervals were able to inhibit tumor growth significantly as reflected by measuring the size of the tumors and by bladders weight.

The average size of the P4-DTA-P3-DTA treated

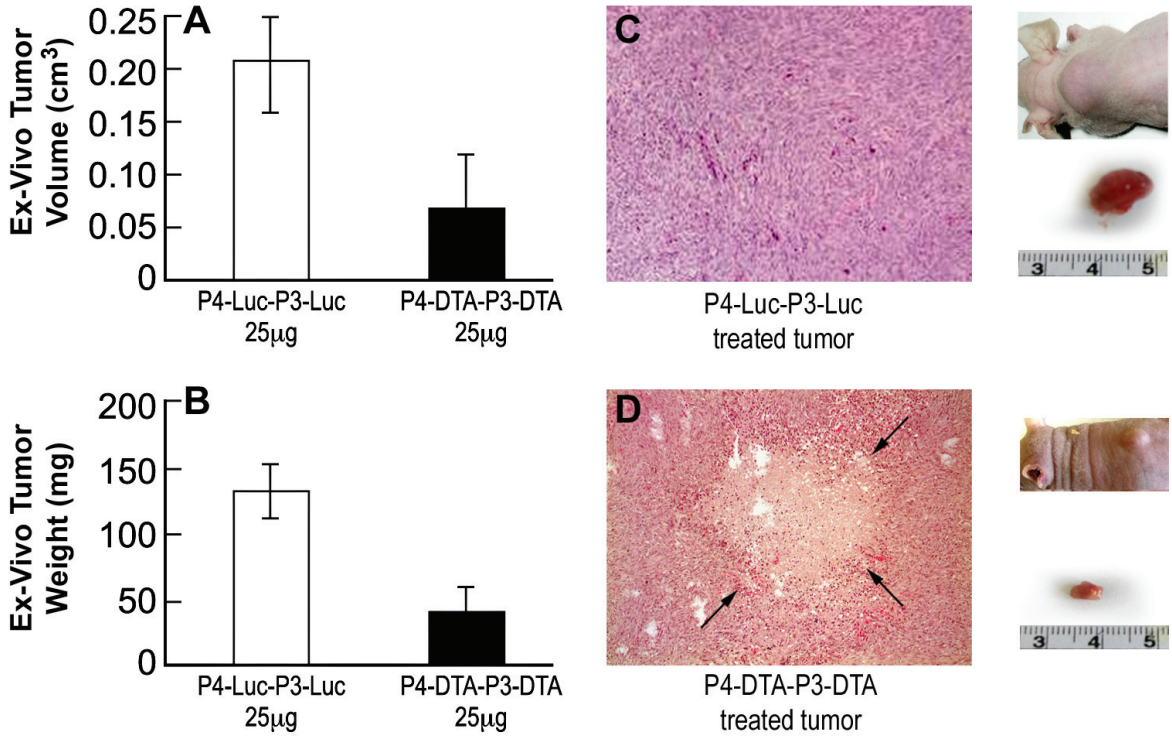

Figure 5. Heterotopic tumors treated by P4-DTA-P3-DTA. Heterotopic bladder tumors treated with P4-DTA-P3-DTA vector (black) or with P4-Luc-P3-Luc control vector (white) were excised and the ex-vivo tumors volume were measured (**A**) and weighted (**B**). **C-D:** Necrosis of heterotopic tumors treated with P4-DTA-P3-DTA: Hematoxylin Eosin (HE) staining (X10) of representative sections of tumors treated with P4-Luc-P3-Luc (**C**), or with P4-DTA-P3-DTA (**D**). The necrotic areas are indicated by arrows (**D**). Inserts are macroscopic photographs of the heterotopic tumors.

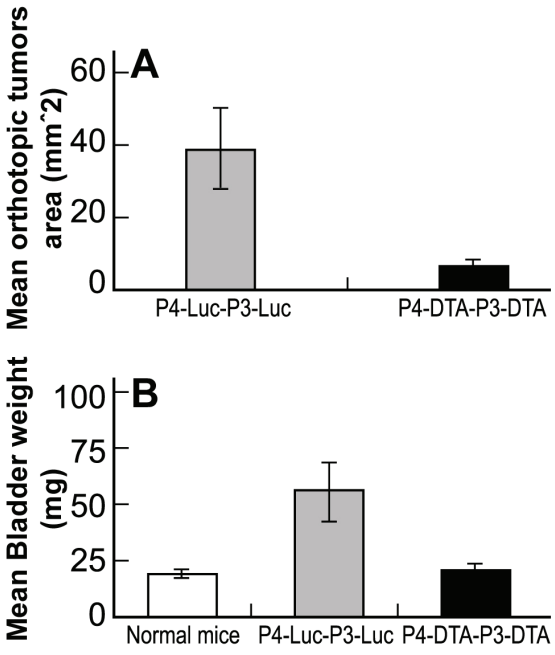

Figure 6. The effect of intravesical treatment with P4-DTA-P3-DTA vector in orthotopic bladder carcinoma: Orthotopic tumors were induced by intravesical instillation of T24P cells, in nude mice bladders. 7 days later, mice of each group (n=6) received an intravesical treatment with 20µg of P4-DTA-P3-DTA, or P4-Luc-P3-Luc. The same treatments were repeated after 3 days, and 4 days later mice were sacrificed. The bladders of both groups were excised, weighted, and the area of the malignant tissue of each bladder was determined by ImagePro Plus software. Another 4 healthy mice were used as control. The total tumor area of each bladder was determined and the mean of the total areas was calculated for each group. The Mean and SD of bladder tumor area (**A**) and weight (**B**) are shown.

tumors at the end of the experiment was 83% smaller (**Figure 6A**) than that of the P4-Luc-P3-Luc treated ones (6.8 ±2.2 mm^2 and 39.046 ±19 mm^2 respectively) ($P<0.001$). As shown in **Figure 7B**, the group treated with the reporter vector showed usually more than one large lesion, with different grades of invasion. In contrast, only small tumors were detected in the P4

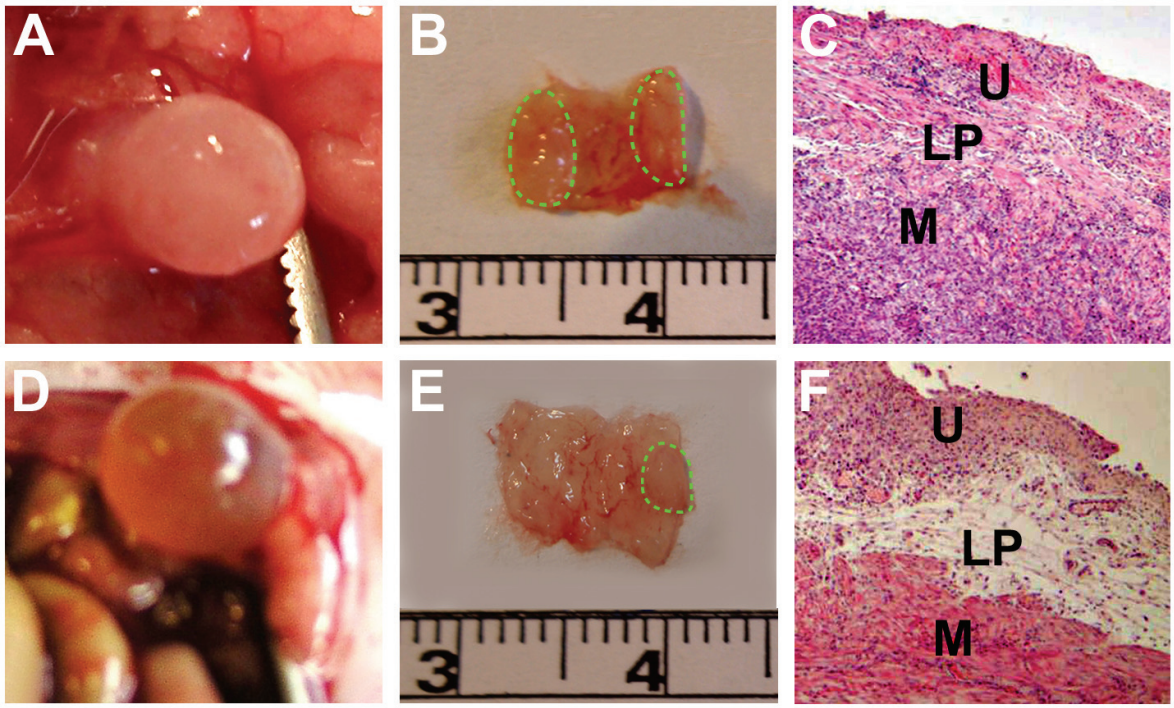

Figure 7. Macroscopic and histopathological views of the orthotopic bladders treated with P4-DTA-P3-DTA: Shown are macroscopic photographs of the whole orthotopic bladders treated with P4-Luc-P3-Luc (**A**), or with P4-DTA-P3-DTA (**D**). Bladders of both groups were excised and the area of the malignant tissue of each bladder is indicated (by grin line) for the P4-Luc-P3-Luc (**B**) and P4-DTA-P3-DTA (**E**). Histopathological microscopic view (H&E X 10) is shown for P4-Luc-P3-Luc treated bladder (**C**) and for P4-DTA-P3-DTA treated bladder (**F**), ('U', urothelium,'LP', lamina propria, 'M', muscle).

-DTA-P3-DTA treated bladders (**Figure 7E**).

Inhibition of tumor growth was also reflected in bladders weight (**Figure 6B**). The mean bladder weight of P4-DTA-P3-DTA treated mice was 22±3.2mg compared to 56±13mg in the control group. The mean bladder weight of the healthy mice was 20±3mg ($P<0.001$).

Expression of DTA and Luc RNA in mouse orthotopic treated bladder tumors

RNA samples from the bladders treated tumors were analyzed by RT-PCR and revealed DTA mRNA expression in the P4-DTA-P3-DTA treated tumors (lanes 3-4) but not in the luciferase treated tumors (lanes 1-2). This indicates that the tumors were efficiently transfected by the P4-DTA-P3-DTA vectors and that the human IGF2-P4 and IGF2-P3 promoters were activated and DTA was produced.
Necrosis in P4-DTA-P3-DTA treated bladder, as a result of DTA activity, is shown in **Figure 8B**.

Discussion

In this study a double promoter expression vector was used, expressing the diphtheria toxin A-fragment (DTA), from two different regulatory sequences, IGF2-P4 and IGF2-P3. This construct was used to transfect and to eradicate tumor cells in culture (*in vitro*) or tumors developed in animal models (*in vivo*) for bladder carcinoma.

IGF2-P3 and IGF2-P4 are highly expressed in bladder carcinoma, but rarely in normal adult tissues [7].

The use of double promoter expressing vectors is highly novel. Tumor cells can express high levels of IGF2-P3 and IGF2-P4, or only one of those transcripts. That way, majority of tumor cells could efficiently express DTA.

Subunit A of the diphtheria toxin (DTA), a highly

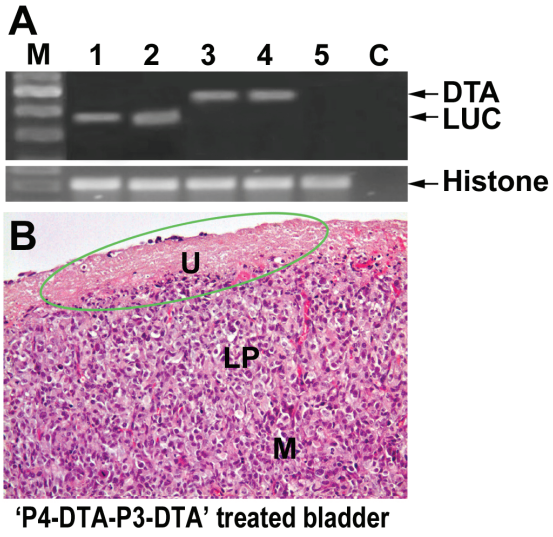

Figure 8. Detection of DTA and Luc transcripts in orthotopic bladder tumors: Mice with orthotopic bladder tumors were intravesically treated twice in 3 days interval and were sacrificed 4 days after the last treatment. Tumors were excised and frozen immediately and 400ng RNA (extracted from the tumors) was used for determination of luciferase and DTA by RT-PCR reaction. (**A**) tumors treated with P4-Luc-P3-Luc (lanes 1-2), or with P4-DTA-P3-DTA (lanes 3-4). Lane 5: untreated orthotopic bladder tumor, 'C': negative control for PCR, 'M': 100bp DNA ladder. The sizes of the PCR products are 468bp and 328bp, for DTA and Luc respectively. The lower panel shows the histone 3.3 internal control. Necrosis of orthotopic bladder tumor treated with P4-DTA-P3-DTA (H&E X 20) is shown (**B**) and the necrotic area is indicated (by green line). ('U', urothelium, 'LP', lamina propria, 'M', muscle).

potent poison, was chosen as an effector molecule. When only the cDNA coding for the A-fragment is expressed, the released toxin from the lysed cells will not be able to enter neighboring cells in the absence of the DT-B fragment [23]. This approach not only will insure high killing activity but will be of great advantage against any unintended toxicity to non-target normal cells. Moreover, introduction of DTA DNA sequence under the control of regulatory sequences of genes differentially expressed in tumors but not in adjacent non-tumor cells will selectively favor the specificity of the treatment.

Over plurality of cancer specific promoters, IGF2 -P3 and IGF2-P4 regulatory sequences were selected for targeting cancer cells. The IGF2-P3 and IGF2-P4 regulatory sequences are expected to be good candidates for specifically inducing the expression of DTA in target tumor cells but not in cells of normal tissue. They are known to be differentially over-activated in various tumor types and to show no or minimum activity in the surrounding normal tissue [24, 25]. This is in addition to the known autocrine/paracrine mode of IGF2 mitogen action in the development of a wide range of human malignancies.

We demonstrated by RT-PCR and ISH that combined expression of IGF2-P3+IGF2-P4, showed enhanced expression profile, in which the majority of tumor samples expressed high levels from at least one of the regulatory sequences and nearly 100% showed positive expression. These results clearly support the rationale of our hypothesis, which DTA could be extensively expressed from more than one specific regulatory sequence.

IGF2 play major role in tumor development. By selective killing of cancer cells expressing IGF2, the treated tumor cells as well as neighboring tumor cells (as IGF2 mediate its effect in autocrine/paracrine manner) are at least partly deprived of their IGF2 supply. Accordingly, destruction of the IGF2 expressing tumor cells not only will eliminate part of the tumor but will also diminish the supply of mitogenic IGF2 to neighboring cells and may lead to arrest of tumor growth and prevent following metastases process [26, 27].

P4-DTA-P3-DTA exhibited superior efficiency *in-vitro* (**Figure 2**), in lysing human UC cells, relative to each single promoter construct carrying either DTA sequence alone (P4-DTA or P3-DTA).

Heterotopic bladder cancer model was used to evaluate tumor growth inhibition of P4-DTA-P3-DTA compared to that of the single promoter vectors. P4-DTA-P3-DTA exhibited superior ability to inhibit tumor development by 68% (P=0.004) compared to P4-DTA or P3-DTA activity (**Figure 4**).

Additional *Ex-vivo* measurements (**Figure 5**), reconfirmed the difference between the P4-DTA-P3-DTA and control group. Thus, eliminates any unrelated difference of the measurements (such as subcutaneous inflammation swelling due to necrosis reaction, etc.).

Transurethral implantation of human UC cells into mice bladders provides a more relevant

tool for investigation of the biology and therapy of bladder cancer than subcutaneous implantation of UC cells (heterotopic model). Therefore we evaluated the feasibility of intravesical therapy of P4-DTA-P3-DTA, in mouse orthotopic model. The average size of the P4-DTA-P3-DTA treated tumors was 83% smaller than that of the P4-Luc-P3-Luc treated ones (**Figure 6A**) and there was also significant difference in mean bladders weight (**Figure 6B**). Only small tumors were detected in the P4-DTA-P3-DTA treated bladders (**Figure 7**), compared to large lesions and with different grades of invasion in the group treated with the reporter vector.

The inhibition of tumor progression resulted exclusively from the toxic effect of the diphtheria toxin. This was confirmed by RT-PCR determining mRNA expression of DTA only in orthotopic tumors treated with DTA expressing vector (**Figure 8A**), and by performance of cellular necrosis in P4-DTA-P3-DTA treated tumors compared to the P4-Luc-P3-Luc treated and non -treated ones (**Figure 8B**).

As IGF2-P4 and IGF2-P3 are expressed at very high levels in bladder cancer, therefore we propose a double promoter expression approach for targeted cancer therapy.

Conclusions

Overall, the double promoter vector exhibited enhanced anti-cancer activity relative to single promoter expression vectors carrying either gene alone. Our findings show that UC may be successfully treated by intravesical instillation of P4-DTA-P3-DTA.

Abbreviation: jetPEI Chemical structure: HO-(CH2)2-(CH2-CH2-NH)n-(CH2)2-OH. The N/P ratio refers to the number of nitrogen residues of jetPEI per DNA phosphate.

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