

# Regulatory sequences of the H19 gene in DNA based therapy of bladder cancer

## Research Article

Patricia Ohana<sup>1\*</sup>, Ofer Gofrit<sup>2</sup>, Suhail Ayesh<sup>1,4</sup>, Wasif Al-Sharef<sup>4</sup>, Aya Mizrahi<sup>1</sup>, Tatiana Birman<sup>1</sup>, Tamar Schneider<sup>1</sup>, Imad Matouk<sup>1</sup>, Nathan de Groot<sup>1</sup>, Eli Tavdy<sup>3</sup>, A. Ami Sidi<sup>3</sup>, and Abraham Hochberg<sup>1</sup>

<sup>1</sup>Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel

<sup>2</sup>Department of Urology, Hadassah University Hospital, Jerusalem, Israel

<sup>3</sup>Department of Urologic Surgery, The Wolfson Medical Center, Holon, and Sackler Faculty of Medicine Tel Aviv, University, Israel

<sup>4</sup>Molecular Genetics Laboratory, Makassed Islamic Charitable Hospital, Jerusalem, Israel

---

\*Correspondence: Patricia Ohana, PhD, Department of Biological Chemistry, The Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel; Tel: 972-2-6585457; FAX: 972-2-5610250; Email: pohana@huji.ac.il

**Key words:** H19 regulatory sequences; transitional bladder carcinoma; diphtheria toxin A; intravesical vector instillation

**Abbreviations:** bladder mucosa, (Ta); diphtheria toxin A,(DT-A); intravesical chemotherapy and immunotherapy, (BCG); luciferase gene, (luc); transurethral resection, (TUR)

The first and second authors contributed equally to this work

Received: 19 May 2004; Accepted: 15 June 2004; electronically published: June 2004

## Summary

The objective of the present study was to develop novel DNA based therapy strategies for bladder cancer. We detected a high expression level of the H19 gene in murine and human bladder carcinoma tissues compared to nearly undetectable levels in the surrounding normal tissues. On the basis of these findings we constructed a plasmid in which H19 regulatory sequences drove the expression of the diphtheria toxin A gene. This plasmid was introduced by intravesical instillation into the bladders of rats with bladder carcinoma (orthotopic model) and into the bladders of two human patients suffering recurrent superficial transitional cell carcinoma, refractory to all commonly used treatments. Very significant tumor growth inhibition was observed in the rat bladder tumors after two intravesical injections of 50 µg of DTA-H19 toxin vector as compared to control animals. Nearly complete ablation of the tumor was determined by video imaging in the two human patients after treating once a week with 2 mg of DTA-H19 plasmid for a total of 9 weeks. Not even a trace of the plasmid could be detected in the bloodstream of the patients. This observation strongly indicates the safety of our treatment. These observations may be the first step of a major breakthrough in the treatment of human bladder carcinoma.

## I. Introduction

Bladder cancer is the fourth most common malignancy in men, and the eighth most common cause of death from cancer. Eighty percent of the bladder tumors are superficial-limited to the bladder mucosa (Ta) or sub-mucosa (T1). These tumors can be removed by transurethral resection (TUR), 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. The target population of this study is

patients with superficial bladder cancer refractory to conventional therapies.

The study of gene expression patterns in normal and in malignant tissues identified differentially expressed genes. The regulatory sequences of genes highly expressed in tumors and not or only marginally expressed in normal tissues may be used to drive the expression of a toxin gene in tumor cells only.

Based upon early studies from our group and others, the transcriptional regulatory sequences of the H19 gene emerged as candidates for cancer gene therapy. H19 is a paternally-imprinted, oncofetal gene that encodes a RNA

(with no protein product) acting as a “riboregulator” (Erdmann et al, 2001), which is expressed at substantial levels in embryonic tissues, in different human tumor types, and marginally or not expressed in the corresponding tissues of the adult (Ariel et al, 1995, 1997, 1998). Its precise function is being debated; however, our recent data suggest a role for H19 in enabling the tumor cells to survive under stress conditions by promoting angiogenesis and cancer progression (Ayesh et al, 2002). While the initial report of our group focused upon bladder tumor (Rachmilewitz et al, 1992), our subsequent findings established H19 expression in other types of tumors, including hepatocellular (Ariel et al, 1998), ovarian, endometrial and testicular cancer (Verkerk et al, 1997; Tanos et al, 1999). The human H19 gene lies within 200 kb downstream of the paternally expressed IGF2 gene. These two genes are frequently coordinately regulated, both in terms of their common expression pattern and reciprocal imprinting. Enhancers located downstream of H19 stimulate transcription of both genes (Leighton et al, 1995). We have shown that H19 is significantly expressed in 84% of human bladder carcinomas and that the expression level is decreasing with loss of tumor differentiation. Independent of tumor grade, the H19 expression level is positively correlated with early tumor recurrence (Ariel et al, 2000).

We used a carcinogen-induced bladder tumor model in rat that parallels superficial papillary TCC in humans. We established that H19 expression is positively correlated with tumorigenesis in this model (Ohana et al, submitted).

We have previously reported the construction of expression vectors carrying the diphtheria toxin A-chain gene, under control of IGF2-P3, IGF2-P4 and H19 regulatory sequences (Ohana et al, 2001; Ayesh et al, 2003). We showed that these constructs are able to selectively kill tumor cell lines and inhibit tumor growth in vitro and in vivo in accordance to the transcriptional activity of the above-mentioned regulatory sequences (Ohana et al, 2001; Ayesh et al, 2003).

We propose tailored transcriptional regulatory sequence selection for bladder cancer gene therapy according to individual patient-specific gene expression profiles. This constitutes the new concept of “patient oriented DNA based therapy” for bladder cancer.

The present study seeks to evaluate the potential utility of the regulatory sequences of the H19 gene for directing tumor-selective expression of toxins, delivered by non-viral vectors in two animal bladder carcinoma models and in human patients. Non-viral vectors appear promising due to their potential in circumventing the main disadvantage of adenoviral vectors, which consists of immune responses directed against adenovirus proteins; this limits their ability to be administered iteratively.

Here we present evidence that treatment of bladder carcinoma in animals and human patients with constructs expressing the diphtheria toxin A (DT-A) gene driven by the H19 regulatory sequences lead to a highly significant suppression of tumor growth in animals and human patients with no apparent toxicity toward the host indicating that these constructs have a high therapeutic

potential and are very promising candidates for bladder cancer therapy in humans.

## II. Materials and methods

### A. Cell culture

The human bladder carcinoma cell line RT112 and the rat bladder carcinoma cell line NBT-II were purchased from ATCC (Rockville, MD). The NBT-II cell line was derived from bladder tumors induced by BBN in male Wistar rats. The cells were grown and maintained in DMEM medium as previously described (Ohana et al, 2001).

### B. Construction of expression plasmids

The luciferase gene reporter constructs were built from the pGL3 basic (Luc-1) vector (Promega, Madison, USA) which lacks both promoter and enhancer sequences. The construct Luc-H19 which contains the luc gene under the control of the human H19 promoter region from nucleotide -818 to +14 was prepared as described in (Ohana et al, 1999). The Luc-H19 plasmid was digested with Xba I and Nco I and the insert of the luciferase gene (luc) was replaced by the Diphtheria toxin A chain (DT-A) coding region to yield the DTA-H19 construct. The DT-A gene was prepared from the pIBI30-DT-A plasmid (kindly donated by Dr. Ian Maxwell, University of Colorado, USA).

The pEYFP-Mito vector (Clontech, CA Palo Alto, USA) encodes for subunit VIII of the human cytochrome-c oxidase fused to the N-terminus of EYFP under the control of CMV promoter.

Large-scale preparations of the plasmids were performed using the EndoFree Plasmid Mega Kit (Quiagen, GmbH Hilden, Germany).

### C. In vitro transfection and luciferase assay

A total of  $0.4 \times 10^6$  cells were plated in a six-well Nunc multidish (30 mm). Transient transfections were carried out using the JetPEI cationic polymer transfection reagent, mean molecular weight of 22 kDa, Polyplus, Illkirsh-France). The transfection was carried out according to the manufacturer's instructions using 3  $\mu$ l of DNA and 6  $\mu$ l of Jet-PEI solution to obtain a PEI nitrogen/DNA phosphate (N/P) ratio of 5. Transfection experiments were stopped after 48 hours and the reporter gene activity in the cells was assessed using the Promega kit “Luciferase Assay System” (E-1500-PROMEGA, Madison, U.S.A). Light output was detected using a Lumac Biocounter apparatus. Protein content was measured by the Bio-Rad protein assay reagent, and the results were expressed as light units/ $\mu$ g protein.

LucSV40 (Luc-4) was used for the determination of the toxic effect of the cotransfected DTA-H19 plasmid, while Luc-1 that lacks any regulatory sequences was used as a negative control to determine the basal non-specific luciferase expression, which was found to be negligible. All experiments were done in triplicates and the results expressed as mean and standard error.

Cells were cotransfected using 3  $\mu$ g of the reporter vector Luc-4 and the indicated amounts of the expression vectors DTA-H19 using the transfection reagent Jet-PEI as described above. Cells were also transfected by 3 $\mu$ g Luc-4 alone. The cell killing capacity of the cotransfected DTA-H19 plasmid was determined by calculating the % decrease in the luc activity in the cotransfected cells compared to that of the cells transfected with Luc-4 only.

### D. Determination of the DTA-H19 plasmid level in urine and blood

The urine samples were centrifuged at 1500 g for 10 min,

obtaining supernatant and cells. Plasmid DNA was isolated and purified from urine (supernatant and cells) and plasma samples using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences IK). The samples were eluted with 35  $\mu$ l of buffer Tris-HCl 10 mM, and PCR analysis was performed using 3  $\mu$ l of the eluted DNA in a reaction volume of 25  $\mu$ l. The purification of total genomic DNA from the urine was carried out using the Master Pure DNA Purification Kit for blood (Epicentre, Madison Wisconsin, USA). The cells were pelleted by centrifugation and resuspended in 80  $\mu$ l of Lysis buffer and treated with protease K (0.8 mg/ml, Sigma) in PK buffer (50 mM Tris-HCl 7.5, 5 mM EDTA) at 55°C for 10 min. The total genomic DNA was purified according to the manufacturer's instructions.

### E. Determination of the level of RNA products of the H19, DT-A and luc genes

Total RNA was extracted from cell lines or tissues using the RNA STAT-60 Total RNA/mRNA isolation reagent (Tel-Test, Inc. Friendswood, TX, USA), according to the manufacturer's instructions. The RNA was treated by RNase-free DNase I (Roche Diagnostics GmbH, Mannheim) for eliminating any contaminating DNA. The cDNA was synthesized from 2  $\mu$ g total RNA in a 20  $\mu$ l reaction volume as described (Ayesh et al, 2003).

The PCR reactions were carried out in 25  $\mu$ l volumes containing 6 ng/ $\mu$ l of each of the forward and the reverse primers using 0.05 units/ $\mu$ l of Taq polymerase (TaKaRa Biomedicals, Japan) according to the manufacturer's instructions. The primer sequences for the rat H19 transcript were (5'-ACTGGAGACTAGGGAGGTCTTAGCA) upstream, and (5'-GCTGTGTGGGTCTGCTCTTCAAGATG) downstream. The PCR was carried out for 30 cycles (98°C for 15 sec, 58°C for 30 sec, and 72°C for 40 sec) and finally 72°C for 5 min. The PCR analysis for DT-A amplification was carried out as described in (Ayesh et al, 2003). The primer sequences used to amplify the luc transcript were (5'-GAGGCGAAGTGTGTGAGA) upstream and (5'-TTTTCCGTCATCGTCTTTCC) downstream. The PCR was carried out for 34 cycles (94°C for 5 min, 94°C for 1 min, 56°C for 30 sec, and 72°C for 30 sec) and 72°C for 5 min. The integrity of the cDNA was assayed by PCR analysis of the ubiquitous, cell cycle independent, histone variant, H3.3. The products of the PCR reaction were run on 2% agarose in TAE electrophoresis running buffer (40 mM Tris acetate, and 2 mM EDTA, pH ~8.5), stained by ethidium bromide and visualized by UV.

### F. DIG-labeled probe synthesis and In situ hybridization

Digoxigenin labeled H19 RNA transcripts were produced by labeling with DIG-11-UTP by SP6, T3, or T7 RNA polymerase in an in vitro transcription reaction (Boehringer Mannheim) as described before (Ayesh et al, 2003).

Paraffin wax blocks of superficial bladder carcinoma of two patients (from the department of pathology, Wolfson Medical Center) were submitted to the study (according to local ethics committee approval). The preparation of the sections for *in situ* hybridization was as described in (Ariel et al, 1998).

### G. In vivo DNA based therapy

#### 1. Heterotopic nude mice model

Confluent RT112 human bladder carcinoma cells were trypsinized to a single cell suspension and resuspended in PBS. 2 x 10<sup>6</sup> cells (in 250  $\mu$ l volume) were subcutaneously injected into

the back of 6-8 weeks old CD-1 female nude mice. 10 days after cells inoculation the developed tumors were measured in two dimensions and subjected to different treatments. Intratumoral injections of 25  $\mu$ g of the toxin construct DTA-H19 and 25  $\mu$ g of the reporter vector Luc-H19 (control group) were performed at days 10, 12 and 14 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. This was carried out in a two-step procedure for the preparation of a standard quantity of 50  $\mu$ g of PEI/DNA complexes, according to the manufacturer's instructions. Tumor dimensions were measured, and the tumor volume was calculated according to the formula width<sup>2</sup> x length x 0.5. The animals were sacrificed 3 days after the last injection, 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 were made using the Image Pro Plus software (Media cybernetics, Silver Springs, USA).

#### 2. Orthotopic rat bladder carcinoma model

Female Wistar rats were anesthetized, and 22 gauge catheters were inserted transurethraly into their bladders. After catheterization the bladder was drained and its mucosa was mildly disrupted by a 15-sec wash with 0.5 ml of 0.1 N HCl, followed by neutralization with 0.5 ml of 0.1 N NaOH. The bladder was then washed with PBS, and NBT-II rat bladder carcinoma cells (2 x 10<sup>6</sup> in 0.5 ml PBS) were instilled into the bladder via the catheter and maintained in the bladder for at least 2 hours by tying the external opening of the urethra. Three or seven days after the instillation of the NBT-II cells the animals were anesthetized and the bladder was recatheterized and drained. The animals underwent intravesical instillation of the proper treatment for 2 hours. At day-3 after the instillation of the cells, one group of 6 rats received 50  $\mu$ g of DTA-H19 and another group of 6 animals received 50  $\mu$ g of Luc-H19 plasmid. The same treatments were repeated at day-7. The *in-vivo*-jetPEI was used as a transfection enhancer agent. 6  $\mu$ l of the jetPEI (N/P ratio = 6) in 250  $\mu$ l glucose 5% (w/v) were mixed with 50  $\mu$ g of DTA-H19 or Luc-H19 plasmid in 250  $\mu$ l of 5% glucose solution. The resulting mixture was vortex-mixed and left for 10-15 minutes at room temperature and subsequently instilled into the rat bladder transurethraly as described above. The animals were sacrificed at the end of the experiment (day-10) each bladder was cut sagittally into three pieces, fixed in formalin and embedded in paraffin. 5  $\mu$ m serial sections prepared from the treated orthotopic bladder tumors were photographed by a digital camera (Olympus, Tokyo, Japan), the high resolution photos were saved as digital images. The malignant area of each slide was measured by the Image-Pro Plus Version 3.0 software.

The efficiency of the *in-vivo* gene transfer into the tumor cells was evaluated by using the reporter vector expressing GFP under the control of the CMV promoter (pEYFP-Mito) complexed with the transfection enhancer PEI at N/P = 6 and N/P = 10, as described above. Twenty-four hours after plasmid instillation the bladders were excised and immediately frozen in liquid nitrogen. A number of successive 10  $\mu$ m sections were cut by a cryostat at least at 3 different depths of the tissue. The slides were fixed for 15 minutes in freshly prepared 4% paraformaldehyde at room temperature. They were washed by PBS solution and mounted in a small drop of 90% glycerol in PBS. Slides were visualized by a Fluorescent microscope (Olympus AX70 Microscope; Olympus, Tokyo, Japan). Fluorescent cells were counted on four different slides of each bladder (41 fields in total), covering systematically the central

and peripheral areas of each tumor. Frozen sections of a bladder transfected with PEI alone were used as control slides.

### H. Toxicity studies in animals

A group of five healthy CD-1 nude mice received three subcutaneous injections of the toxin construct DTA-H19 complexed with PEI in their dorsa every two days, another group of healthy mice (n=5) were treated with PEI only serving as control. The mice were sacrificed ten days following the last plasmid injection. They were weighed, and blood samples for the study of renal (creatinine, urea) and liver function (serum glutamic-oxaloacetic transaminase, serum glutamic-pyruvic transaminase, alkaline phosphatase, and gamma-glutamyl transpeptidase) were taken. Samples of liver, lung and kidney were fixed in 4% buffered formaldehyde and processed for histological examination.

A group of four normal Wistar rats underwent intravesical instillation of 50 µg of DTA-H19/PEI. Another group of five rats were treated with PEI alone. Three non-treated healthy rats were used as control group. The treatment was repeated twice with three days intervals. The rats were sacrificed ten days after the last plasmid treatment, and the same parameters as described for the mice were monitored for the rats. The level of the plasmid DTA-H19 was measured by PCR in the blood and urine samples collected 6, 24, 72 and 120 hours after the third plasmid instillation treatment.

### I. Study design and treatment of two patients

Two male adult patients (57 and 56 years old) suffering from refractory superficial bladder cancer participated in the study. The patients provided written informed consent. The study protocol was approved by the local ethics committee (Wolfson Medical Center), and the ethical committee of the Israel Ministry of Health. The study was conducted according to the Declaration of Helsinki and followed the principles of good clinical practice.

The two patients had multiple (8 and 10 respectively) transurethral resections of bladder tumors for Ta, G1-2 during the last five years. Both patients received 6 courses of BCG, one course of BCG with interferon alpha and thermo-chemotherapy with the Synergo device (14) with no response and repeated recurrences. *In-situ* hybridization on the paraffin blocks from the last TURBT using the riboprobe Dig-H19, showed high levels of H19 RNA. The first patient was treated with an intravesical instillation of 2 mg of the toxin vector DTA-H19 complexed with 240 µl of JetPEI (N/P ratio = 6) dissolved in a final volume of 10 ml 5% glucose (w/v). After instillation the catheters were blocked to allow a contact time of 2 hours. This treatment was repeated once a week for a period of 6 weeks. The second patient received an intravesical instillation of the DTA-H19 plasmid (5 mg) 18 hours before transurethral resection of multiple bladder tumors, while marker lesions were left. Detection of DT-A DNA was carried out by PCR analysis in tumor samples obtained from the TUR. The second patient received the first treatment of 2 mg of the toxin vector DTA-H19, followed by 2 treatments of 4 mg and another 3 treatments of 2mg of the toxin vector. Patients were closely monitored for adverse events during the whole study period. Hematology, serum chemistry, urinalysis (kidney and liver functions), and video-cystoscopy were performed every four weeks. DT-A DNA was analyzed in the blood and in urine samples (at different times following the treatment) by PCR analysis as described above.

## III. Results

### A. Heterotopic nude mice bladder carcinoma model

We have previously shown that the regulatory sequences of H19 are highly transcriptionally active in the

human bladder carcinoma cells RT112 (Ohana et al, 1999, 2001). In accordance to that, we have also found that the DTA-H19 construct even at low concentration was able to drive the expression of the DT-A toxin thereby reducing the luc activity in cells cotransfected with LucSV40 and with the DT-A vector as a measure for cell death. We have evaluated the therapeutic potential of the toxin vector DTA-H19 in a heterotopic model of bladder cancer in nude mice. 14 days after subcutaneous RT112 bladder carcinoma cells inoculation, measurable subcutaneous tumors had developed. One group of mice was intratumoral injected with 25 µg of the H19-DTA plasmid /tumor and the tumors of another group were treated with 25 µg of the reporter vector Luc-H19, in both treatments PEI was used as a transfection enhancer reagent. **Figure 1** shows that two injections of DTA-H19 were able to inhibit any further tumor growth as compared to the Luc-H19 treatment. While the average tumor size of the toxin treated tumors did not change, the Luc-H19 treated tumors continued to increase their volume 2.5 fold during the 7 days after the start of the treatment. The growth rates of tumors according to tumor volume measured *in vivo*, were significantly reduced (p= 0.01) compared with the growth rates of control treated groups (**Figure 1**). Computerized histologic measurements of the RT112 tumors showed that the toxin treated tumors have significantly (p=0.005) relatively larger necrotic areas (42% ± 10.2) compared to the Luc-H19 treated tumors (27% ± 11.8).

A group of healthy CD1 nude mice were treated with a subcutaneous injection of 25 µg of DTA-H19/PEI and another group with PEI alone every three days. During the course of the treatment the members of the two groups didn't show any signs of toxicity. The DTA-H19 treated mice did not loose weight till they were sacrificed 10 days after the third injection. Liver and renal function tests indicated no change compared to values obtained from untreated animals. No traces of the plasmid DTA-H19 were found by PCR analysis of blood samples collected after the mice were sacrificed.

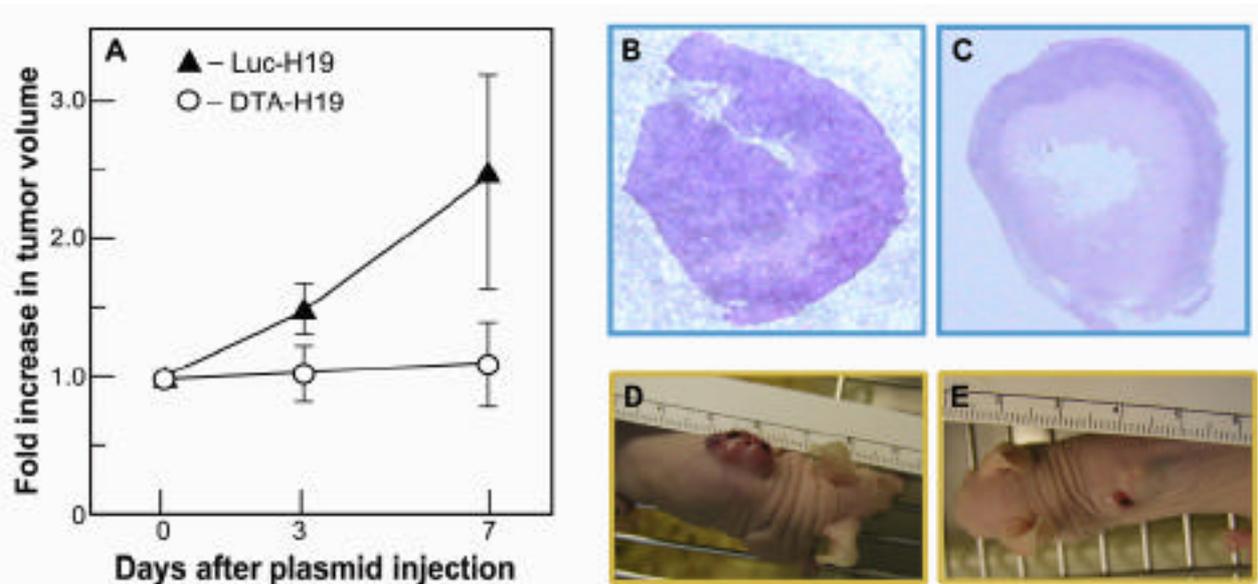
### B. Validation of the orthotopic rat bladder carcinoma model

The transurethral implantation of murine bladder cancer cells into the rat bladder (orthotopic model) provides a more relevant model for the investigation of the biology and therapy of bladder cancer than subcutaneous implantation of bladder cancer cells (heterotopic model). Accordingly, a rat model for bladder cancer was developed by intravesical instillation of NBT-II rat bladder carcinoma cells onto the wall of the rat bladder *in-vivo*. To evaluate the potential use of H19 regulatory sequences for the therapy of this rat bladder carcinoma model, we determined the levels H19 RNA in the tumors developed in the bladders. One week was enough for the rats to develop TCC after the intravesical inoculation of the NBT-II cells. Hence, seven days after the inoculation the rats were sacrificed, their bladders were excised and snap frozen. The level of H19 RNA was determined by RT-PCR analysis in the total RNA extracted from the bladders. The results of the PCR analysis shown in (**Figure 2**) indicated high levels of H19 RNA in bladder

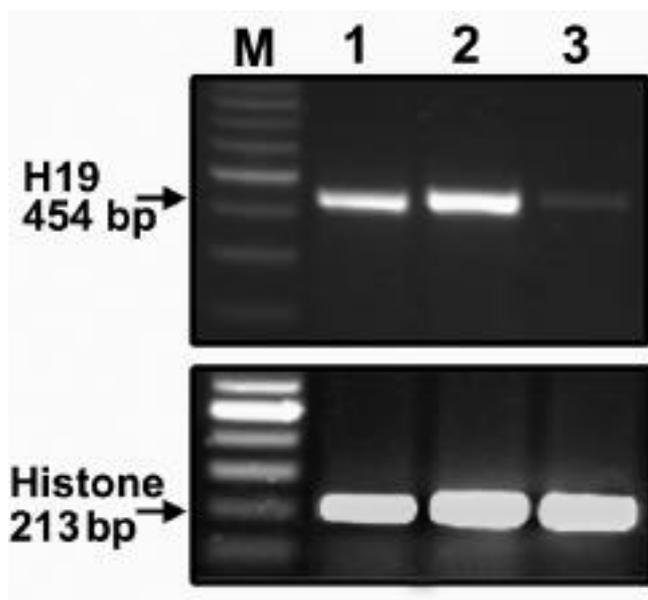
tumors (lanes 1-2). It is interesting to note that the level of H19 RNA in the bladder tumor is significantly higher than in the carcinoma cells NBT-II used for the inoculation (lane 3). We have also previously shown that H19 RNA is undetectable in rat normal bladder tissue (results not shown).

### C. *In-vitro* DT-A expression under the control of human H19 regulatory sequences in rat cells

The *in-vitro* toxic potential of H19-DTA was determined after cotransfection of the rat NBT-II cells with 3  $\mu\text{g}$  of LucSV40 and the indicated concentrations of H19-DTA (**Table 1**). The luciferase activity in the cotransfected cells was determined and compared to that in cells transfected by LucSV40 alone. The relative reduction of the luciferase activity in the cotransfected cells is the result of the H19 driven DT-A expression causing cell killing. **Table 1** shows that low amounts of the DTA-H19 plasmid (0.25  $\mu\text{g}$ ) was able to drive the expression of the DT-A toxin reducing luc activity in the



**Figure 1. DTA-H19 inhibits tumor growth in nude mice-** A-The expression vectors DTA-H19 or Luc-H19 (control group) were administrated twice (on day 0 and day 3) by direct intratumoral injection after subcutaneous implantation of human bladder carcinoma cells in the back of nude mice. Histology sections of nude mice tumors from a Luc-H19 (B) or DTA-H19 (C) treated animal (8 X and 20 X magnification respectively) showing extensive necrosis of the tumor tissue only in the DTA-H19 treated tumor. Nude mice bearing bladder tumors after Luc-H19 (D) and DTA-H19 (E) treatment.

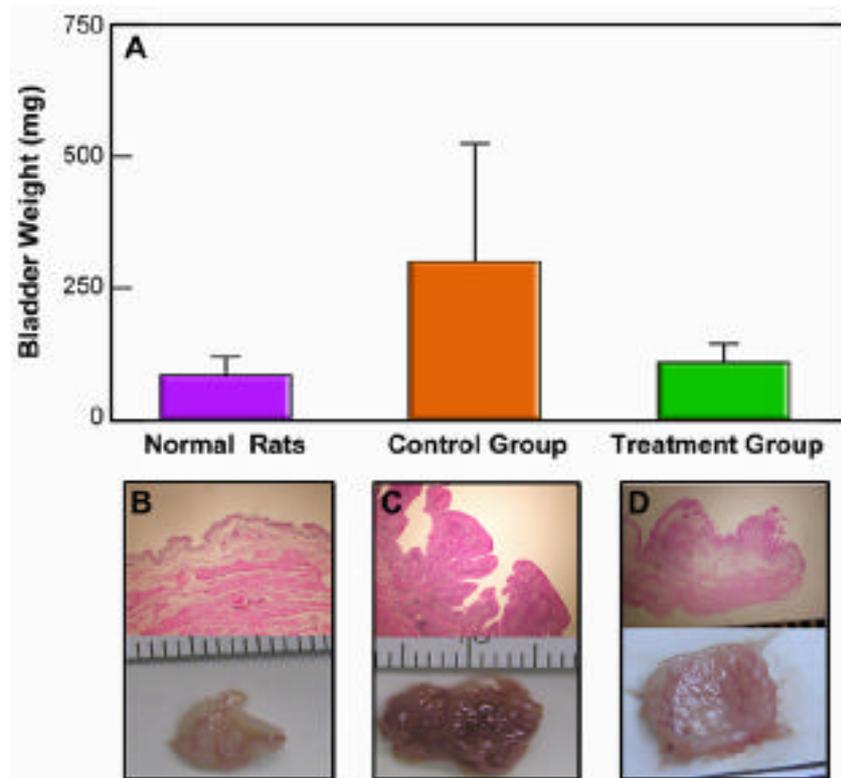


**Figure 2. The level of H19 transcript in NBT-II rat bladder carcinoma cells and in bladder tumors induced by intravesical instillation of NBT-II cells.** RNA samples from rat orthotopic bladder tumors (lanes 1-2) and from NBT-II cells (lane 3) were analyzed by RT-PCR for H19 transcript expression. 200 ng DNase treated RNA were used for each reaction. M (100-bp ladder). The upper panel is the 454 bp H19 cDNA and the lower panel is the histone internal control.

**Table 1.** Response of rat bladder carcinoma cells to DTA-H19 plasmid

System	Relative luciferase activity (%) Versus control
Luc-1	1.40
Luc-H19	12.25
Luc-4	100.00
Luc-4 + 0.25 µg DTA-H19	12.64
Luc-4 + 1.0 µg DTA-H19	6.41

NBT-II rat bladder carcinoma cells were cotransfected with 3 µg of the control vector Luc-4 and the indicated amounts of the DTA-H19 toxin vector. Luciferase activity is expressed as a percentage of that observed after transfection with the positive control plasmid alone, set at 100 %. The values represent the averages of 3 independent experiments, each performed in triplicate determinations, with an SD < 10 %.



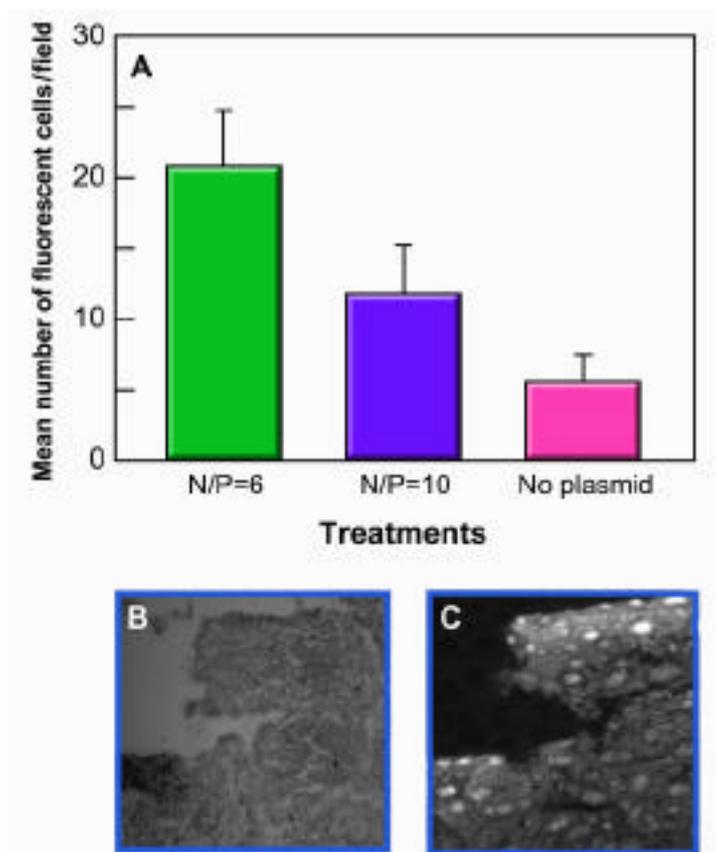
**Figure 3. Effect of intravesical administration of DTA-H19 vector on the orthotopic bladder carcinoma in rats-** The expression vectors DTA-H19 or Luc-H19 (control group) complexed with PEI were administrated intravesically into rat bladders, which were previously implanted with syngeneic bladder carcinoma cells as described in “Materials and Methods”. Various groups of normal healthy rats received the same treatments separately. (A) The mean bladder weight of the DTA-H19 and Luc-H19 treated bladders, measured at the end of the treatment, after the animals were sacrificed. Hematoxylin/eosin stained sections (40 X magnification) and macroscopic bladders appearance of the normal (B) control (C) and DTA-H19 treated rats (D).

cells cotransfected with the LucSV40 by almost 88 %. The total amount of the plasmids used in the cells cotransfected with DTA-H19 was almost unchanged compared to the amount of the Luc-SV40 plasmid alone.

#### D. Treatment of the orthotopic bladder carcinoma in rats

The DTA-H19 construct was shown to be highly active in the NBT-II cells *in-vitro* (Table 1). Moreover, the H19 transcript is expressed at high levels in tumors that developed after implanting NBT-II cells into the bladder of syngeneic rats. Therefore, the orthotopic rat model for bladder is an excellent model for determining the DTA-H19 therapeutic potential for the treatment of

human bladder cancer. The treatment with the toxin vector DTA-H19 was started four days after NBT-II inoculation. A group of four rats received 50 µg of DTA-H19, another group of six rats received 50 µg of Luc-H19 and three healthy rats were treated either with the toxin vector, the reporter vector or saline by intravesical instillation. In all the treatments the DNA transfection was enhanced by using the polycation PEI. Four days later the same treatments were repeated, and the animals were sacrificed three days later; their bladders were weighed and processed for assessment of tumor sizes. Figure 3 shows that two injections of 50 µg of H19-DTA with a four days interval were able to inhibit the tumor growth as indicated by a significant decrease in bladder weight as compared to



**Figure 4. Determination of efficiency of transfection in the orthotopic model of bladder cancer-** Orthotopic tumor in rat bladder was intravesical instilled with a plasmid pEYFP-Mito containing the coding region of Green fluorescence protein (GFP) complexed with the transfectant PEI (a 22 kDa linear form of polyethylenimine) at the indicated N/P ratio. GFP expression was visualized using epifluorescence microscopy (Olympus AX70 Microscope; Olympus, Tokyo, Japan). (A) Shows the mean number of GFP-positive cells/field (each field represents 300  $\mu$ m) in the tumor cryosections 48 hours after the DNA/PEI intravesical instillation. (B) Cross-section of orthotopic rat bladder tumor counterstained with hematoxylin/eosin. (C) Fluorescence micrograph of GFP-transfected tumor cryosections of the same tumor section as shown in (B), 48 hours after transfection of PEI/DNA complexes. Magnification X 40.

the bladder weights of the group treated with Luc-H19. The mean bladder weight of DTA-H19 treated rats was  $130 \pm 10.8$  mg compared to  $382 \pm 106$  mg in the Luc-H19 treated group ( $p = 0.014$ ). The mean bladder weight of healthy rats treated with the plasmids or saline was  $83 \pm 20$  mg. We have also microscopically estimated the tumor area by an accurate computerized technique (see "Materials and Methods"). The average size of the DTA-H19 treated tumors at the end of the experiment was 95 % smaller than that of the Luc-H19 treated ones ( $2.8 \pm 2$  mm<sup>2</sup> and  $63 \pm 42$  mm<sup>2</sup> respectively). The group treated with the reporter vector showed more than one large TCC lesion, with different grades of invasion (data not shown). In contrast, only smaller papillary tumors were detected in the DTA-H19 treated bladders.

A group of healthy rats ( $n=5$ ) were treated by intravesical instillation of the toxin vector DTA-H19 complexed with PEI, another group ( $n=4$ ) was treated with PEI alone and an additional group of 3 untreated healthy rats was used as control. The DTA-H19/PEI and the PEI treated rats continued to gain weight in a similar way as the untreated animals. Liver and renal function tests indicated no change compared to those obtained from normal rats after the animals were sacrificed 10 days after the last treatment. No traces of the plasmid DTA-H19

were found by PCR analysis of blood samples collected after the rats were sacrificed, indicating that there is no systemic absorption of the plasmid after intravesical instillation. Analysis of the urine samples showed that the plasmid was still detected 72 hours after plasmid instillation.

#### E. Determination of efficiency of transfection in the orthotopic model of bladder cancer

One week after NBT-II cells implantation, we analyzed the gene-transfer efficiency of the orthotopic bladder tumors after intravesical instillation of 50  $\mu$ g of the pEYFP-Mito reporter vector as a complex with PEI at a ratio of PEI nitrogen to DNA phosphate either N/P = 6 or N/P = 10. The detection of the fluorescent yellow-green protein expressed from pEYFP-Mito under the control of the CMV promoter, revealed high levels of the protein inside the cells of the tumor. We have calculated the number of GFP-positive cells in the tumor cryosections 24 hours after the DNA/PEI intravesical instillation. **Figure 4** shows that the level of GFP-transgene expression in the tumors treated with the complex DNA/PEI at a ratio of 6 is higher than those treated with DNA/PEI at a ratio of 10.

### F. *In vivo* expression of DT-A toxin gene and luc reporter gene in an orthotopic model of bladder cancer

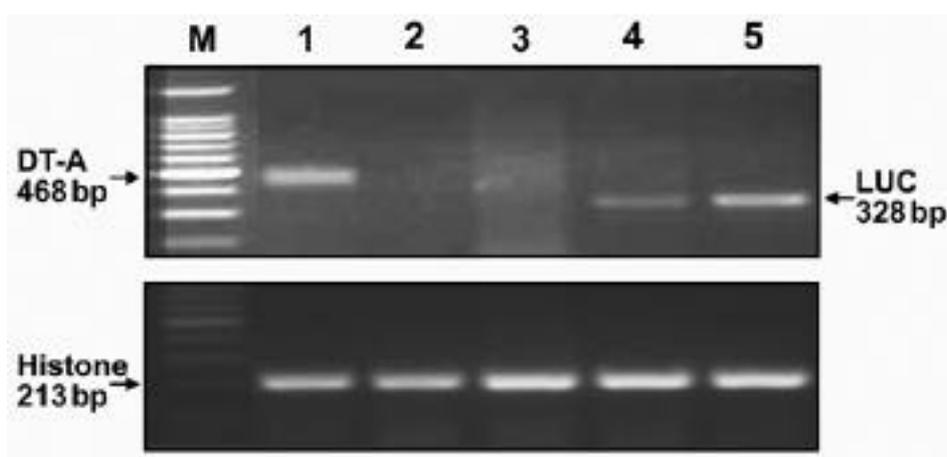
One week after NBT2 cells inoculation, we analyzed the gene-transfer efficiency of the vectors into the orthotopic tumors after intravesical instillation of 50 µg of either DTA-H19 or the reporter vector Luc-H19. The rats received the treatment as a complex with PEI (N/P = 6). 72 hours after the treatment the rats were sacrificed and their bladders were excised and snap frozen. The levels of DT-A and luc mRNAs were determined by RT-PCR in the total RNA extracted from the bladders. High levels of DT-A (**Figure 5-lane 1**) and luc transcripts (**Figure 5-lanes 4-5**) were detected, while no expression of DT-A was detected in the liver (**Figure 5-lane 2**) and only marginal expression in the kidney could be observed (**Figure 5-lane 3**). This indicates that the tumors were efficiently transfected by the plasmid and that the H19 promoter was active, which led to DT-A or luc expression inside the transfected cells.

### G. Treatment of two human patients with the therapeutic vector DTA-H19

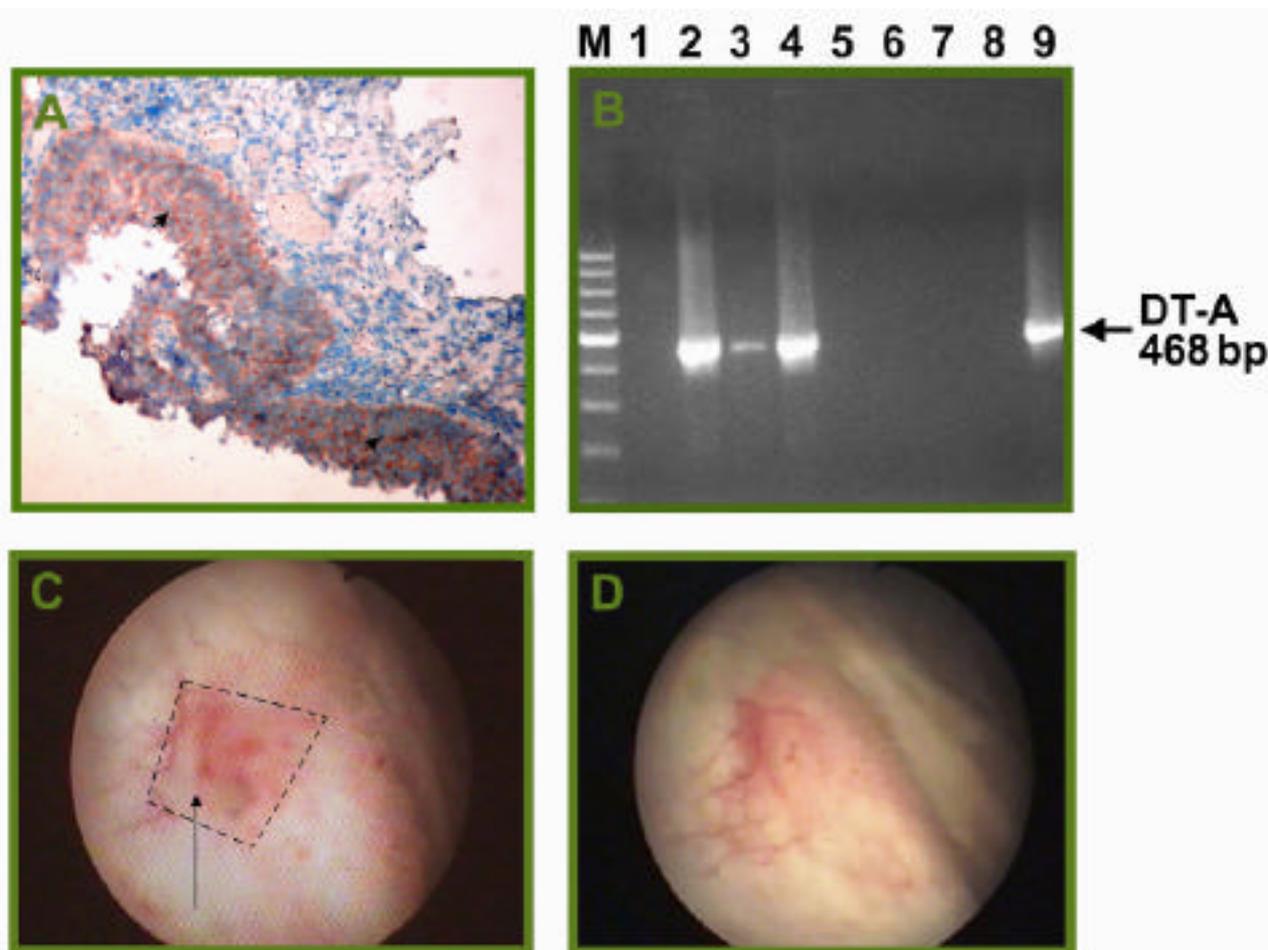
We tested the toxin vector DTA-H19 in two patients who were inflicted with recurrent superficial transitional cell carcinoma refractory to the commonly used treatments such as BCG, intravesical chemotherapy and chemothermotherapy. The patients volunteered after testing H19 expression in bladder biopsy samples by ISH analysis. **Figure 6A** shows high level of the H19 transcripts in a TCC tissue sample from the first patient. This TCC tissue sample shows strong hybridization signals in the cytoplasm of the TCC cells (+3 intensity). The patient was treated with 2 mg of the DTA-H19 construct (instilled intravesically) once a week for a total of 9 weeks. The treatment course was uneventful without any side effects beside one episode of UTI (E. coli) after the third treatment in the first patient. Moreover, not even a trace of the plasmid DTA-H19 could be detected in the

bloodstream of the patient by PCR analysis (**Figure 6B, lanes 5-8**), while high levels of the toxin vector were found in the urine samples tested 2 hours after the first and the second treatment (**Figure 6B, lanes 2 and 4**). This observation strongly indicates the safety of our treatment. It is interesting to note that traces of the construct were found in the urine sample up to one week after the first treatment (**Figure 6B, lane 4**). Video-cystoscopy performed after the third treatment in the first patient showed no adverse reactions of the bladder and a drastic reduction in the tumor size by more than 75 % compared to the tumor size before the treatment (**Figure 6C, D**).

In order to evaluate the efficiency of the plasmid uptake by the tumor tissue, the second patient was treated with 5 mg of the toxin vector DTA-H19 18 hours before TUR of multiple bladder tumors, while marker lesions were left. **Figure 7A** shows the presence of the plasmid DTA-H19 in a tumor sample obtained from UTR. This result indicates an efficient uptake of the toxin vector into the tumor tissue. The therapeutic treatment was started two weeks after the TUR by giving 2 mg of DTA-H19 vector. The presence of the DTA-H19 vector was monitored in urine samples collected starting 2 hours after the treatment and every 24 hours for 96 hours (**Figure 7B, lanes 1-5**). The results of the PCR analysis outlined in Fig. 7-B show that traces of DNA could be still detected till 48 hours after the plasmid instillation (lane 3). The same analysis was carried out in the urine samples collected after the third treatment, in which the patient received 4 mg of the plasmid (**Figure 7B, lanes 6-10**). These results indicated that although the patient received a higher dose of the toxin vector, the kinetic pattern of plasmid secretion in the urine was similar to that observed in the urine samples collected after the first treatment. The urine samples collected from the patient 2 and 30 hours after the fifth treatment with the toxin vector were centrifuged and were examined for the presence of the plasmid in the pelleted cells and in the supernatant by PCR analysis.



**Figure 5. Detection of DT-A and luc transcripts in some of the DTA-H19 and Luc-H19 treated rat bladder tumors.** Rats instilled with these vectors were killed after 72 h and the bladders, livers and kidney were collected. 400 ng of DNase treated RNA samples were used for the RT-PCR reactions. DTA-H19 treated bladder tumor (lanes 1). Liver and kidney tissues from a DTA-H19 treated rat (lane 2 and 3 respectively). Luc-H19 treated bladder tumors (lanes 4-5), M (100 bp ladder). The lower panel shows the histone internal control.



**Figure 6.** Treatment of a human patient (HM) with the therapeutic DTA-H19 vector (A) ISH detection of H19 transcript in tissue section of bladder carcinoma from the patient, demonstrating diffuse and high H19 expression (head arrows indicated). (B)- Detection of the DTA-H19 plasmid in urine and blood samples by PCR analysis. Urine collected in the first and second week of treatment before and 2 h after plasmid instillation (lanes 1-2, 3-4 respectively). Blood collected in the first and second week of treatment before and 2 h after plasmid instillation (lanes 5-6, 7-8). M (100 bp ladder) (C)- Video-cystoscopy performed before starting the treatment (see the dotted tumor area) and 3 weeks after the third treatment with the toxin vector (D).

**Figure 7C** shows that most of the plasmid DTA-H19 that was present in the urine sample collected 2 hours after the treatment was found inside the exfoliated bladder cells (lanes 1-3), and the fact that DTA-H19 was detected in the genomic DNA extracted indicates that the plasmid entered the nucleus (lane 4). 30 hours after the treatment the plasmid was found only in the cells of the urine samples, but was undetectable in the supernatant fraction. Video-cystoscopy performed three weeks after the completion of the 6<sup>th</sup> treatment in the second patient showed no adverse reactions of the bladder and a reduction of the tumor size by more than 75 % (**Figure 7E**) compared to the tumor size before treatment initiation (**Figure 7D**). **Figure 7E** shows that the DTA-H19 mediated inhibition of tumor growth was also accompanied by extensive necrosis.

#### IV. Discussion

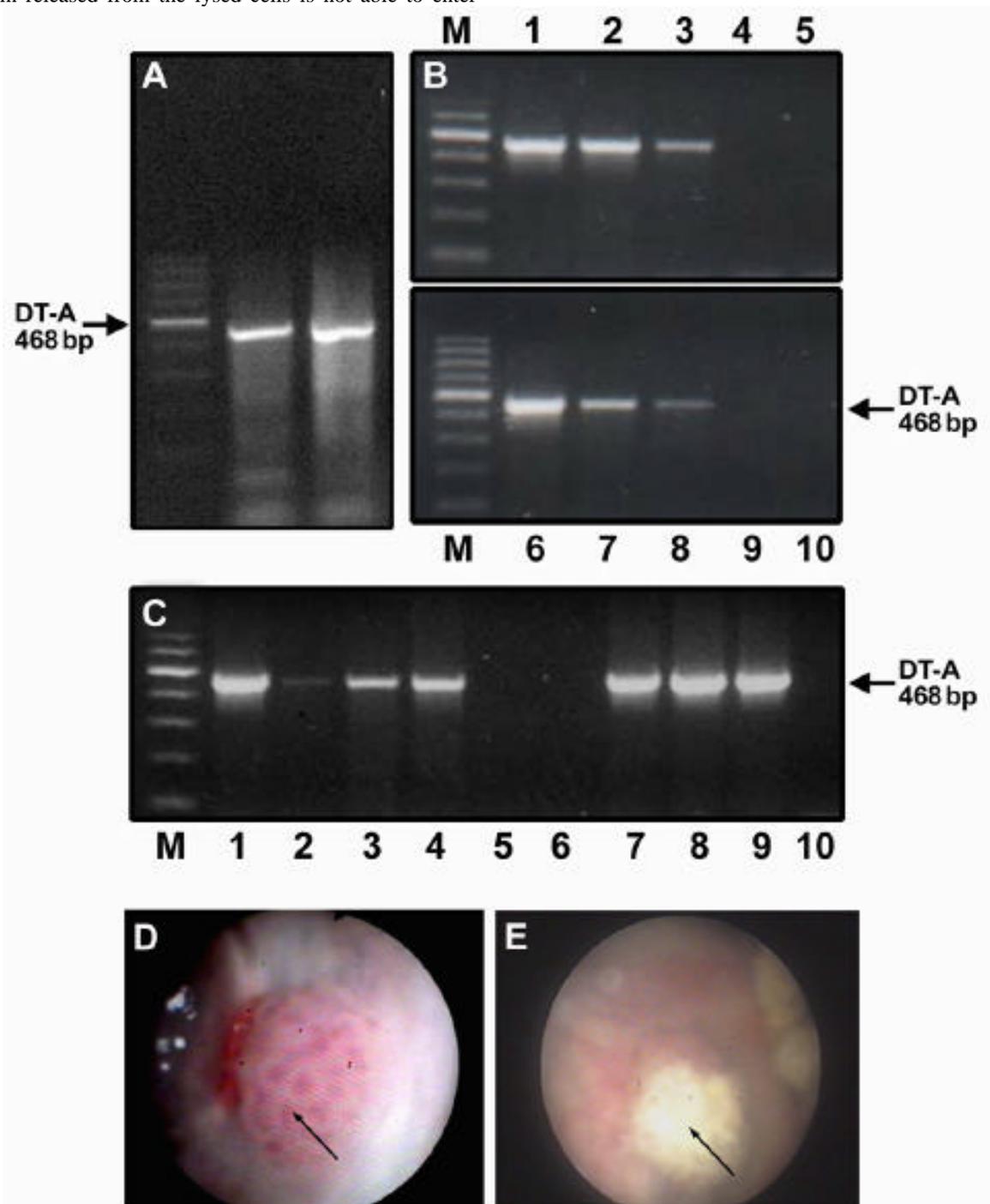
The present work shows the successful use of the regulatory sequences of the H19 gene for the development of DNA based therapy of human superficial bladder carcinoma. The development of anti-tumor gene therapy depends on the use of a combinatorial approach for the

targeted delivery and specific expression of effective anti-tumor agents. In this work a tumor-selective promoter was used in conjunction with a cytotoxic gene to achieve targeted tumor cell destruction. Trials in animal models showed that tumor specific promoters exhibit a clear advantage compared to strong viral promoters such as CMV promoter currently used in clinical trials (Wu et al, 2003). While most tumor-specific promoters are relatively weak resulting in insufficient transgene expression levels, the H19 promoter is known to be highly activated in various tumor types and to show no or only marginal activity in the surrounding normal tissue (Ohana et al, 2001; Ayeshe et al, 2003).

The goal of the present study was to evaluate the therapeutic potential of expression vectors carrying the "A" fragment of the diphtheria toxin (DT-A) gene under the control of the H19 regulatory sequences in two different animal models and in human patients. We have previously shown that these constructs are able to selectively kill tumor cell lines and inhibit tumor growth in vitro and in vivo (Ohana et al, 2001). The choice of the DT-A as a toxin gene ensured not only high killing

activity but its use has a great advantage in avoiding unintended toxicity to normal cells, since the DT-A protein released from the lysed cells is not able to enter

neighboring cells in the absence of the DT-B fragment (Maxwell et al, 1992).



**Figure 7. Evaluation of plasmid delivery and therapeutic effect in the second patient (ER).** (A) Detection of DT-A DNA was carried out by PCR analysis in tumor samples obtained from the TUR performed 18 hours after the patient received 5 mg of DTA-H19 plasmid. (B) Two weeks after the TUR the patient received a dose of 2 mg of DTA-H19 vector. The presence of the DTA-H19 vector was monitored in urine samples collected starting 2 hours after the treatment and every 24 h till 96 h (lanes 1-5 respectively). In a second treatment the patient received a dose of 4mg of DTA-H19 vector, the urine samples were collected every 24 h till 96 h (lanes 6-10 respectively). M (100 bp ladder). (C) Detection of DT-A plasmid was carried out by PCR analysis in urine samples collected from the patient 2 (lanes 1-4) and 30 h (lanes 5-8) after the fifth treatment. Non-centrifuged urine (lanes 1 and 5); supernatant obtained after urine centrifugation (lanes 2 and 6); cell lysate (lanes 3 and 7); genomic DNA extracted from the cell pellet (lanes 4 and 8). The DTA-H19 plasmid was used as positive control (lane 9), negative control (lane 10). (D) Video-cystoscopy performed before the treatment and three weeks after the completion of the 6<sup>th</sup> treatment, showing a large necrotic area replacing the tumor region (E).

The plasmid vectors have advantages over viral vectors (Kouraklis, 2000) including lack of immunogenicity and cytotoxicity allowing repeated treatments. We used the cationic polymer PEI (Jet-PEI), a linear polyethylenimine derivative as a transfection promoter agent. The Jet-PEI works by compacting the DNA into positively charged particles capable of interacting with anionic proteoglycans at the cell surface thereby facilitating the entering of the DNA by endocytosis (Mislick and Baldeschwieler, 1996).

Here we present evidence that intratumoral injection of the construct DTA-H19/PEI into tumors induced in nude mice by subcutaneous injection of RT112 bladder carcinoma cells, led to a significant reduction of the growth rate of the tumors. No further tumor growth was observed already after the first injection with the toxin vector compared to tumors treated with the reporter vector Luc-H19 ( $P = 0.01$ ) (**Figure 1**). The cytotoxic effect of DTA-H19 was also obvious by the increase in cellular necrosis of the DTA-H19 treated tumors compared to the Luc-H19 treated ones. However, it is very important to note that no signs of toxicity were detected in healthy mice treated subcutaneously with DTA-H19 complexed with PEI or with PEI alone.

The therapeutic potential of the toxin vector was evaluated in a rat orthotopic bladder tumor model. This model adequately serves in preclinical trials of human gene therapy. Orthotopic grafts are superior to the subcutaneous transplantation models with respect to the evaluation of the gene transfer methods and the assessment of the efficiency of *in vivo* gene transfer. Using the orthotopic bladder carcinoma model we evaluated the feasibility of intravesical DNA based therapy utilizing the cationic polymer PEI as a transfection promoter agent. Transurethral intravesical instillation delivery enables local administration and efficient delivery of therapeutic genes to bladder cancers. We have shown high levels of expression of H19 transcript in the rat bladder carcinoma cells NBT-II and even higher levels in the malignant tissue formed by implantation of these cells into the bladder (**Figure 2**). This is in accordance with our previous reports showing that during stress conditions like those prevailing during tumor development, an increase in the level of H19 transcript occurred (Ayesh et al, 2002).

We have previously reported that the human H19 regulatory sequences are also active in murine cells (Ohana et al, 2001; Ayesh et al, 2003). These findings are in accordance with the results presented in **Table 1**, showing that the human H19 regulatory sequences were able to drive the DT-A expression in the rat bladder carcinoma NBT-II cells. Therefore, these cells proved to be suitable for the generation of the orthotopic animal model used in this study to examine the antitumor therapeutic potential of the H19-DTA vector *in vivo*. The mean weight of the bladders carrying the DTA-H19 treated tumors was 76 % smaller than that of the Luc-H19 treated bladders (**Figure 3**). Moreover, a computer based assessment of the tumor size after sacrifice adopted to accurately assign and compare the tumor areas, showed that much smaller papillary TCC tumors were detected in the DTA-H19 treated bladder tumors compared to the

Luc-H19 treated ones ( $2.8 \pm 2 \text{ mm}^2$  versus  $63 \pm 43 \text{ mm}^2$ ). We showed that the orthotopic bladder tumors were efficiently transfected by the reporter vector that express the EYFP fluorescent protein under the control of CMV promoter, while fluorescent cells were found not only in the surface cells but also in the deeper layers of the tumor. Higher transfection efficiency was obtained when the vector was complexed with PEI at a ratio of  $N/P = 6$  compared to  $N/P = 10$  (**Figure 4**). Hence, a PEI/DNA ratio of 6 was used in the preclinical animal experiments and in the human clinical study. Gene delivery and gene expression in bladder tumors from rats treated with DTA-H19/PEI or with the reporter vector Luc-H19/PEI were analyzed by RT-PCR (**Figure 5**). High levels of DT-A and luc transcripts were still detected in the bladder tumors 72 hours after plasmid instillation while no expression of the DT-A gene was detected in the liver. The H19 promoter was activated leading to DT-A expression in the transfected cells, resulting in growth inhibition of DTA-H19 treated tumors. Most importantly, the results obtained showed that healthy animals that were treated with the vehicle PEI either alone or with the toxin construct showed no signs of toxicity in those animals. Moreover, no traces of the plasmid were found in the blood, indicating that there is no danger for systemic exposure.

After investigating the efficiency, safety and toxicity of H19 DNA based therapy in animal models, we performed a pilot clinical study in two compassionate patients with recurrent superficial bladder cancer for characterization of the treatment and clinical response of two human patients. Both patients suffered from refractory bladder cancer after failure of multiple intravesical treatments. Samples of bladder tumors removed by transurethral surgery were submitted to histological and molecular diagnosis. After demonstrating high levels of H19 RNA in the TCC of both patients by *in situ* hybridization analysis, both patients were treated with the toxin construct DTA-H19. Taking advantage of the anatomy of the bladder we evaluated the feasibility and safety of intravesical vector instillation using a transurethral catheter. Because our preclinical studies demonstrated that the transfection efficacy of the vector instilled into the bladder is enhanced by the polycation PEI, we administered the DTA-H19 construct in combination with the transfection-enhancing reagent Jet-PEI using the  $N/P$  ratio of 6 previously defined for the rat model. We showed that this treatment was well tolerated and devoid of any detectable systemic toxicity in both patients treated with 2 and 4 mg of the toxin vector respectively. This was supported by the results showing no trace of DTA-H19 plasmid in the blood while it is secreted into the urine even 72 hours after the treatment. PCR analysis of the pelleted cells and the supernatant of the urine strongly indicated that the DTA-H19 plasmid is presence in the exfoliated tumor cells. Efficient uptake of the plasmid was determined in the tumor tissue of the second patient. Evidences for biological activity of the transgene was strongly demonstrated by the video-cystoscopy performed after the third treatment in the first patient showing no adverse reactions of the bladder but reduction in the tumor size by more than 75 % (**Figure 6D**) as compared to the

video-cystoscopy performed before starting the treatment with the DTA-H19 vector. Similar results were obtained with the second patient, the tumor was reduced > 75 % after 6 weeks of treatment with the DTA-H19 toxin vector. Moreover, in the first patient no recurrence of TCC occurred even eight months after the first plasmid treatment. No adverse side effects were detected in both patients. This study may form a platform for the design of an extensive phase I and II study on a larger number of human patients.

Our treatment seems to be a very efficient one, which can increase the quality of life of many patients. The results obtained in the present study may represent the first step of a major breakthrough in the treatment of human bladder carcinoma.

### Acknowledgements

This work was partly supported by the Tchoresh grant.

### References

- Ariel I, Lustig O, Schneider T, Pizov G, Sappir M, de Groot N, Hochberg A (1995) The imprinted H19 gene as a tumor marker in bladder carcinoma. *Urology* 45, 335-338.
- Ariel I, Ayesh S, Perlman EJ, Pizov G, Tanos V, Schneider T, Erdmann VA, Podeh D, Komitowski D, Quasem AS, de Groot N, and Hochberg (1997) The product of the imprinted H19 gene is an oncofetal RNA. *Mol Pathol.* 50, 34-44.
- Ariel I, Miao H.Q, Ji X.R, Schneider T, Roll, D, de Groot N, Hochberg A, Ayesh, S (1998) Imprinted H19 oncofetal RNA is a candidate tumour marker for hepatocellular carcinoma. *Mol Pathol.* 51, 21-25.
- Ariel I, Sughayer M, Fellig Y, Pizo G, Ayesh S, Podeh D, Libdeh BA, Levy C, Birman, T, Tykocinski ML, de Groot N, Hochberg A (2000) The imprinted H19 gene is a marker of early recurrence in human bladder carcinoma. *Mol. Pathol.* 53, 320-323.
- Ayesh S, Matouk I, Schneider T, Ohana P, Laster M, Al-Sharef W, de Groot N, Hochberg A (2002) The possible physiological role of H19 RNA. *Mol Carcinog.* 35, 63-74.
- Ayesh B, Matouk I, Ohana P, Sughayer MA, Birman T, Ayesh S, Schneider T, de Groot N, Hochberg A (2003). Inhibition of tumor growth by DT-A expressed under the control of IGF2 P3 and P4 promoter sequences. *Mol. Ther.* 7, 535-541
- Erdmann VA, Barciszewska MZ, Szymanski M, Hochberg A, de Groot N, Barciszewski J (2001) The non-coding RNAs as riboregulators. *Nucl Acids Res.* 29, 189-193.
- Kouraklis G (2000) Gene therapy for cancer from the laboratory to the patient. *Dig. Dis. Sci.* 45, 1045-1052.
- Leighton PA, Ingram RS, Eggenschwiler J, Efstratiadis A, Tilghman SM (1995) Disruption of imprinting caused by deletion of the H19 gene in mice. *Nature* 375, 34-39.
- Maxwell IH, Glode LM, Maxwell F (1992) Expression of diphtheria toxin A-chain in mature B-cells: a potential approach to therapy of B-lymphoid malignancy. *Leuk. Lymphoma* 7, 457-462.
- Mislick KA, Baldeschwieler JD (1996) Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proc. Natl. Acad. Sci.U.S.A.* 93, 12349-12354.
- Ohana P, Kopf E, Bibi O, Ayesh S, Schneider T, Laster M, Tykocinski M, de Groot N, Hochberg A (1999) The expression of the H19 gene and its function in human bladder carcinoma cell lines. *FEBS Lett.* 454,81-84.
- Ohana P, Bibi O, Matouk I, Levy C, Birman T, Ariel I, Schneider T, Ayesh S, Giladi H, Laster M, de Groot N, Hochberg A (2001) The use of H19 regulatory sequences for targeted gene therapy in cancer. *Int. J. Cancer* 98, 645-650
- Rachmilewitz J, Goshen R, Ariel I, Shneider T, de Groot N, Hochberg A (1992) Parental imprinting of the human H19 gene. *FEBS Lett.* 309, 25-28.
- Rath-Wolfson L, Moskovitz B, Dekel Y, Kugel V, Koren R. (2003) Combined intravesical hyperthermia and mitomycin chemotherapy: a preliminary in vivo study. *Int. J. Exp. Pathol.* 84, 145-152.
- Tanos V, Prus D, Ayesh S, Weistein D, Tykocinski ML, de Groot N, Hochberg A, Ariel I (1999) Expression of the imprinted H19 oncofetal RNA in epithelial ovarian cancer. *Eur. J. Obstet. Gynecol. Reprod. Biol.* 85, 7-11.
- Verkerk AJ, Ariel I, Dekker MC, Schneider T, van Gurp RJ, de Groot N, Gillis AJ, Oosterhuis JW, Hochberg A, Looijenga LH (1997) Unique expression patterns of H19 in human testicular cancers of different etiology. *Oncogene* 14, 95-107.
- Wu L, Johnson M, Sato M (2003) Transcriptionally targeted gene therapy to detect and treat cancer. *Trends in Mol. Med.* 9, 421-429.



A. Ami Sidi

First row from left to right: Suhail Ayesh, Abraham Hochberg, Imad Matouk

Ofer Gofrit

Second row from left to right: Tatiana Birman, Patricia Ohana