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The H19-IGF2 Role in Bladder Cancer Biology and DNA-Based Therapy

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1. Introduction

The H19-IGF2 locus within the imprinted cluster of the human chromosome 11, has been implicated in a variety of disorders and cancer pre-disposition including bladder cancer. BBN induced bladder cancer model in rats has identified both H19 and IGF2 among differentially expressed genes that are induced in response to carcinogen exposure. In this chapter, the role of both H19 and IGF2 genes in cancer will be handled in general with special focus on bladder cancer. Although IGF2 role in human cancers is relatively well established, recent data from our laboratory and others have just revealed a critical role for H19 RNA in the process of tumorigenicity including that of the bladder. H19 functions as a stress modulator, being induced by hypoxia, and a survival factor that is involved in several fundamental processes of tumorigenesis. Furthermore, we uncovered a molecular mechanism that integrates H19, p53 and HIF1-ǂ to hypoxic stress response. Placing the H19 gene product in this deadly circuit undoubtedly will have major impacts in its utility as a target for cancer gene therapy.

Regulatory sequences of both H19 and IGF2 have already been used to successfully target expression of a toxic protein, diphtheria toxin A (DT-A), in carcinoma cells in culture, in several xenograft, orthotopic animal models, and in chemically induced BBN model of bladder cancer. In case of H19, it is successfully used in patients with bladder carcinoma for a period of over 5 years and recently a clinical trial phase I/IIa using this therapeutic approach has been successfully completed. It is also successfully used in other types of human cancers but will not be handled in the current chapter.

We will discuss also novel approaches, to create a new family of plasmids. In one approach a cytotoxic gene is driven by two different regulatory sequences, selected from the cancer-specific promoters H19, IGF2-P3 and IGF2-P4 carried on a single construct. In a second
approach a single promoter is used to drive two cytotoxic genes having synergistic effect on a single construct. Both approaches show superior tumor growth inhibition activity, in preclinical studies of bladder cancer.

Bladder Cancer is the fourth most common cancer in men accounting for about 7% of all cancer cases and 3% of all cancer related mortality. Each year, more than 50,000 new patients are diagnosed with bladder cancer in the USA and about 10,000 die from this disease (Jemal et al., 2010). Carcinogens activity on a susceptible epithelium is believed to be the cause of bladder cancer. Many industrialized chemicals are causally related; benzidine, 1-naphthylamine, 4-aminobiphenyl, etc. However, the commonest cause of bladder cancer nowadays, is by far cigarette smoking accounting for about half of all bladder cancers (Burch et al., 1989). Whether bladder cancer arises from a single transformed cell (clonogenic theory) or from multiple transformed cells (field change theory), is still under debate.

Painless hematuria is the hallmark of bladder cancer. This dramatic symptom urgently brings the patient to see a doctor. Therefore, most bladder tumors are diagnosed during the lifetime of the patient. Bladder tumors can also present with irritative urinary symptoms (urinary urgency, frequency and dysuria). Bladder tumors can be diagnosed by ultrasonography, intravenous urography, or computerized tomography. The resolution of these radiologic techniques is low, and only a 5 to 10 mm lesion can be detected. Cystoscopy done by inserting an optical instrument into the bladder can diagnose bladder tumors as small as 1mm. Urinary cytology is an important adjunct to cystoscopy, especially for the diagnosis of the flat lesion carcinoma in-situ (CIS).

Most bladder tumors arise from the epithelial lining the urinary system-the transitional epithelium (urothelium), and are therefore, transitional cell carcinomas (or urothelial carcinomas). Bladder cancer is a heterogeneous disease with wide variations in molecular pathogenesis, morphology and prognosis. They are classified according to their depth of invasion into the bladder wall (stage) and according to the degree of histological anaplasia (grade).

As in most other types of cancer, it is believed that 4-6 DNA hits are required for malignant transformation (Duggan et al., 2004). These include deletions, mutations and loss of heterozygosity (LOH) of genetic material that carries tumor suppressor genes or proto-oncogenes, and epigenetic changes such as CpG methylations that modify gene expression. There are at least 2 major pathogenic pathways leading to 2 completely different bladder cancers. One pathway leads a low grade, papillary bladder cancer (about 75% of all bladder tumors). This type of tumor has a proliferative ability but no ability to invade the epithelial lamina propria and muscle of the bladder, to metastasize and to kill the patient. The second pathway leads to a high grade, solid tumor that has an invasive and metastatic potential (about 15% of the tumors). Possibly, there is a third pathway that leads to a high grade papillary tumor (about 10% of the tumors) (Goebell & Knowles, 2010). While low grade tumors tend to recur but almost never endanger the life of the patient, high grade tumors are often lethal.

It is believed that low grade tumors develop following this pathway: Urothelial hyperplasia → urothelial atypia → low grade TCC. The most prominent molecular change in this group is mutation in FGFR3, found up to 88% of these tumors (van Rhijn et al., 2002). In most cases these are activation mutation that probably supports tumor proliferation by stimulating the RAS-MAPK pathway. The frequency of FGFR3 mutations in high grade tumors is much lower. Activation of the phosphatidylinositol 3-kinase (PI3K) pathway by a wide range of mechanisms is also typical to low grade tumors (Goebell & Knowles, 2010).
High grade bladder tumors develop following this pathway: Urothelial atypia → dysplasia → CIS → Invasive TCC → metastatic disease. The most prominent molecular changes in high grade tumors involve inactivation of the p53 and RB pathways. p53, whose activity is augmented in the presence of DNA damage, arrests cells at G1-S checkpoint by inducing the transcription of the CDK inhibitors-p21/Waf1, and GADD45. Then the cell may either correctly repair its DNA or undergo apoptosis. Deletions or mutations in the p53 pathway are found in about 70% of the high-grade tumors.

The Retinoblastoma gene codes for an 110kDa nuclear phosphoprotein acting as a tumor suppressor that also arrest cells at G1. It is often mutated by a truncating mutation of the carboxyl terminal. Mutations or deletions in Rb gene are found in 30% of the patients with advanced bladder cancers. They result in uncontrolled cellular proliferation even without mitogenic signals. Hypermethylation of the Rb promoter region can have the same effect. Similarly, loss of the cyclin dependent kinase inhibitor p16 either by mutation, deletion, or by promoter hypermethylation, as documented in 20-45% of the bladder cancers prevents Rb activation by hyper-phosphorylation, or leads to uncontrolled cell cycle progression (Schultz, 1998).

Epithelial to mesenchymal transition (EMT) is an important process typical to high grade tumors. It is characterized by down regulation of the adhesion molecule E-cadherin and of proteins associated with cell polarity along with up regulation of fibronectin, vimentin and matrix metalloproteases (MMPs). EMT is induced by cytokines like the TGFβ and is associated with increased invasion, migration and angiogenesis.

Alterations in chromosome 9 are the most common cytogenetic findings in bladder cancer. Of these, the most common are deletions and LOH in the short arm, home of the tumor suppressor genes and cell-cycle regulators CDKN2A (encoding for p16 and p14ARF) and CDKN2B (encoding for p15). In a rather consistent manner high grade tumors demonstrate LOH of 3p, 8p, 13q, and 17p, while low grade tumors demonstrate LOH of chromosome 9 only (Knowles et al., 2001).

Animal models are critical in the understanding of bladder cancer pathogenesis and in the quest for new treatments. Most animal models in bladder cancer are in rodents. Although various models exist, we’ll focus on carcinogen induced bladder cancer model.

1.1 Carcinogen induced bladder cancer in mice and rats
In this model, the rodent is given a carcinogen, most commonly in the drinking water. BBN (N -butyl-N- (4-hydroxybutyl) nitrosamine) is a carcinogen given to the rodents in a concentration of 0.05% in the drinking water. It induces bladder tumors in 95% of the rodents after 25 weeks of administration (Okada et al., 1975). The tumors produced by the carcinogen resemble human bladder cancer in histology, etiology, and in kinetics (25 rat weeks equal 10 human years- the believed incubation period of human bladder cancer). Molecular events occurring during chemical carcinogenesis can be followed (Ariel et al., 2004). Tumor development and the response to novel treatments can be assessed non-invasively, without scarifying the animal using ultrasonography (Gofrit et al., 2006). The main disadvantages of this model are necessity to handle a carcinogen and the long period required for tumor production.

In our lab we used this model to “fish up” genes involved in bladder tumorigenesis using microarray analyses. We identified both H19 and IGF2 among differentially expressed genes that are induced in response to carcinogen exposure (Elkin et al., 1998, Ariel et al., 2004)
In the following sections we will present the role of both H19 and IGF2 genes in tumorigenicity. Then we will discuss pre-clinical and clinical data for the successful treatment of bladder cancer by DNA-based drug developed in our laboratory based on both the H19 and IGF2 regulatory sequences to drive the expression of a toxic protein, diphtheria toxin A (DT-A). This approach also proved to be successful in other cancer types; ovarian (Mizrahi et al., 2010), prostate (submitted) in human patients, and pre-clinically under development in glioblastoma (under preparation), lung (Hasenpusch et al., 2011), and colorectal liver metastasis (Ohana et al., 2005). This chapter will focus only on bladder cancer. Pre-clinical data using novel approaches for the treatment of bladder cancer with improved cytotoxic effect will be presented as well.

2. The H19-IGF2 locus and tumorigenesis

The IGF2 and H19 genes are both located on the short arm of chromosome 11 and are reciprocally imprinted. Genomic imprinting of the IGF2 and H19 genes has been shown to play a role in the regulation of the IGF2 and H19 expressions during embryonic development and in cancer. The role of genomic imprinting in tumor development is not well understood and it is beyond the scope of this chapter. Over-expressions of H19 and IGF2 genes in many tumors may or may not be associated with loss of imprinting.

2.1 The pivotal role of H19 RNA in tumorigenesis

H19 is an oncofetal gene that expresses only RNA and not protein, being expressed in the embryo, repressed in the adult, and re-expressed in a variety of human tumors, for review (Matouk et al., 2005). H19 is emerging as one of the key players in cancer biology. We and others have demonstrated an essential role of H19 RNA in tumor development, and the association and contribution of H19 RNA with various aspects of tumorigenic process. This contradicts the initial proposal that H19 gene product has a tumor suppressive activity (Hao et al., 1993).

Our strategy to delineate the role of H19 RNA in tumor development is based on determining if tumor development is dependent on H19 expression through both over-expression and knockdown approaches in different tumor models including bladder cancer. To shed light into its mechanism of action our strategy is based on identifying upstream effectors and also downstream targets by applying the global gene expression profiling to identify genes modulated by both H19 over-expression and knockdown. Here again bladder cancer model is included.

Our results, supported by results from others, reveal that H19 RNA harbors oncogenic properties, enhancing the development of carcinogenesis. In this section we’ll present major findings that support this issue and highlight its relevance to bladder cancer where possible.

2.1.1 H19 RNA is essential for human tumor growth

Although H19 over-expression sometimes associated with loss of imprinting have been reported in a large arrays of human cancers, direct evidence of its tumorigenic role was lacking. Using two cell line models including bladder carcinoma, we provided evidences, that H19 is critical for tumor development. Our in vivo results show that bladder carcinoma formed from UMUC3 cells in which the H19 RNA have been knocked down , induce a very significant retardation of tumor growth. Similar results were reproduced using other carcinoma model (Matouk et al., 2007).
Moreover we showed that ectopic H19 expression enhances the tumorigenic potential of bladder carcinoma cells in vivo. Tumors induced from T24P bladder carcinoma cell line ectopically over-expressing H19 RNA, differ significantly in their growth properties and growth kinetics in vivo relative to the control, and are well vascularized with evidences of tumor hemorrhage (Matouk et al., 2007). H19 RNA also enhances entry to S-phase of the cell cycle of bladder cancer cells under serum starved condition, but not under normal cell culture condition (Ayesh et al., 2002). Further supports for the tumorigenic properties have been reported in other cancer models. H19 over-expression of ectopic origin confers a proliferative advantage for breast epithelial cells in a soft agar assay and in several combined immunodeficient mice (Lottin et al., 2002). c-Myc induces the expression of the H19 RNA. c-Myc binds to the E-boxes near the imprinting control region to facilitate histone acetylation and transcriptional initiation of the H19 gene, to potentiate tumorigenesis (Barsyte-Lovejoy et al., 2006). The H19 is reported to be a target gene for the hepatocyte growth factor (HGF), further signifying the potential role of H19 RNA in hepatocellular carcinoma development (Adriaenssens et al, 2002). Furthermore, H19 RNA is important for entry into S-phase after serum starvation recovery by E2F binding to its promoter (Berteaux et al, 2005). Recently, it was reported that the Retinoblastoma tumor suppressor gene is a target gene for miR-675 which is produced from exon-1 of the H19 gene (Tsang et al., 2010).

2.1.2 H19 is induced by hypoxia – the P53 brakes and the HIF-1α engine

It is well established that every solid tumor encounters hypoxic regions beyond certain diameters. Hypoxia is a major trigger for tumor angiogenesis, metastasis, chemo-resistance and also associated with poor prognosis at least in some types of human cancers. All of these conditions as discussed below are associated with an increase of H19 RNA expression. Over-expression of H19 RNA, is accompanied with up-regulation of a 95 kDa membrane glycoprotein (p95) observed in a variant of breast and lung carcinomas that are multi-drug resistance (Doyle et al., 1996). Moreover, results show that the level of H19 RNA is elevated in the multidrug resistance variant of HCC cell lines. Here doxorubicin resistance phenotype is related to H19 over-expression (Tsang et al., 2007).

H19 provides a novel and clinically useful diagnostic marker for prognosticating human bladder carcinoma. More striking is the predictive value of H19 for tumor recurrence. We have found that in transitional cell carcinoma of the bladder with tumors that express H19 in most cells have shorter median disease-free survivals (Ariel et al., 2000). We have identified downstream targets modulated by H19 over-expression in the T24P bladder carcinoma cell line (Ayesh et al., 2002); comparing the mRNA levels of many genes between cells containing high levels of H19 RNA (from H19 expressing plasmid) to that of the same cells lacking H19 RNA, showed a clear preference towards genes promoting cellular migration, angiogenesis and metastasis.

All of these observations prompted us to explore the effect of hypoxia on H19 expression and to delineate the mechanism of action involved. Indeed, under hypoxic conditions, we have reported that in bladder carcinoma cell lines T24P and UMUC3, and hepatocellular carcinoma cell line Hep3B the H19 RNA is significantly elevated (Matouk et al., 2007). Following these initial studies we screened about thirty different carcinomas cell lines of different lineages and origins for their ability to induce H19 RNA in hypoxic stress (Matouk et al., 2010). We observed very different patterns of response to hypoxia. To gain insight into
the possible mechanism associated with the H19 response to hypoxia, we searched for a common denominator among these cell lines. It is well established today that the tumor suppressor signaling pathway of p53 can be activated by stress signals such as hypoxic stress and can either trans-activate or trans-repress its target genes to influence the cellular response. The key processes regulated by p53 pathway include cell cycle arrest, apoptosis, DNA repair, senescence, metastasis and angiogenesis, depending on cell types, nature of the inducer, cell intrinsic environment, and the activities of other signal transduction pathways. These observations suggest a possible association between the status of p53 (wild type or mutant) and H19 responsiveness to hypoxic stress. Moreover the involvement of the wild type tumor suppressor gene p53 in the down regulation of the H19 promoter activity which lacks a p53 consensus site and a TATA box was previously shown by (Dugimont et al., 1998). Taking all of these observations into account and the availability of IARC TP53 mutation database, we explored the possible involvement of p53 in determining H19’s behavior in hypoxic response.

We recently demonstrated a tight correlation between H19 RNA elevation by hypoxia and the status of the p53 tumor suppressor. In cells harboring wild type p53 (p53<sup>wt</sup>) H19 RNA is not induced upon hypoxia, whereas in cells carrying a mutated p53 (p53<sup>mt</sup>) the H19 message is significantly induced most strongly in p53-null cells. Furthermore through both over-expression and knockdown approaches we identified HIF1-α as the factor that is responsible for H19 elevation under hypoxic stress (Matouk et al., 2010).

H19 functions, consequently, as a stress modulator and a survival factor and is involved in several fundamental processes, including epithelial-mesenchymal transition (EMT), malignant transformation, cell-cycle transition, metastasis and neo-angiogenesis. EMT is an important process on the way to the malignant phenotype; notably- H19 up-regulation occurs in the stroma as well as in the epithelium. In the metastatic tumor stage, which bears a striking similarity to the embryonic stage, H19 involvement appears to be essential: adherent and cohesive cells lose their anchorage, migrate under stressful conditions to remote sites and replicate with neovascular support. Thus, H19 is a central figure in the cancer embryonic shift (Matouk et al., 2008).

In the light of our study, a molecular mechanism that integrates H19, p53 and HIF1-α to hypoxic stress response is uncovered. As hypoxia readily occurs in the majority of solid tumors driving critical steps in tumor development and metastasis and resistance to therapeutic modalities, placing the H19 gene product in this deadly circuit undoubtedly will have major impacts in its utility as a target for cancer gene therapy. Indeed a DNA-based drug depending on H19 regulatory sequence and diphtheria toxin is now in clinical trial with promising results (Ohana et al., 2004, Sidi et al., 2008, Mizrahi et al., 2010). We’ll concentrate on bladder cancer.

2.1.3 Targeted therapy for bladder cancer mediated by a plasmid expressing DTA under the control of H19 regulatory sequences – clinical data

During the past few years we have developed a DNA based therapy strategies for treating tumors expressing H19 RNA. The successful development of anti-tumor gene therapy depends on the use of a combinatorial approach aimed at targeted delivery and specific expression of effective anti-tumor agents. We exploit the unique H19 transcriptional regulatory sequences for directing tumor-selective expression of toxins. For this purpose we use non-viral vectors due to their potential to circumvent the main disadvantage of
adenoviral vectors, caused by immune responses directed against adenovirus proteins which limit their ability to be administered iteratively. As a toxic gene, we used the diphtheria toxin A chain (DT-A), which has suitable properties for achieving efficacious cancer cell killing. DT-A peptide catalyzes ADP-ribosylation at the diphtamide residue of the cellular translation elongation factor 2 (eEF-2), inhibiting protein synthesis and causing cell death. While a very low level of DT-A expression suffices for cell killing, DT-A released from the lysed cells is not able to enter the neighboring cells in the absence of the DT-B chain.

All preclinical studies needed to set up the stage for using this approach to treat bladder cancer patients will not be handled in this chapter, and are reviewed elsewhere (Matouk et al., 2005).

**Clinical studies**

The goals of treatment are to reduce tumor recurrence, decrease the risk of disease progression, avoid cystectomy (bladder sparing treatment), and improve survival. Preventing progression to muscle invasive disease is of key importance because even with aggressive treatment, including radical cystectomy, as few as 50% of patients with muscle invasive disease will survive 5 years (Dalbagni et al., 2001).

The primary factors that influence risk of disease progression include: 1. the number of tumors at primary diagnosis; 2. recurrence rate in a previous period or an early recurrence at 3 months after the first resection; 3. size of the tumor (tumors larger than 3 cm are more likely to recur than smaller tumors); and stage and grade of the tumor.

The initial clinical development plan for DTA-H19/PEI for bladder cancer is in the intermediate-risk patient population who has failed prophylactic therapy with either BCG or chemotherapy. In the Phase 1/2a study, the safety and preliminary efficacy was examined in this population. Having determined that the highest dose tested in this trial, the 20 mg dose of DTA-H19, was well tolerated and elicited complete responses in 2 of 5 evaluable patients, the Phase 2b clinical protocol will assess the safety of this regimen in a larger patient population as well as the efficacy in a marker tumor clinical trial design.

**Compassionate Use in Bladder Cancer.** Two patients had recurrent superficial TCC of the bladder and had failed multiple courses of Bacille Calmette-Guérin (BCG) and chemotherapy, and two additional patients that underwent nephrourectomy due to a diagnosis of recurrent superficial TCC that showed BCG intolerance. The investigations in the first two bladder cancer patients demonstrated that intravesical instillation of DTA-H19/PEI is safe up to a dose of 5 mg in a single administration or a cumulative dose of 70 mg intravesically. No local or systemic adverse effects considered attributable to DTA-H19 treatment were observed throughout treatment. In addition, DTA-H19 DNA was not detectable in the circulation by PCR analysis of blood samples taken after the first and second week of treatment, and 2 hours after plasmid administration. DTA-H19 DNA was detectable in a tumor biopsy taken 18 hours after intravesical administration and in voided urine for 1 week after treatment. Tumor regression (75% reduction in marker tumor size) was observed in marker tumors of both patients. One of the 2 compassionate patients was treated over a nearly 5 year period with 22 intravesical administrations of either a 2 mg or 4 mg dose of DTA-H19/PEI for a cumulative dose of 70 mg of plasmid DNA. Treatments were well tolerated, and although the marker tumor persisted, it did not increase in size, stage or grade during the 14-month period before it was finally resected.
along with one other new low grade papillary tumor. No increase in stage or grade of TCC was observed.

The patient that had a nephroureterectomy due to a diagnosis of high-grade TCC in the renal pelvis was treated with 6 weekly 10 mg injections of DTA-H19/PEI via the left nephrostome at a total volume of 15 ml each. After completion of the 6 plasmid infusions the patient underwent nephroscopy that revealed the following: renal pelvis with no presence of tumors and several papillary tumors on the left bladder wall in the trigon. Findings were biopsied, analyzed, and diagnosed as low grade TCC. Four months later, the patient underwent nephrography during which no tumors were observed. Urine cytology confirmed the absence of tumor cells.

There were no significant events or side effects throughout the patient’s treatments. Overall, this was a good tumor response in a patient with only one kidney who was considered to be anephric and with dialysis during the last year. The treatment was well-tolerated by the patient, and there was no evidence of any negative systemic or urinary tract effect. During the treatments, the patient was fully functional, continued to work, and did not suffer any effect to his quality of life (QOL). The second patient that had nephroureterectomy had multiple recurrences with the appearance of multi-focal lesions in the bladder. The patient underwent additional resection of a number of lesions that were localized inside and on the walls of the bladder. Since the patient was not a candidate to receive BCG treatment due to his compromised immune system, and after being refractory to Mitomycin C or Synergo, and refusal to undergo cystectomy, he was offered treatment with DTA-H19/PEI. He was treated with 20 mg of DTA-H19/PEI twice a week for the period of 4 weeks and once a week for another 2 weeks. Cytoscopy conducted at the end of the treatment showed an improvement in the number of lesions and in the general appearance of the bladder. The histological diagnosis of the biopsy showed low grade Ta.

**Phase 1/2a Clinical Trial in Conventional Treatment Refractory Bladder Cancer Patients.** A Phase 1/2a clinical trial was designed to determine the maximum tolerated dose (MTD) and assess the safety and preliminary efficacy of 5 different doses (2 mg, 4 mg, 6 mg, 12 mg, and 20 mg of DTA-H19) of DTA-H19/PEI given as 6 intravesical infusions into the bladder of patients with superficial bladder cancer (stages Ta and carcinoma in situ (CIS)) who had failed intravesical therapy with BCG. Patients had a diagnosis of superficial Stage Ta or CIS, grade 1 or 2 superficial bladder cancer that was confirmed by histopathology and that expressed H19 which was shown by in situ hybridization (ISH). Treatments were given weekly for 3 weeks followed 1 week later by safety and disease assessments, then another 3 weekly instillations were performed. Each dose cohort received the same dose for all treatments. Doses were escalated if none of the first 3 patients in the preceding dose cohort experienced a dose-limiting toxicity (DLT) after the first 3 weekly intravesical treatments. A DLT was defined as any grade 3 or greater toxicity by the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) that was considered to be related to the investigational product during the first 3 weekly intravesical treatments. Prior to initiating treatment, papillary tumors were resected leaving a single marker tumor. Videocystoscopy was performed 4, 8, and 12 weeks after the start of treatment for a safety evaluation of the bladder and also to record the presence or absence of the marker tumor and any other lesions suspicious for TCC of the bladder. If the marker tumor was still present at the Week 12 assessment, it was to be resected. If any new lesions were observed at Week 12, they were also to be resected. Patients whose disease had not progressed (i.e., no new tumors, increase in the size of the marker tumor by at
A total of 18 patients were enrolled in this study. No DLTs were observed in this study. As the highest dose of product tested was the 20 mg dose of DTA-H19/PEI, this dose was considered the MTD for this study. The most frequently reported adverse events (AEs) considered at least possibly related to investigational products for any dose cohort were mild to moderate in severity and were most commonly renal and urinary disorders.

Of the 18 patients evaluable for tumor response at Week 12, a total of 4 patients had a complete response (CR) [complete disappearance of the marker tumor and no recurrence) including 2 of the 3 patients in the 2 mg dose cohort, and 2 of 6 patients in the 20 mg dose cohort. In addition, 2 patients (one in each of the 2 mg and 12 mg dose groups) had an incomplete partial response [IPR - complete disappearance of the marker tumor but with new tumor(s) occurring] suggesting that DTA-H19/PEI did have an effect on these marker tumors as well. Other responses included 1 partial response (PR - reduction in size of marker tumor by 50% and no new tumors present) (12 mg dose cohort) and 5 patients with SD (marker tumor still present but has not increased in size by more than 50% and no new tumors) (3 patients in the 4 mg dose cohort, 1 patient in the 12 mg dose cohort, and 1 patient in the 20 mg dose cohort). Thus, in this small study, there was evidence of tumor ablation over the dose range from 2 to 20 mg of DTA-H19/PEI. The 20 mg dose of DTA-H19/PEI was selected for evaluation in the Phase 2b clinical trial because this dose had an acceptable safety profile, showed objective tumor responses, and as the mechanism of action of DTA-H19/PEI is tumor-specific cytotoxicity, theoretically the highest safe dose has the greatest likelihood of an efficacious outcome.

2.2 Insulin-like growth factor 2 (IGF2) and tumorigenesis
IGF2 expression is driven by four different promoters (P1-P4) that produce 4 different transcripts all of which give rise to the same mature protein, a 67-amino acid polypeptide. The four promoters are activated in a development-dependent and tissue-specific manner. In fetal liver, promoters P2–P4 are active, of which P3 is the most active promoter, and promoter P1 is inactive. However, in liver tissue, shortly after birth, the IGF2 promoter P1 is exclusively active. The imprinting of the human IGF2 gene is promoter-specific. The P2, P3 and P4 promoters display monoallelic activity in embryonic, neonatal and postnatal liver specimens, whereas in adult, P1 is transcribed from both alleles.

The IGF2 peptide is a member of the insulin-like growth-factor family and is known to play an important role in the growth and differentiation of various tissues (Rechler 1990). This family also includes IGF1, insulin and relaxin. IGF2 is released to the extracellular fluid where it interacts with different cell membrane receptors and binding proteins. IGF2 binds three different types of receptors: IGF type 1 (IGF-1R), insulin receptor (IR) and IGF-2/mannose 6-phosphate receptors (IGF-2R/M6P). The receptors, however, differ completely in structure and function (Yu and Rohan 2000). Ligand binding to IGF-1R and IR mediates mitogenic and anti-apoptotic effects. IGF-2R/M6P has tumor suppressor function and it mediates IGF2 degradation (Morison and Reeve 1998, Randhawa and Cohen 2005).

IGF2 can promote different functions depending on the cell type in which it is acting, and is a strong mitogen for a wide variety of cancer cell lines. It acts on the cell division cycle (DNA replication and mitosis) and on the cell growth (cellular enlargement), possibly by interfering with control cell checkpoint proteins. Moreover, IGF2 functions as an anti-
apoptotic agent. For example, it blocks c-Myc and SV40 T-antigen induced apoptosis in Raf-1 fibroblast cells (Ishii et al., 1993, Morali et al., 2000).

The mitogenic and metabolic actions of IGF2 in embryonic development and tumorigenesis are mediated by the IGF-1R and/or IR-A and are tightly regulated at different levels. These levels include the IGF-receptors availability, IGF2 interaction with its receptors and binding proteins (IGFBPs) and its degradation following internalization of the IGF2 after binding to its receptor, especially IGF-2R. Moreover, the IGF2 mRNA and protein levels are regulated by different ways. Any abnormality at one or more of these levels can be correlated to tumorigenesis. Over-expression of growth factors, or their receptors is a common event in malignancy and provides the underlying mechanisms for one of the hallmarks of cancer, namely uncontrolled proliferation (Hanahan and Weinberg 2000).

Transgenic mice, over-expressing the IGF2 gene, developed spontaneous tumors at a high frequency (Bates et al., 1995, Moorehead et al., 2003, Rogler et al., 1994), suggesting that over-expressed IGF2 may be involved not only in the progression of tumors but also in the initiation of neoplasia. IGF2 over-expression is significantly correlated to the increased tumor progression and proliferative activity as well as to decreased patient survival (Kawamoto et al., 1998, Rogler et al., 1994, Takanami et al., 1996).

Several mechanisms can potentially result in IGF2 over-expression in cancer, including, loss of imprinting (LOI) of the maternal allele, loss of heterozygosity (LOH) with paternal duplication, amplification of the IGF2 gene and abnormally activated signaling pathway leading to transcriptional up-regulation of the active alleles reviewed by (Hahn et al., 2000). IGF2 imprinting is relaxed in many different types of tumors, including bladder cancer (Byun et al., 2007).

The link between IGF2 and metastasis may be the basis for the identification of IGF2 and IGF-IR as predictors of poor outcome in many types of cancer. In the rat model of bladder cancer induced by BBN, we observed over-expression of igf2 in the tumor, relative to the low level of expression in the normal tissue (Ariel et al., 2004). Moreover we detected high levels of IGF2 mRNA expression from P3, P4 or both promoters in TCC samples. Whereas normal bladder samples showed no expression from either promoter. The human IGF2-P3 and IGF2-P4 promoters are highly active in bladder carcinoma. We showed that these constructs were 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, when they are used to drive the expression of DTA (Ayesh et al., 2003, Amit et al., 2011).

3. Double promoter vectors: Novel approaches for the treatment of bladder cancer with improved cytotoxic effect

3.1 Double promoter DTA-expressing vectors
We have shown that IGF2 or H19 are significantly expressed in 50-84% of human bladder carcinoma respectively (Elkin et al, 1995) but not in normal bladder. Whereas combined expression (e.g. H19 and IGF2-P3/P4) was detected at high levels in nearly 100% of human bladder cancer samples. By that proving that the double promoter vectors are suitable for treating all bladder cancer patients. P3 and P4 were able to express the DTA in tumor cells in vitro, and inhibited tumor growth of mice heterotopic model, proving that both promoters
could be used successively, in addition to H19 promoter, as part of the double promoter constructs (Ayesh et al., 2003, Amit et al., 2010, Amit et al., 2011).

Double promoter expressing vectors were created, carrying on a single construct two separate DNA sequences expressing the diphtheria toxin A-fragment (DTA), from two different regulatory sequences, selected from cancer-specific promoters H19, IGF2-P3 and IGF2-P4. This novel approach, create a new family of plasmids regulated by two regulatory sequences, which in their natural genome position are both proximately located and are reciprocally imprinted.

These vectors were then used to transfect and to eradicate tumor cells in culture or to inhibit tumor growth (in vivo), in heterotopic and orthotopic CD1 nude mice, bladder tumor models.

The activity of the double promoter vectors was tested and compared to the activity of the single promoter vectors. The double promoter vectors exhibited superior activity compared to the single promoter vectors. Furthermore, an augmented-than-additive activity was exhibited, compared to combination activity of the single promoter vectors, in cell lines and in heterotopic bladder cancer mice (Amit et al., 2010, Amit et al., 2011).

3.2 A single promoter driving two cytotoxic genes with synergistic effect

Because it is unlikely that gene transfer reaches every cell of a cancer, DNA based therapy approaches are thought to require the induction of a ‘bystander’ effect. An interesting approach for this purpose is cytokine DNA based therapy. TNF-α is a multifunctional and immuno-regulatory cytokine that exhibits direct tumor cell cytotoxicity, possesses antiangiogenic properties, and enhances antitumor immunity by activating immune cells such as dendritic cells and T cells. Systemic delivery of the TNF-α protein has had limited success clinically because of severe dose limiting toxic effects. This limitation can be overcome by the use of a gene delivery approach, combined with a tumor specific promoter to express TNF-α in the tumor tissue.

In this approach, an enhanced cytotoxic effect could be achieved that could also overcome the resistance developed by tumor cells to either one of the toxin. It was reported that several cell lines – none are bladder cancer- are resistant to Diphtheria toxin and therefore would not be affected by the pH19-DTA vector. Adding TNF-α to the existing system was in agreement with supporting evidence of some publications showing a synergistic effect in cell cytotoxicity mediated by TNF-α and diphtheria toxin. This was shown on ovarian cancer cell lines sensitive or resistant to both diphtheria toxin and TNF-α and on renal cell carcinoma cell lines (Morimoto et al., 1991, Mizutani et al., 1994). Using a construct in which both TNF-α and DTA expressions are driven by H19 tumor specific promoter, would overcome the dose limiting toxic effects of the systemic delivery of TNF-α protein.

So, we investigated a plasmid carrying, in addition to DTA, the gene for human hTNF-α. The pH19-TNF-IRES-DTA plasmid was built while the construct carries a viral IRES sequence (from the ECMV virus) 3’ of the TNF. This IRES construct is 619 pb long and responsible the synthesis of DTA from the m-RNA transcript.

3.2.1 Synergistic effect in the killing activity of DTA and TNF in vitro using different cell line models

In vitro the cytotoxic effect in cells treated with the pH19-TNF-IRES-DTA plasmid was determined by luciferase assay (Ohana et al., 2004). To test for potency of this construct, cells
were also treated with a plasmid carrying either the DTA or TNF under the control of the H19 promoter. Cells from human, mouse and rat origin expressing H19 RNA were co-transfected with 2 μg/well of LucSV40 and the indicated concentrations of pH19-DTA, pH19-TNF or pH19-TNF-IRES-DTA plasmids. Luciferase activity was determined and compared to that of cells transfected with LucSV40 alone (Figure 1). In order to rule out the possibility of a false positive result due to the combined plasmid’s structure effect, we reversed the TNF sequence in the pH19-TNF-IRES-DTA plasmid to eliminate the expression of TNF (pH19-TNFrev-IRES-DTA). The killing potency of the pH19-TNF-IRES-DTA plasmid was significantly higher compared to the pH19-DTA plasmid alone, even at very low concentrations. As the concentrations got higher the difference was diminished (Figure 1).

It should be noted that the use of the pH19-DTA plasmid alone was sufficient to cause a substantial decrease in luciferase activity, leading to 80-90% decrease in high concentrations, whereas the use of pH19-TNF alone showed little decrease, if any, and in some cases even an increase. When expressed in conjunction with the DTA domain, it clearly enhances cell death.

**Fig. 1.** Enhanced killing activity of pH19-TNF-IRES-DTA vector in different bladder carcinoma cell lines.

The killing potential of the pH19-DTA (blue), pH19-TNF (pink) or pH19-TNF-IRES-DTA (orange) vectors in human UMUC-3 and HT-1376, mouse T-50 and rat NBT-II was measured as a reduction of luciferase activity induced by LucSV40. Cells were co-transfected with 2 μg/well of LucSV40 and the indicated concentrations of pH19-DTA, or pH19-TNFα, or pH19-TNF-IRES-DTA. The pH19-TNFrev-IRES-DTA (green) served as a control.

### 3.2.2 In-vivo tumor growth inhibition by the pH19-DTA, pH19-TNF or pH19-TNF-IRES-DTA vectors in a carcinogen rat bladder cancer model

In vivo we utilize the carcinogen (BBN) induced rat bladder cancer model. Rats were treated with the above mentioned plasmids used in the in-vitro studies. An additional control
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plasmid carrying the gene of Luciferase under the regulation of the H19 promoter (pH19-Luc) was included. The therapeutic plasmid was given at two different times in order to investigate the correlation of the treatment efficiency to the severity and invasiveness of the disease. In the first, the plasmid treatments started after the rats received BBN for 20 weeks. By this time, the rats had developed visible tumors in their bladders. In the second, the plasmid treatment started only 16 weeks after the beginning of BBN administration in which tumors were visible only by histopathological examination.

Fig. 2. Enhanced killing activity of pH19-TNF-IRES-DTA vector in the BBN rat bladder cancer model depending on time schedule of the treatments.

Tumor index (volume X weight) of rat’s bladders as measured after treatment with three injections of pH19-DTA(green), pH19-TNF(brown) or pH19-TNF-IRES-DTA(red), and pH19-Luc(blue). A. Results of tumor index of rat’s bladders which started treatments after 20 weeks. B. Results of tumor index of rat’s bladders which started treatments after 16 weeks.

Each rat, in both experiments and in each group, received intravesical injection of 50 µg of the plasmid administered 3 times with an interval of 3 days between treatments. Four days after the last treatment, all rats were sacrificed and bladders were removed, weighed, photographed, excised and taken either for histological analysis or for DNA/RNA preparation. Tumor index was derived as mentioned. Figure 2 shows the results of the in vivo experiments, where rats were treated from these two time points and onward.

We decided to try and estimate the tumor’s volume by picturing each tumor at several angles, allowing us to use image analysis software in order to receive a reasonable
estimation. This was multiplied by the bladder’s weight in order to give us a new, more comprehensive index of the tumor’s status. It can be seen in the results that when starting the treatments after 20 weeks of BBN administration, virtually no effect is achieved in any of the plasmids. On the other hand, starting the treatments after 16 weeks showed a remarkable inhibition of tumor progression compared to the control. When administrated with pH19-DTA the tumor was delayed by approximately 30%, while when using the pH19-TNF-IRES-DTA vector about 50% inhibition was measured. No effect was seen when pH19-TNF was used. These results suggest that when treatment begins in an early stage it can be a highly potent one.

4. Concluding remarks and future perspectives

Our ability to understand the biology of bladder cancer at the molecular level utilizing the ever growing biotechnologies is an important step for understanding a wide range of signaling events in both healthy cells and in the context of carcinogenesis. Substantial progress has been made in this avenue. Many new genes that are involved in bladder carcinogenesis have been identified. It is clear the H19-IGF2 locus is playing a central role in this aspect. As the role of IGF2 in embryogenesis and tumorigenesis is relatively well understood, H19 as a stress modulator is recently emerging to be involved in several fundamental processes of tumorigenesis including that of the bladder. Yet the exact molecular mechanisms that integrate H19 to such diverse events and circuits that are malfunctioning in cancer need further investigations.

In our lab, different regulatory sequences, selected from cancer-specific promoters of H19, IGF2-P3 and IGF2-P4 linked to the potent toxin (DTA), have been successfully used to drive cytotoxicity to cancer cells in vitro, in vivo, in different tumor models, and more importantly in bladder cancer patients, at least in the case of H19, with promising results. Similar approaches are also used in other types of human cancers including ovarian, hepatocellular, and pancreatic cancers and are clinically encouraging. Preclinically, this approach also shows promising results in lung cancer, glioblastoma and colorectal cancer metastasis to the liver. We are working on novel approaches to increase the cytotoxicity of the therapeutic plasmids, and to increase the numbers of patients that can benefit from this therapy. Furthermore, and given the central role of hypoxia and also p53 in the resistance of conventional therapeutic options and the involvement of the IGF2-H19 locus, we are developing sequential treatments of the therapeutic plasmids with conventional chemotherapeutic drugs with encouraging results. Moreover, the preclinical utility of short interfering RNA to knockdown both H19 and IGF2 is under development.

5. Acknowledgement

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6. References


This book is an invaluable source of knowledge on bladder cancer biology, epidemiology, biomarkers, prognostic factors, and clinical presentation and diagnosis. It is also rich with plenty of up-to-date information, in a well-organized and easy to use format, focusing on the treatment of bladder cancer including surgery, chemotherapy, radiation therapy, immunotherapy, and vaccine therapy. These chapters, written by the experts in their fields, include many interesting, demonstrative and colorful pictures, figures, illustrations and tables. Due to its practicality, this book is recommended reading to anyone interested in bladder cancer.

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