THE JOURNAL OF GENE MEDICINE
J Gene Med 2005; **7**: 366–374.
Published online 1 November 2004 in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/jgm.670

RESEARCH ARTICLE

Regulatory sequences of H19 and IGF2 genes in DNA-based therapy of colorectal rat liver metastases

Patricia Ohana[1]*†
Pinhas Schachter[2†]
Basim Ayesh[1]
Aya Mizrahi[1]
Tatiana Birman[1]
Tamar Schneider[1]
Imad Matouk[1]
Suhail Ayesh[1]
Peter J. K. Kuppen[3]
Nathan de Groot[1]
Abraham Czerniak[2]
Abraham Hochberg[1]

[1]*Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel*

[2]*Department of Surgery 'A', Wolfson Medical Center, Holon, Israel*

[3]*Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands*

*Correspondence to: Patricia Ohana, Department of Biological Chemistry, The Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel. E-mail: pohana@huji.ac.il

†These authors contributed equally to this work.

Received: 10 February 2004
Revised: 13 April 2004
Accepted: 21 July 2004

Abstract

Background Malignant tumors of the liver are among the most common causes of cancer-related death throughout the world. Current therapeutic approaches fail to control the disease in most cases. This study seeks to explore the potential utility of transcriptional regulatory sequences of the H19 and insulin growth factor 2 (IGF2) genes for directing tumor-selective expression of a toxin gene (A fragment of diphtheria toxin), delivered by non-viral vectors.

Methods The therapeutic potential of the toxin vectors driven by the H19 and the IGF2-P3 regulatory sequences was tested in a metastatic model of rat CC531 colon carcinoma in liver.

Results Intratumoral injection of these vectors into colon tumors implanted in the liver of rats induced an 88% and a 50% decrease respectively in the median tumor volume as compared with the control groups. This therapeutic action was accompanied by increased necrosis of the tumor. Importantly, no signs of toxicity were detected in healthy animals after their treatment by the toxin expression vectors.

Conclusions DT-A was preferentially expressed in liver metastases after being transfected with H19 or IGF2-P3 promoter-driven DT-A expression plasmids, causing a very significant inhibition of tumor growth as a result of its cytotoxic effect. Our findings strongly support the feasibility of our proposed therapeutic strategy, which may contribute to open new gene therapeutic options for human liver metastases. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords DNA-based therapy; DT-A gene; H19 gene; IGF2 gene; liver metastases; CC531 colon carcinoma cells

Introduction

Malignant tumors of the liver are among the most common causes of cancer-related death throughout the world [1]. Neoplasms of many organs frequently metastasize to the liver, particularly colon malignancies. Colon cancer is estimated to account for about 11% of the overall cancer incidence in 2003 (Cancer facts and figures, American Cancer Society, Inc.). For patients with liver cancer, surgical resection remains the only curative method, although the proportion of patients suitable for a potentially curative resection is less than 30% and the local recurrence rate following

resection remains high (60–70%) [2]. Current therapeutic approaches fail to control the disease in most cases. Thus, new avenues of therapy should be pursued and early results of immunotherapy and gene therapy look promising. The present study seeks to explore the potential utility of tumor-selective transcriptional regulatory sequences for directing tumor-selective expression of toxins, delivered by non-viral vectors. Non-viral vectors appear promising due to their potential to circumvent the main disadvantage of adenoviral vectors, caused by immune responses directed against adenovirus proteins, and limits their ability to be administered iteratively.

Based on early studies from our group and others, transcriptional regulatory sequences of the H19 gene have emerged as candidates for cancer gene therapy. H19 is a paternally imprinted, oncofetal gene that encodes a RNA (with no protein product) acting as 'riboregulator' [3], which is expressed at substantial levels in embryonic tissues, in different human tumor types, and marginally or not expressed in the corresponding tissue of the adult [4–6]. Its precise function is being debated; however, our recent data suggest a role for H19 in enabling the cells to survive under stress conditions by promoting angiogenesis, metastasis and cancer progression [7]. While the initial report of our group focused upon bladder tumor [8], our subsequent findings established the presence of H19 in other types of tumors, including hepatocellular [5], ovarian, endometrial and testicular cancer [9]. The human H19 gene lies within 200 kb downstream of the paternally expressed IGF2 genes, which are frequently coordinately regulated, both in terms of their common expression pattern and reciprocal imprinting. Shared enhancers located downstream of H19 stimulate transcription of both genes [10]. The imprinted gene IGF2 is highly expressed in a variety of cancers, but is expressed at a very low level in most normal tissues [11]. The IGF2 gene has four different promoters (P1–P4), with P3 and P4 promoters preferentially active in embryonic tissues and in human cancers [12]. IGF2 appears to be the most predictive marker for colon metastasis to the liver, where high expression in the primary tumor is associated with a >50 times risk of developing liver metastasis [13]. IGF2 and IGF-1R are expressed in the invasion margins of 70% and 83% of liver colon metastasis cases, respectively [14].

Recently, we have characterized the expression profile of H19 in human liver metastases originating from colorectal, pancreatic, ovarian, breast and gastric cancer. Our results, using quantitative *in situ* RNA hybridization analysis, showed that H19 is highly expressed in various human liver metastases (unpublished data).

We have previously described the construction of expression vectors carrying the diphtheria toxin A-chain gene under the control of IGF2-P3, P4 and H19 regulatory sequences, and showed that these constructs selectively kill tumor cells and inhibit tumor growth *in vitro* and *in vivo* [15,16].

On the basis of the results presented in this communication, we propose to use regulatory sequences of the imprinted genes H19 and IGF2 for the development of DNA-based therapy of human colorectal cancer liver metastases. The therapeutic potential of vectors carrying the DT-A gene driven by H19 and P3 regulatory sequences was tested in a metastatic model of rat CC531 colon carcinoma, that has been shown to share many characteristics with human colon adenocarcinomas [17,18]. The data presented here showed dramatic reduction of subcapsular-induced liver tumors after intratumoral injection of the toxin vector. Our data indicate that constructs in which the DT-A gene is driven by H19 or IGF2 regulatory sequences have a high therapeutic potential and are good candidates for liver metastases therapy.

Materials and methods

Cell culture

The CC531 cell line, derived from a dimethyl hydrazine induced adenocarcinoma Wag rat colon, was used to establish our working animal model. The cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM), as previously described [15].

Plasmids and construction of expression plasmids

All the luciferase gene reporter constructs were built from the pGL3 basic (Luc-1) vector (Promega) which lacks both promoter and enhancer sequences. The construct Luc-H19 which contains the reporter gene under the control of the human H19 promoter region from nucleotide -818 to $+14$ was prepared as described [19]. 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 human P3 regulatory sequence was subcloned from the Hup3 vector (a kind gift from Prof. P. E. Holthuizen, University of Utrecht, The Netherlands [16]) into the Luc-1 vector. The Luc-P3 construct was prepared by digesting the P3 promoter (-747 to $+140$) from (Hup3) plasmid by *Sac*I/*Sal*I and its insertion between the *Sac*I/*Xho*I restriction sites of the pGL3 basic vector. The DT-A-containing vector DTA-P3 was designed by replacing the luciferase gene in Luc-P3 by the DT-A as described above.

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

In vitro transfection and luciferase assay

A total of 0.4×10^6 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 manufacture's instructions using 3 µl of DNA and 6 µl of JetPEI solution to obtain an N/P ratio of 5. Transfection experiments were stopped after 48 h and reporter gene activity was assessed. Luciferase activity was measured using the Promega kit 'Luciferase Assay System' (E-1500; Promega, Madison, USA). Light output was detected using a Lumac Biocounter apparatus. Protein content was measured by the Bio-Rad (Hercules, CA, USA) protein assay reagent, and the results were expressed as light units/µg protein.

LucSV40 (Luc-4) was used as a reference for maximal luciferase activity, as it contains the SV40 promoter and enhancer, while Luc-1 that lacks any regulatory sequences was used as a negative control to determine the basal non-specific luciferase expression, which was found to be negligible. All experiments were carried out in triplicate and the results expressed as mean and standard error.

In vitro activity and specificity of the regulatory sequences (cell killing assay)

Cells were cotransfected using 3 µg of the reporter vector Luc-4 and the indicated amounts of the expression vectors DTA-H19 or DTA-P3, respectively, using the transfection reagent JetPEI as described above. Cells were also transfected by 3 µg of Luc-4 alone. *In vitro* activity of the regulatory sequences was determined by calculating the % decrease in the luc activity in the cotransfected cells compared with that of the cells transfected with Luc-4 only.

RNA isolation and cDNA synthesis

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 with RNase-free DNase I (Roche Diagnostics GmbH, Mannheim, Germany) to eliminate any contaminating DNA. The cDNA was synthesized from 2 µg total RNA in 20 µl reaction volume as described [16].

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

The PCR reactions were carried out in 25 µl volumes in the presence of 6 ng/µl of each of the forward and the reverse primers using 0.05 units/µl of Taq polymerase (TaKaRa Biomedicals, Japan) according to the manufacturer's instructions. The primer sequences used to amplify the rat H19 transcript was (5′-ACTGGAGACTAGGGAGGTCTCTAGCA) upstream and (5′-GCTGTGTGGGTCTGCTCTTTCAAGATG) downstream. The polymerase chain reaction (PCR) was carried out for 30 cycles (98 °C for 15 s, 58 °C for 30 s, and 72 °C for 40 s) and finally 72 °C for 5 min. The PCR analysis for DT-A amplification was carried out as described [16]. The primer sequences used to amplify the luc transcript were (5′-GAGGCGAACTGTGTGTGAGA) 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 s, and 72 °C for 30 s) 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.

Dig-labeled probe synthesis and *in situ* hybridization

We used a PCR strategy to generate DNA for synthesis of the Dig-labeled P3 RNA probe as described previously [16].

Paraffin wax rat colon carcinoma sections were prepared from the metastatic model of rat CC531 colon carcinoma. The non-radioactive *in situ* hybridization washings and treatments were as described previously [5]. The intensity of staining was indicated as (+1) for weak, (+2) for moderate and (+3) for strong signals. The degree of staining was referred to as focal (20–70% of the cells) and diffused (>70% of the cells).

In vivo DNA-based therapy

Wag/Rij male rats (12 weeks old, 260–280 g) purchased from Harlan (Zeist, The Netherlands) were used for all the experiments. All the surgical procedures and the care given to the animals were approved by the Local Committee for Animal Welfare.

Induction of CC531 tumors

After anesthetization of the rat, the liver was surgically exposed and 100 µl tumor cell suspension containing 50 000 tumor cells in phosphate-buffered saline (PBS) were injected subcapsularly into the left and right parts of the median lobe of the liver, using a 27-gauge needle.

Treatment of the CC531 tumor-bearing rats

Two weeks after tumor induction, the rats underwent surgery, and 50 µg of the reporter vectors (Luc-H19 or Luc-P3) or the toxin vectors (DTA-H19 or DTA-P3) were directly injected into the tumors. The reporter or toxin

vectors were injected into tumors of the right and left parts of the median lobe of the same liver. *In vivo* JetPEI, 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 μg of PEI/DNA complexes, according to the manufacturer's instructions. In the cases of intratumoral injection of naked DNA, 50 μg of plasmid DNA in 100 μl of PBS were directly injected into the tumor. Four days following the first treatment the rats underwent surgery, and naked DNA was injected for the second time. Four days after the second treatment, the animals were sacrificed, the liver taken, the size of the tumors was measured and taken for histological analysis. The rats treated with DTA-P3 received only one intratumoral injection of either the toxin or the reporter vector. The 5 μm serial sections were prepared from the treated colon metastasis and were photographed by a digital camera (Olympus); the high resolution photos were saved as digital images. The malignant tissue of each slide was identified microscopically and its area measured by the Image-Pro Plus version 3.0 software (Media Cybernetics, 1993–1997). The total area of tumor masses calculated for each set of slides sectioned from one animal should reflect the size of the tumors in that animal.

For statistical analysis of the differences between means, we used the Mann-Whitney test, and differences were considered significant at $p < 0.05$.

Results

Validation of the orthotopic rat colon carcinoma model

To evaluate the possible use of H19 and IGF2 regulatory sequences for the therapy of an orthotopic rat model of metastatic colon carcinoma, we determined the levels of H19 and IGF2 (rP2) (equivalent to hP3) RNA in the tumors, as compared with their levels in normal tissue. Rat colon carcinoma CC531 cells were implanted under the liver capsule in the left and in the right side of the median liver lobe. Subcapsular tumors were allowed to grow for 14 days. The rats were sacrificed by anesthesia overdose on day 15 and tumors were dissected out and weighed. The tumors dissected from the livers and regions of the liver that were not in contact with the metastases were subjected to molecular analysis and histology processing. Total RNA was extracted from the frozen tissue and the level of H19 RNA was determined by RT-PCR analysis. The results of the PCR analysis shown in Figure 1 indicated high levels of H19 RNA in liver metastases (lanes 3 and 4) while H19 RNA levels in normal liver or in liver parenchyma that was not involved in metastases were marginal (lanes 1 and 2, respectively).

The *in situ* hybridization technique (ISH) was used to determine the level and localization of the Igf2 rP2 transcript in the orthotopically implanted colon metastasis and in the host liver tissue as well. High levels of rP2 transcript were detected in the metastatic tissue, whereas no or very low levels were detected in the adjacent host liver tissue (Figure 2). The intensity of hybridization signals in the cytoplasm of the metastasis cells varied from +1 to +3. On the other hand, no rP2 expression was detected in the normal liver tissue except for very low background (<+1).

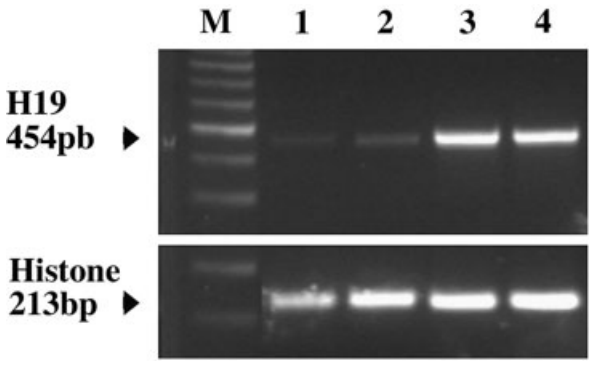

Figure 1. The level of H19 transcript in normal liver and in tumor liver metastases induced by subcapsular injection of CC531 cells. RNA samples from normal liver (lane 1), from non-tumor area of the liver (lane 2) and from liver metastases (lanes 3 and 4) were analyzed by RT-PCR for H19 transcript expression. 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

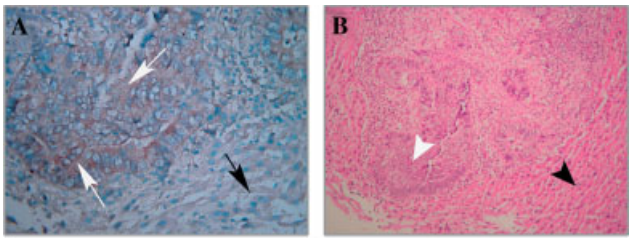

Figure 2. The level and distribution of the rP2 Igf2 transcript in colon metastases in rat liver induced by the subcapsular injection of CC531 cells. (A) ISH detection of rP2 transcript. The white arrows point to the positive hybridization signals within the cytoplasm of the tumor cells and the black arrow shows the host liver tissue with no rP2 transcript expression. The nuclei of the cells were counterstained by Giemsa stain (blue). (B) Hematoxylin/eosin staining of the metastases shown in (A). The white arrowhead shows the metastases and the black one shows the normal host liver

In vitro DT-A expression under the control of human H19 and hP3 regulatory sequences

The *in vitro* therapeutic potential of H19-DTA and P3-DTA was determined after cotransfection of the CC531 cells with 3 μg of LucSV40 and the indicated concentrations of H19-DTA (Figure 3A) or DTA-P3 constructs (Figure 3B). The luciferase activity driven by LucSV40 in the cotransfected cells was determined and

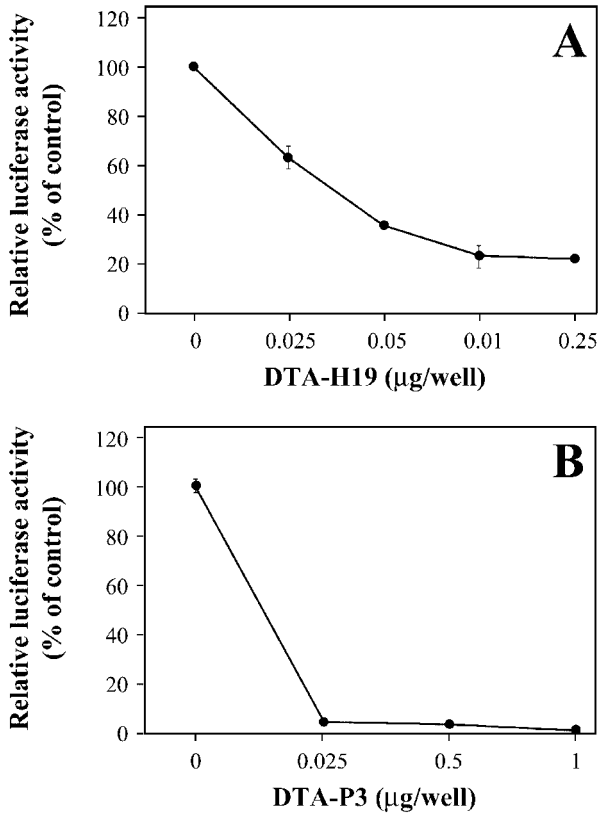

Figure 3. Effect of the therapeutic vectors DTA-H19 and DTA-P3 on CC531 cells. The CC531 colon carcinoma cells were cotransfected with 3 μg of LucSV40 and the indicated concentration of DTA-H19 (A) or DTA-P3 (B) (X-axis) using the *in vitro* JetPEI transfection reagent. Luciferase activity is expressed as a percentage of that measured after transfection with the positive control plasmid LucSV40 alone

compared with that in cells transfected by LucSV40 alone. The relative reduction of the luciferase activity in the cotransfected cells is the result of the H19- and hP3-driven DT-A expression which caused cell killing. H19 and hP3 are able to drive the expression of the DT-A gene and thus reduce luc activity in the CC531 cells in a dose-response manner. The human H19 and hP3 promoters were found to be active in CC531 cells even more than the SV40 promoter (data not shown). Consequently, low concentrations of the DTA-H19 or DTA-P3 plasmids were able to drive the expression of the DT-A toxin reducing luc activity in the cells cotransfected with the LucSV40 constructs as a measure for cell death (Figures 3A and 3B). The toxin vectors DTA-H19 and DTA-P3 at a concentration of 0.25 μg/well reduced the luc activity (induced by 3 μg LucSV40) by 78% ($p < 0.0041$) and >95% ($p < 0.001$), respectively.

Treatment of the orthotopic colon tumors in rat liver

The DTA-H19 and the P3-DT-A constructs were shown to be highly active in the CC531 cells *in vitro* (Figure 3). Moreover, the H19 and the rP2 transcripts are expressed at high levels in the metastasis developed by implanting the CC531 cells into the liver of syngeneic rats. Therefore, the orthotopic rat model for colon metastasis in liver seems to be an excellent model for determining the DTA-H19 and the DTA-P3 therapeutic potential for the treatment of colon metastasis in the human liver. A surgical procedure was performed to expose the liver of the Wag/Rij male rats in order to subcapsularly implant the CC531 cells as well as to treat the developed metastases 2 weeks later. Before starting the therapeutic treatment, the initial tumor size was determined by a caliper. It was possible therefore to calculate the fold increase of the tumor size at the end of the treatment. This will correct for the unequal sizes of the developed tumors before starting the treatment. At the end of the experiment, the tumors were excised and it was possible to determine their *ex vivo* parameters by a caliper and to prepare serial sections for the computer-based assessment of the tumor size (see 'Materials and methods'). Thus it was possible to represent the results of the two toxin vectors by different means.

Figure 4A shows that two injections of 50 μg of DTA-H19 with a 4-day interval either injected as naked DNA or complexed with a 22 kDa linear form of polyethylenimine (PEI) were able to inhibit the tumor growth. While the average tumor size of the toxin-treated tumors did not change, the Luc-H19-treated tumors in the liver of the same animal continued to grow to more than 4-fold during the 8 days after the first treatment with DTA-H19 (Figure 4A). The mean *ex vivo* tumor volume of the DTA-H19/PEI-treated group is 78% smaller than that of the Luc-H19/PEI-treated group, while the tumors treated with naked DTA-H19 were almost 90% smaller than the mean *ex vivo* volume of the control group ($p = 0.019$; Figure 4B). We added a more accurate estimation of the actual tumor size by microscopic observation of serial sections of the colon tumors. The tumor area assessment of the serial sections from each DTA-H19-treated tumor (injected as naked DNA) showed that the mean tumor area was 52% smaller than those treated with Luc-H19 ($p < 0.05$; Figure 4C). Since the use of PEI as an enhancer of transfection did not improve the therapeutic effect of the toxin vector, we decided to continue our experiments using naked DNA constructs only. The results obtained after treating the developed colon tumors with DTA-P3 (Figures 5A, 5B and 5C) were similar to those obtained with the toxin vector expressed under the control of H19 regulatory sequences. One treatment with 50 μg of DTA-P3 was able to completely inhibit further growth of the tumor, as the average tumor size did not change after the application of the DTA-P3 vector. On the other hand, the Luc-P3-treated tumors in the liver of the same animal continued to grow more than 3-fold during the 4 days after the treatment ($p = 0.05$; Figure 5A). The mean *ex vivo* tumor volume of the DT-A-P3-treated group is 50% smaller than that of the Luc-P3-treated group ($p = 0.04$; Figure 5B). The tumor area assessment of the serial sections from each

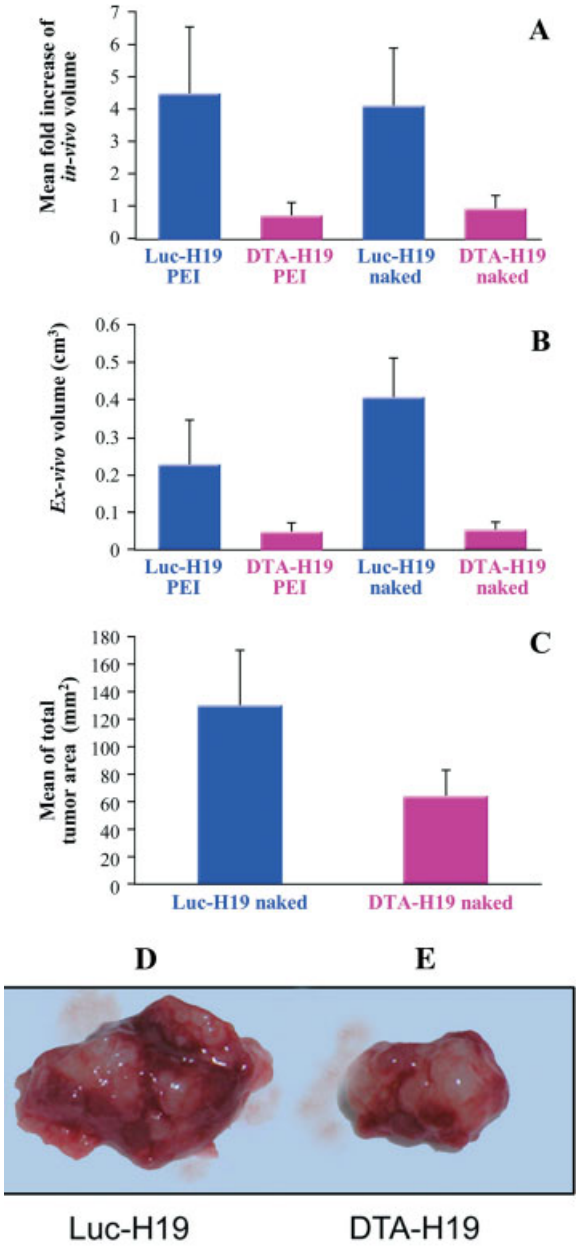

Figure 4. Effect of direct intratumoral injection of the plasmid DTA-H19 on the growth of colon metastases in the rat liver. Twelve rats received an injection of CC531 cells in the left and right parts of the median liver lobe. Two weeks later the rats received two injections of 50 μg of DTA-H19 or Luc-H19 with an interval of 4 days either injected as naked DNA (n = 6) or complexed with PEI (n = 6). The tumor dimensions were measured prior to the treatments and after sacrifice. (A) The mean fold increase in the DTA-H19- and Luc-H19-treated tumors. (B) The mean *ex vivo* tumor volumes of the DTA-H19 and Luc-H19, measured at the end of the treatment, after the animals were sacrificed. (C) The tumors of the naked DNA treated rats were cut into serial sections and the area of the malignant tissue of each section was calculated by using the Image Pro Plus version 3.0 software. Macroscopic *ex vivo* Luc-H19- and DTA-H19-treated tumors in separate lobes of the same liver (D + E), respectively

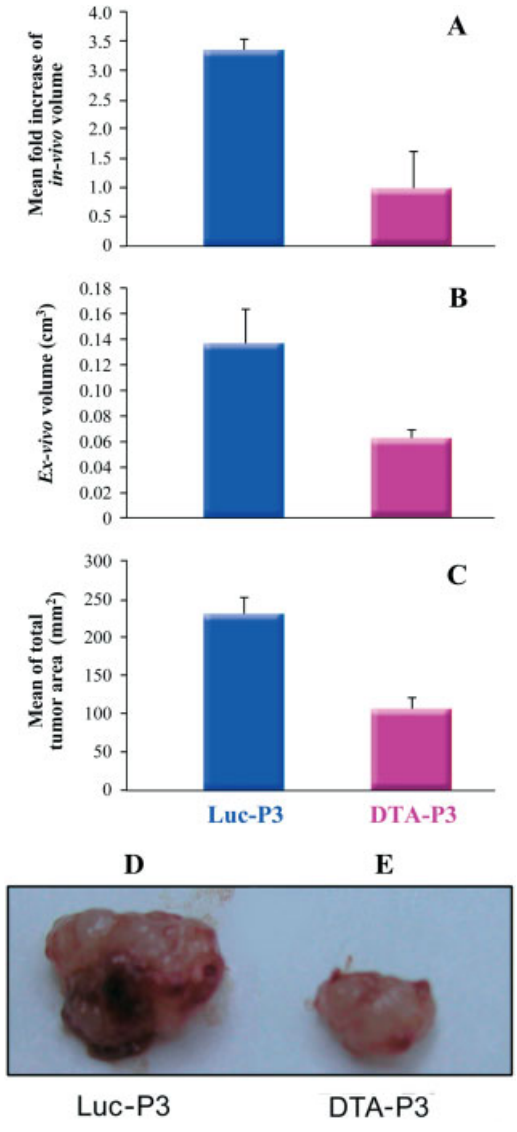

Figure 5. Effect of direct intratumoral injection of the plasmid DTA-P3 on the growth of colon metastases in the rat liver. Nine rats received an injection of CC531 cells in the left and right parts of the median liver lobe. Two weeks later the rats received one naked 50 μg injection of DTA-P3 (left median lobe) or Luc-P3 (right median lobe), and were sacrificed after 4 days. The tumor dimensions were measured prior to the treatment and after sacrifice. (A) The mean fold increase in the DTA-P3- and Luc-P3-treated tumors measured *in vivo*. (B) The mean *ex vivo* tumor volumes of the DTA-P3 and Luc-P3, measured after the sacrifice of the animals. (C) The tumor area was determined as described in Figure 4C. Macroscopic *ex vivo* Luc-P3- and DTA-P3-treated tumors in separate lobes of the same liver (D + E), respectively

treated tumor also revealed 50% smaller tumor areas when treated by DTA-P3 than by Luc-P3 ($p < 0.05$; Figure 5C).

The difference between the sizes of the DTA-H19- or DTA-P3- and Luc-H19- or Luc-P3-treated tumors was also revealed macroscopically (Figures 4D and 4E and Figures 5D and 5E). Moreover, the DTA-H19- and DTA-P3-mediated inhibition of tumor growth was accompanied by many foci of necrosis inside the tumor. However, only very few foci of necrosis were seen in Luc-H19- or Luc-P3-treated tumors (data not shown).

Expression of DT-A and luc after injection of the DTA-H9 and Luc-H19 plasmids into the tumors

Two weeks after inoculation with CC531 cells, we analyzed the gene transfer efficiency of the orthotopic tumors after intratumoral injection of 50 µg of either DTA-H19 or the reporter vector Luc-H19. Animals were sacrificed 48 or 72 h after plasmid injection, and the tumors were excised and snap frozen. The levels of the DT-A and luc mRNAs in the colon metastases and in the liver tissue were determined by RT-PCR of the total RNA extracted. Figure 6 showed higher levels of DT-A transcripts in those tumors from rats 72 h after plasmid injection (lanes 2, 3) as compared with those sacrificed 48 h after plasmid injection (lanes 1, 4), while no luc expression was detected in the non-treated liver tissue (lane 8). 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.

The livers of four healthy rats were treated with the toxin plasmids as previously described. No signs of systemic toxicity or weight loss were noted. Histological examination of the liver and the kidney showed no pathological changes (data not shown).

Treatment of the orthotopic colon tumors in rat liver with the DTA-H19 construct in a long-term experiment

The therapeutic effect of the DTA-H19 toxin vector was tested in the treatment of rats carrying colon liver metastases in a long-term experiment. Figure 7 shows that three injections of 50 µg of DTA-H19 for a period of 26 days were able to inhibit the tumor growth. The mean *ex vivo* tumor volume of the DTA-H19-treated group is 43% smaller than that of the Luc-H19-treated group ($p = 0.05$).

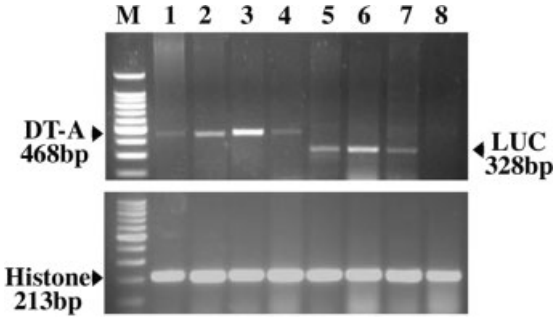

Figure 6. Detection of DT-A and luc mRNA expression in some of the DTA-H19 and Luc-H19-treated tumors. 400 ng of DNase-treated RNA samples were used for the RT-PCR reactions. DTA-H19-treated tumors as naked DNA (lanes 1–4), Luc-H19-treated tumor as naked DNA (lane 5), Luc-H19/PEI-treated tumors (lanes 6–7), non-treated liver tissue (lane 8), M (100 bp ladder). The lower panel shows the histone internal control

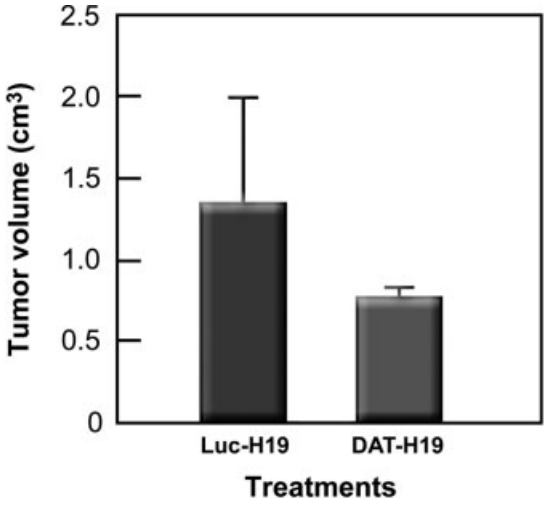

Figure 7. Effect of direct intratumoral injection of the plasmid DTA-H19 on the growth of colon metastases in the rat liver in a long-term experiment. Rats carrying liver metastases received three injections of DTA-H19 or Luc-H19 in the left and right median lobes of the liver with an interval of 1 week. Four days after the third plasmid treatment the animals were sacrificed. The mean *ex vivo* volumes of the tumors treated with DTA-H19 (n = 7) and Luc-H19 (n = 7) were measured at the end of the treatment, after the animals were sacrificed

Discussion

The present work proposes to use regulatory sequences of the genes H19 and insulin growth factor 2 (IGF2) for the development of DNA-based therapy of human colorectal cancer liver metastasis. 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. Various gene therapy strategies are currently under evaluation for the treatment of advanced colorectal cancer, some of them exploiting differences in gene expression levels between liver metastases and normal hepatic tissue for targeted treatment. In the approach pursued in this work tumor-selective promoters were used in conjunction with cytotoxic genes to achieve targeted tumor cell destruction. Testing in animal models showed that tumor-specific promoters exhibit a clear advantage compared with a strong constitutive promoter such as the CMV promoter currently used in clinical trials [20]. While most tumor-specific promoters are relatively weak resulting in insufficient transgene expression levels, the H19 and IGF2-P3 promoters are known to be differentially activated in various tumor types and to show no or minimum activity in the surrounding normal tissue [15,16]. This is in addition to the known autocrine/paracrine mode of IGF2 mitogen action in the development of a wide range of human malignancies [21]. Accordingly, killing the IGF2-expressing tumor cells will eliminate not only part of the tumor, but will also diminish the supply of the mitogenic IGF2 to neighboring tumor and non-tumor cells.

The goal of the present study was to evaluate the therapeutic potential of expression vectors carrying the A″ fragment of diphtheria toxin (DT-A) under the control of the H19 or IGF2-P3 regulatory sequences in an orthotopic rat model of metastatic colon carcinoma. We have previously shown that these constructs are able to selectively kill tumor cell lines and inhibit tumor growth *in vitro* and *in vivo* [15,16]. The choice of DT-A as a toxin gene ensured not only high killing activity, but its use has a great advantage in excluding unintended toxicity to normal cells, since the released DT-A protein from the lysed cells will not be able to enter neighboring cells in the absence of the DT-B fragment [22].

In order to determine the validity of this approach for therapy of rat colon tumors in the liver, both RT-PCR and ISH techniques were applied to determine the level of H19 and IGF2-P3 expression. We have shown high levels of expression of H19 or rP2 transcript in the malignant tissue but no or marginal levels in the host liver tissue (Figures 1 and 2). The pattern of rP2 expression in the rat liver resembles that of the human hP3 IGF2 expression in the human colon metastases in liver [14]. The high level of H19 RNA found in the rat liver metastases is in accordance with the results obtained from our study on the expression profile of H19 in human liver metastasis originating from colorectal tumors (A. Hochberg, unpublished data).

We have previously reported that the human H19 and IGF2-P3 regulatory sequences are also active in murine cells [15,16]. These findings are in accordance with the results presented in Figure 3, showing that the human H19 and IGF2-P3 regulatory sequences were able to drive the DT-A expression in the rat colon carcinoma CC531 cells. Therefore, these cells proved to be suitable for the generation of the orthotopic animal model used in this study to examine the anti-tumor therapeutic potential of H19-DTA and DTA-P3 expression vectors *in vivo*. The use of these plasmid vectors avoids any immunological consequences resulting from successive injections by viral vectors. Moreover, transient transfection of the tumor cells is sufficient for the toxin expression and cell killing. It was previously reported that direct injection of a luciferase-expressing plasmid induced luciferase expression in liver tumors [23]. We compared the therapeutic efficiency of intratumoral injection of naked DTA-H19 toxin vector to that of the complex DNA/JetPEI, a linear polyethylenimine derivative widely used in *in vivo* gene delivery approaches [24]. Gene delivery and expression in liver metastases from rats injected with naked DTA-H19 or with the reporter vector Luc-H19 (either naked or complexed with PEI) were analyzed by RT-PCR (Figure 6, lanes 1–4 and lanes 6–7, respectively). High expression of DTA and luc transcripts was detected in both of the groups with no clear preference for any of the two methods used for transfection (Figure 6). The results presented in Figures 4 and 5 indicated that this level of transfection was sufficient to obtain a clear therapeutic effect, while the use of DNA complexed with PEI had no advantage over the use of naked DNA. The H19 and the hP3 promoters were activated leading to DT-A expression in the transfected cells. Therefore, the inhibition of the growth of DTA-H19- or DTA-P3-treated tumors (Figures 4 and 5) is a direct result of the action of the DT-A. Intratumoral injection of the toxin vectors under the control of H19 or IGF2-P3 regulatory sequences induced a 90% and 50% decrease, respectively, in the median tumor volume as compared with the control groups that were treated with the correspondent reporter vector. Although the liver metastases were not totally ablated, the treatment with the toxin vectors stopped nearly completely the further growth of the metastases (Figures 4B and 5B). The inhibition in tumor growth was also demonstrated in a group of rats treated with the toxin vector DTA-H19 compared with the control group in a long-term experiment. The decrease in the median tumor volume in this experiment (43%) was smaller than that obtained with the short-term experiment. The reduced efficacy of the long-term treatment may be the longer time intervals between the treatments with the plasmids (7 days compared with 4 days). This treatment schedule avoids complications due to frequent surgical procedures.

Our observation that no systemic toxicity was detected as a result of the treatment described strongly support the feasibility of our proposed therapy strategy. These preliminary results demonstrate the advantage of the use of tumor-specific promoters to drive the expression of the DT-A gene in tumor tissues only, avoiding the severe toxicity that could be caused by the use of a non-specific promoter.

In summary we have demonstrated that the animal model described is suitable for studying the therapeutic potential of the DTA-H19 and DTA-P3 constructs. DT-A was preferentially expressed in liver metastases which were transiently transfected with H19 or IGF2-P3 promoter-driven DT-A expression plasmids, causing a great inhibition of tumor growth as a result of tumor cells killing. The DTA-H19 and DTA-P3 constructs caused no macroscopic or microscopic signs of unwanted toxicity to healthy animals injected by these toxin vectors.

Based on the findings presented here, we propose a patient-oriented approach to treat colon metastases in the liver. Given the clinical and genetic diversity of liver metastases, it may prove difficult to find a one-for-all promoter sequence to be used in their therapy. Instead, we propose tailored transcriptional regulatory sequence selection for DNA-based therapy of liver metastases according to individual patient-specific gene expression profiles. According to this approach the patient may be simultaneously treated with a combination of toxin-expressing plasmids some of which are under the control of the H19 and IGF2 regulatory sequences, and others under the control of the regulatory sequences of other differentially expressed genes. This approach should be further tested in appropriate animal models.

Acknowledgements

This work was supported in part by Bikura – Israel Science Foundation.

References

1. Lotze MT, Flickinger JC, Carr B (eds). *Cancer: Principles and Practice of Oncology*, Lippincott: Philadelphia, 1993; 883–914.
2. Scheele J, Stang R, Altendorf-Hoffman A. Hepatic metastases from colorectal carcinoma. *Br J Surg* 1990; **77**: 1241–1246.
3. Erdmann VA, Barciszewska MZ, Szymanski M, *et al*. The non-coding RNAs as riboregulators. *Nucleic Acids Res* 2001; **29**: 189–193.
4. Ariel I, Lustig O, Schneider T, *et al*. The imprinted H19 gene as a tumor marker in bladder carcinoma. *Urology* 1995; **45**: 335–338.
5. Ariel I, Miao HQ, Ji XR, *et al*. Imprinted H19 oncofetal RNA is a candidate tumour marker for hepatocellular carcinoma. *Mol Pathol* 1998; **51**: 21–25.
6. Ariel I, Ayesh S, Perlman EJ, *et al*. The product of the imprinted H19 gene is an oncofetal RNA. *Mol Pathol* 1997; **50**: 34–44.
7. Ayesh S, Matouk I, Schneider T, *et al*. The possible physiological role of H19 RNA. *Mol Carcinog* 2002; **35**: 63–74.
8. Rachmilewitz J, Goshen R, Ariel I, *et al*. Parental imprinting of the human H19 gene. *FEBS Lett* 1992; **309**: 25–28.
9. Tanos V, Ariel I, Prus D, *et al*. H19 and IGF2 gene expression in human normal hyperplastic and malignant endometrium. *Int J Gynecol Cancer* 2004; in press.
10. Leighton PA, Ingram RS, Eggenschwiler J, *et al*. Disruption of imprinting caused by deletion of the H19 gene in mice. *Nature* 1995; **375**: 34–39.
11. Ooasa T, Karasaki H, Kanda H, *et al*. Loss of imprinting of the insulin-like growth factor II gene in mouse hepatocellular carcinoma cell lines. *Mol Carcinog* 1998; **23**: 248–253.
12. Li X, Nong Z, Ekstrom C, *et al*. Disrupted IGF2 promoter control by silencing of promoter P1 in human hepatocellular carcinoma. *Cancer Res* 1997; **57**: 2048–2054.
13. Barozzi C, Ravioli M, D'Errico A, *et al*. Relevance of biologic markers in colorectal carcinoma: a comparative study of a broad panel. *Cancer* 2002; **94**: 647–657.
14. Kawamoto K, Onodra H, Kan S, *et al*. Possible paracrine mechanism of insulin-like growth factor-2 in the development of liver metastases from colorectal carcinoma. *Cancer* 1999; **85**: 18–25.
15. Ohana P, Bibi O, Matouk I, *et al*. The use of H19 regulatory sequences for targeted gene therapy in cancer. *Int J Cancer* 2001; **98**: 645–650.
16. Ayesh B, Matouk I, Ohana P, *et al*. Inhibition of tumor growth by DT-A expressed under the control of IGF2 P3 and P4 promoter sequences. *Mol Ther* 2003; **7**: 535–541.
17. Marquet RL, Westbroek DL, Jeekel J. Interferon treatment of a transplantable rat colon adenocarcinoma: importance of tumor site. *Int J Cancer* 1984; **33**: 689–692.
18. Hagenaars M, Ensink NG, Basse PH, *et al*. The microscopic anatomy of experimental rat CC531 colon tumour metastases: consequences for immunotherapy? *Clin Exp Metastasis* 2000; **18**: 189–196.
19. Ohana P, Kopf E, Bibi O, *et al*. The expression of the H19 gene and its function in human bladder carcinoma cell lines. *FEBS Lett* 1999; **454**: 81–84.
20. Wu Lily, Johnson M, Sato M. Transcriptionally targeted gene therapy to detect and treat cancer. *Trends Mol Med* 2003; **9**: 421–429.
21. Toretsky J, Helman LJ. Involvement of IGF-II in human cancer. *J Endocrinol* 1996; **149**: 367–372.
22. Maxwell IH, Glode LM, Maxwell F. Expression of diphtheria toxin A-chain in mature B-cells: a potential approach to therapy of B-lymphoid malignancy. *Leuk Lymphoma* 1992; **7**: 457–462.
23. Baque P, Pierrefite-Carle V, Gavelli A, *et al*. Naked DNA injection for liver metastases treatment in rats. *Hepatology* 2002; **35**: 1144–1152.
24. Ferrari S, Moro E, Pettenazo A, *et al*. ExGen 500 is an efficient vector for gene delivery to lung epithelial cells in vitro and in vivo. *Gene Ther* 1997; **4**: 1100–1106.