**Introduction**

H19 is an imprinted non-coding RNA gene, which is located within the imprinted cluster on chromosome 11p15.5 in human and its homologous region chromosome 7 in mice, and shares common regulatory sequences with other genes within the cluster including insulin-like growth factor-2 (IGF2) [1]. Extensive investigation including from our own group, have unveiled exciting findings on the mode of imprinting and expression of the H19 gene [2, 3]. H19 gene is either highly expressed and/or shows aberrant allelic pattern of expression in a large array of human cancers, while not expressed in the corresponding normal tissues [2, 3]. H19 expression is also induced by a variety of carcinogens [2, 3]. In previous works, we showed that H19 and IGF2 possess diagnostic, prognostic and therapeutics values in many types of human cancers [2, 3]. Elevated H19 expression was reported in both primary and metastatic tumors, in morphogenesis and epithelial-mesenchymal transition (EMT), in migration and angiogenesis, in inflammatory diseases, and wound healing, and in multidrug resistant [3, 4].

**Original Article**

Transcriptional targeting of glioblastoma by diphtheria toxin–A driven by both H19 and IGF2-P4 promoters

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**Abstract:** Background: The H19-IGF2 locus is either highly expressed and/or shows aberrant allelic pattern of expression in a large array of human cancers, while rarely expressed in the corresponding normal tissue. Preclinical, clinical studies and human compassionate using a DNA plasmid containing H19 and/or IGF2-P4 regulatory sequences that drive the expression of an intracellular toxin [diphtheria toxin A-fragment (DTA)] have demonstrated promising results in several types of carcinomas. Recently we reported that a single construct that expresses DTA under the control of both H19 and IGF2 P4 promoters showed superior efficacy in vitro as well as in vivo, in comparison to a single promoter construct in bladder carcinoma. Here we extended this approach to glioblastoma and tested the antitumor efficacy of the double promoter DTA-expressing vector (H19-DTA-P4-DTA) in vitro as well as in heterotopic animal model. H19 gene expression was tested by in-situ hybridization (ISH) and by quantitative Real-Time PCR (qRT-PCR) in samples of diffuse glioma. Methods: IGF2-P4 gene expression was tested by qRT-PCR as well. Results: Both H19 and IGF2-P4 transcripts were highly expressed in high grade gliomas. Furthermore, significant H19 expression in other types of primary brain tumors as well as in brain metastases was detected by ISH. Both A172 and U87 human glioblastoma cell lines showed high expression of IGF2-P4 while the A172 cell line showed high expression of H19 RNA as well. H19-DTA-P4-DTA exhibited superior cytotoxic activity compared to the single promoter expression vectors, in U87 and A172 glioblastoma cell lines in vitro and showed antitumoral efficacy in heterotopic glioblastoma animal model. Conclusions: Our findings indicate antitumoral efficacy against glioblastoma of the targeted double promoter vector H19-DTA-P4-DTA, both in-vitro and in-vivo. Thus, its test in orthotopic animal model of glioblastoma as well as in clinical trials is warranted.

**Keywords:** H19, IGF2-P4, glioblastoma, targeted therapy, H19-DTA-P4-DTA
Targeting glioblastoma by H19-DTA-IGF2-P4-DTA

Thus, the expression of H19 and/or IGF2 in a large array of human tumors and at different stages of tumor development, their hypoxic induction and their association with mutant forms of p53 make them an ideal new target for cancer therapy, including diffuse astrocytoma, which show frequent p53 mutations [31], and glioblastoma, which show unique hypoxia-driven angiogenesis (microvascular proliferation) and necrosis (pseudopalisading necrosis) [32].

Several anti-tumoral vectors were constructed in our lab during recent years, which are based on the use of regulatory sequences of either H19 gene [33] or IGF2-P4 [34] or a combination [35], conjugated to the gene for the A fragment of diphtheria toxin (DTA), namely H19-DTA [33], P4-DTA [34] and H19-DTA-P4-DTA [35], respectively. Our group has already showed the relative efficacy and safety of the use of these vectors, both in-vitro and in-vivo in several types of carcinomas [33-39], as well as in FDA approved phase I/II clinical study of bladder transitional cell carcinoma [40], and human compassionate of bladder transitional cell carcinoma, colon carcinoma metastatic to liver [3], inoperable ovarian cancer associated with massive ascites [41], and carcinoma of the exocrine pancreas (unpublished data). In addition, we have developed a very efficient siRNA, specifically targeting the H19 RNA. Preclinical results using heterotopic models of urothelial and hepatocellular carcinomas are promising, and indicate the ability of H19 siRNA to retard tumor growth in both models (unpublished data).

The aim of the present study was to establish that there is high expression of both H19 and IGF2 in brain tumors, and to show anti-tumoral efficacy of DTA based vectors, mainly H19-DTA-P4-DTA, against glioblastoma, both in-vitro and in-vivo.

Materials and methods

Brain tumor samples

Paraffin sections cut from archival material (paraffin blocks) of 34 variable brain tumors (Table 1) were subjected to in-situ hybridization (ISH) for H19 as previously described [35, 42-44]. Also, Banked frozen glioma samples were subjected to quantitative RT-PCR for both H19 (5 samples) and IGF2-P4 (3 samples). An exemption from the local ethical (Helsinki) com-
mittee at Hadassah Hebrew University Medical Center was secured.

**In situ hybridization (ISH)**

The non radioactive ISH washing and treatments were done as previously described [35, 42-44]. Briefly, each section was rehydrated by 30 μl of the hybridization solution containing about 30 ng of DIG labeled RNA probe at 52°C. The ISH was performed on successive paraffin sections of brain tumors tissue for H19 transcripts. The intensity of hybridization signal was indicated as (0) for no staining, (+1) for weak, (+2) for moderate and (+3) for strong staining signals. The area of the hybridization signal was referred to as (0) for no staining, up to one third of the cells (+1), one to two thirds (+2), and more than two thirds (+3). Therefore, total scoring (intensity+ quantity) for each sample varied from 0 (no expression) to 6 (very high expression). Low expression was set as total scoring of 0 < X < 3 and high expression was set as total scoring of 3 ≤ X ≤ 6.

**Cell culture**

The human glioblastoma cell lines A172, U87, and the mouse glioblastoma cell line GL261 were obtained from the American Type Culture Collection (ATCC; Rockville, MD). Cells were grown to confluence in a humidified incubator with 5% CO2 in polystyrene culture flasks and were maintained in Dulbecco’s Modified Eagle’s Medium-F12 (DMEM-F12) (1:1) medium containing 10% Fetal Calf Serum.

**RNA isolation, cDNA synthesis and PCR**

For in vitro experiments we evaluated the expression level of H19, IGF2-P3 and IGF2-P4 using semi-quantitative PCR. Reverse transcription of total RNA was performed as previously described [35, 45]. 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 according to the kit instructions (Takara). The forward (5'-CCGGCTTCCTGCTGAACA) and reverse (5'--TTCCGATGGTGTCTTTGATGT) primers designed for the detection of H19 RNA are spanning exons 2-3 and from exon 5 respectively so that no genomic H19 gene could be amplified. The primers designed for the detection of IGF2-P4 RNA were designed to bind at exon 6 (5'-TTCGCATGGTGCTCTTGATGT) and reverse (5'-CCGCTTCCTCTCTGCCCAGCG) for the P4 transcript in the forward direction and the reverse primer (5'- CAGCAATGCAGCACGAGGCGAAGCC) was designed to bind the 3' end of exon 7 and the 5' end of exon 8. The integrity of the cDNA

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**Table 1. H19 in-situ hybridization results (as average intensity and quantity) for variable brain tumors**

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Number</th>
<th>H19 average intensity</th>
<th>H19 average quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal brain (control)</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pilocytic astrocytoma, WHO grade I</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Diffuse astrocytoma, WHO grade II</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Oligoastrocytoma, WHO grade II</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Anaplastic astrocytoma, WHO grade III</td>
<td>3</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Primary glioblastoma, WHO grade IV</td>
<td>9</td>
<td>1.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Secondary glioblastoma, WHO grade IV</td>
<td>3</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Oligodendroglioma, WHO grade II</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anaplastic oligodendroglioma, WHO grade III</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Diffuse large B-cell lymphoma (primary)</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Diffuse large B-cell lymphoma (secondary)</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Medulloblastoma, WHO grade IV</td>
<td>1</td>
<td>2.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Meningioma, WHO grade I</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Atypical meningioma, WHO grade II</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Rhabdoid meningioma, WHO grade III</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Schwannoma (acoustic), WHO grade I</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Metastatic urothelial carcinoma</td>
<td>1</td>
<td>2.5</td>
<td>3</td>
</tr>
<tr>
<td>Metastatic lung carcinoma</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Metastatic malignant melanoma</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
was assayed by RT-PCR analysis of the ubiquitous, cell cycle independent, histone variant, H3.3. The PCR products were separated by electrophoresis on 2% gel agarose, and detected by ethidium bromide dye.

**Quantitative real time PCR (qRT-PCR)**

Banked frozen glioma samples and human glioblastoma cell lines (including A172 and U87) were subjected to RT-PCR (Applied Biosystems 7000 Real-Time PCR system) for both H19 and IGF2-P4. Normal human brain tissue as well as neuronal stem cells (NPCs) and embryonic stem cells (ESC) were used as control samples. For H19 analysis, starting from 0.2 ng (9 x 10^7 copies) up to 0.2 x 10^-7 ng (≤ 9 copies of plasmid DNA) were used. For IGF2-P4 analysis, starting from 0.2 ng (3 x 10^7 copies) up to 0.2 x 10^-7 ng (≤ 3 copies of plasmid DNA) were used. Total RNA was prepared using Trizol (Sigma). cDNA was prepared from 1 μg of total RNA using MMLV reverse transcriptase (Applied Biosystems) and random hexamers according to the manufacturer’s instructions for first-strand cDNA synthesis. The reaction mixture included 1 μl of cDNA, 0.75 μl of TaqMan probe and primers, and 7.5 μl of the master mix buffer containing nucleotides and Taq polymerase, (Taqman Master Mix, Applied Biosystems), in a total volume of 15 μl. Gene amplification was carried out using the GeneAmp 7000 Sequence Detection System (Applied Biosystems). Amplification included one stage of 10min at 95°C, followed by 40 cycles of a two-step loop: 20s at 95°C and 1min at 60°C. The gene expression results were normalized to the 18S rRNA gene.

**In vitro targeted therapy**

The in-vitro cytotoxicity of the vectors was determined by cotransfection of A172 or U87 human glioma cell lines with 2 μg of LucSV40 and serial concentrations of H19-DTA, P4-DTA, or H19-DTA-P4-DTA as described before [35]. Briefly, the in vitro jetPEI™transfection reagent compact the plasmid DNA into positively charged particles capable of interacting with anionic proteoglycans at the cell surface and entering cells by endocytosis. The transfection procedure was done as recommended by the manufacturer (Polyplus-transfection, France). A total of 0.1 x 10^6 cells/well were grown overnight in a twelve-well Nunc multidish (75 mm). For each well, 2 μg plasmid DNA and 4 μl of the jetPEI (N/P = 5) were diluted separately with 50 μl of 150 mM NaCl each, and vortex-mixed gently. The jetPEI solution was added at once to the plasmid DNA solution, the mixture was vortex-mixed for 10 seconds and the mixture was incubated for 15 minutes at room temperature. The 100 μl jetPEI/DNA mixture was then applied drop-wise onto the serum containing medium of each well. The transfection experiment was terminated after 48 hours. The cells were harvested and the luciferase activity was determined using the luciferase Assay System kit (Promega). The light output was measured using a Lumac Biocounter apparatus. The total protein content of the lysates was determined by the Bio-Rad protein assay reagent and the results were normalized to the total protein and expressed as Light units/μg protein. LucSV40 (Luc-4) was used as a positive control for the efficiency of transfection as it contains the SV40 promoter and enhancer, while Luc-1 that lacks any regulatory sequences was used as a negative control to determine the basal nonspecific luciferase expression level, which was found to be negligible in all of the cell lines. All experiments were done in triplicates and the results expressed as mean and standard error. The H19-DTA, P4-DTA and H19-DTA-P4-DTA cytotoxic activity was determined by calculating the % of decrease in the cotransfected LucSV40 activity compared to that of LucSV40 transfected alone in the same cell type normalized to total protein and expressed as light units/μg protein.

**In vivo targeted therapy in heterotopic nude mice animal model**

All surgical procedures and the care given to the animals were approved by the local committee for animal welfare. Animals were kept in the Hebrew University’s animal facility with water and food ad librum (all experimental research on animals follow internationally recognized guidelines).

Cells preparation, tumor inoculation and targeted therapy were performed as previously described [35]. Briefly, confluent U87 human glioblastoma cells were trypsinized to a single cell suspension and resuspended in PBS. 3 x 10^6 cells (in 150 μl volume) were subcutaneously injected into the back of female CD1 nude mice, 6-8 weeks old. 10 days after cell inoculation the developing tumors were measured in two dimensions and randomized to different treatments. Animals were separated to different groups of the same size (n= 6). Since in-vitro
Targeting glioblastoma by H19-DTA-IGF2-P4-DTA

Experiments showed an advantage of the double promoter DTA expression vector (H19-DTA-P4-DTA) over other vectors we have tested its antitumoral efficacy. Intratumoral injections of 25 μg of either DTA expressing construct (treatment group) or Luc expressing construct (control group) were given 10, 12 and 14 days after cells inoculation. In vivo Jet-PEI, a 22 kDa linear form of polyethylenimine (PEI) was used as a transfection enhancer reagent. PEI/DNA complexes of N/P ratio of 6 were prepared in a solution of 5% w/v glucose according to the manufacturer's instructions. Each tumor was measured, and volume was calculated according to the formula width^2 × length × 0.5. The animals were sacrificed 3 days after the last treatment, the tumors were excised and ex-vivo weight and volume were measured. Samples were fixed in 4% buffered formaldehyde and processed for histological examination for evidence of necrosis and persistent tumor.

Results

H19 RNA is highly expressed in variable brain tumors and glioblastoma cell lines

Our preliminary data (Table 1) is indicating high expression (>3) of H19, demonstrated by ISH performed on paraffin sections from 34 variable brain tumors including high grade diffuse astrocytic neoplasms, low and high grade meningioma, medulloblastoma, primary and secondary diffuse large B-cell lymphoma, and metastatic urothelial and lung carcinomas (Figure 1). High expression is indicated in most primary (6 out of 9), and secondary (2 out of 3) glioblastomas. Although the cohort is too small for statistical analysis, low grade diffuse gliomas (WHO grade II) appear to show low expression of H19, while high grade diffuse gliomas (WHO grade III and IV) show high expression.

Furthermore, we assessed both H19 and IGF2-
Targeting glioblastoma by H19-DTA-IGF2-P4-DTA

P4 expression in banked frozen glioma samples and human glioblastoma cell lines (including A172 and U87) by qRT-PCR analyses. Normal human brain tissue as well as neuronal stem cells (NPCs) and embryonic stem cells (ESC) were also included in the analyses as control. Relatively high expression level of H19 was indicated in 3 out of 4 high grade gliomas (3 glioblastomas and one anaplastic astrocytoma) and one low grade diffuse glioma (oligoastrocytoma). One glioblastoma and human glioblastoma cell lines (A172 and U87) showed low expression. Expression of IGF2-P4 was indicated in 2 out of 3 glioblastomas, but to lower extent than for H19. One glioblastoma and one human glioblastoma cell line (A172) showed low expression. Some of the results are shown in Figure 2.

Furthermore, H19 as well as IGF2 (P3 and P4) expression was tested in several glioma cell lines using semi-quantitative PCR (Figure 3). Our results indicate that the A172 human glioblastoma cell line expressed both H19 and IGF2 genes at relatively higher levels than U87 human glioblastoma cell line and GL261 mouse glioblastoma cell line.

The double promoter vector H19-DTA-P4-DTA encounters an enhanced cytotoxic activity relative to others in vitro

All tested vectors were able to drive the expression of the DTA gene and thus significantly reduces LucSV40 activity, in a dose-dependent manner in both A172 and U87 cell lines (Figure 4). However, the double promoter construct H19-DTA-P4-DTA exhibited far enhanced cytotoxic effect relative to each of the single promoter constructs. Luciferase activity was determined and compared to that of cells transfected with LucSV40 alone. The total amount of DNA cotransfected in samples receiving both single promoter constructs was twice than the cells transfected with H19-DTA-P4-DTA alone. Nevertheless the H19-DTA-P4-DTA vector exhibited enhanced cytotoxicity, relative to the combined activity of both single promoter constructs (Figure 4C).
We used the double promoter construct, H19-DTA-P4-DTA to assess its tumor growth inhibition activity, in vivo, using heterotopic animal model for glioblastoma, induced by U87 cells. U87 cells were subcutaneously injected into the back of 6-7 weeks old CD-1 female mice in order to develop a model for heterotopic glioblastoma. 10 days after subcutaneous cell inoculation, the mice developed measurable tumors. The therapeutic potency of the vectors was tested by direct intratumoral injection of 25 μg of the DTA expression vector H19-DTA-P4-DTA, or of the control H19-Luc-P4-Luc. Tumor size was determined and the in vivo fold increase of tumor size was calculated prior to each treatment and before sacrifice. Three injections of the double promoter plasmid H19-DTA-P4-DTA at two-day intervals significantly inhibited tumor development by 61% (P=0.004) compared to H19-Luc-P4-Luc treatment (Figure 5). The double promoter construct thus exhibited enhanced ability to inhibit tumor development in vivo.

To confirm the difference between the H19-DTA-P4-DTA and H19-Luc-P4-Luc groups, tumors were excised and their ex vivo volume and weight was determined. Mice treated with H19-DTA-P4-DTA exhibited a 57% (P=0.01) reduction of the ex vivo tumor volume (Figure 6A) and a 38% (P=0.005) reduction of the ex vivo tumor weight (Figure 6B) compared to H19-Luc-P4-Luc treated mice. The consistency of the results, in measurements of ex vivo tumors as well, eliminates any unrelated difference of the measurements (such as subcutaneous swelling due the inflammatory reaction, etc.).

Discussion

Our work indicates the efficacy of a double promoter expressing vector, carrying on a single construct two separate DNA sequences expressing the diphtheria toxin A-fragment (DTA), from two different regulatory sequences, selected from the cancer-specific promoters H19 and IGF2-P4. This construct was used to induce cy-

The double promoter vector is highly potent in suppressing tumor growth in heterotopic glioblastoma model in vivo

Figure 4. In vitro enhanced protein synthesis inhibition activity of H19-DTA-P4-DTA in A172 human glioblastoma cell line (A) and U87 human glioblastoma cell line (B). The cells were cotransfected with 2μg of LucSV40 and the indicated concentrations of the vectors are shown on the x-axis. The decrease in LucSV40 activity was determined by comparison to the same cell type transfected with LucSV40 alone as a measure of cytotoxicity and shown as percentages on the y-axis. The double promoter construct H19-DTA-P4-DTA exhibited enhanced efficiency in lysing the glioblastoma cells, relative to each of the single promoter constructs. H19-DTA-P4-DTA exhibited superior efficiency in lysing the U87 cells, relative to the combined activity of both single promoter-constructs (C).

Figure 5. In vivo inhibition of heterotopic glioblastoma tumors in response to H19-DTA-P4-DTA treatments. Inhibition of tumor growth in response to H19-DTA-P4-DTA treatment is shown. Tumor size of tumors treated with the DTA expressing vector, or with control luciferase expressing vectors were determined prior to each treatment and before sacrifice. The fold increase in tumor volume was calculated relative to the initial volume at the day of the first treatment.

Figure 6. (A) In vivo inhibition of heterotopic glioblastoma tumors in response to H19-DTA-P4-DTA treatments. Tumor volume of tumors treated with the DTA expressing vector, or with control luciferase expressing vectors were determined prior to each treatment and before sacrifice. The fold increase in tumor volume was calculated relative to the initial volume at the day of the first treatment. (B) Tumor weight of tumors treated with the DTA expressing vector, or with control luciferase expressing vectors were determined prior to each treatment and before sacrifice. The fold increase in tumor weight was calculated relative to the initial weight at the day of the first treatment.
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totoxicity both in vitro and in vivo in glioblastoma heterotopic cancer model.

Although our cohorts are too small for statistical analysis, our preliminary data is indicative of significant expression of H19 RNA in variable brain tumors, as demonstrated by H19-ISH performed on paraffin sections (Table 1 and Figure 1), quantitative RT-PCR performed on banked frozen glioma samples (Figure 2); and semi-quantitative RT-PCR performed on several glioma cell lines (Figure 3). There is also an expression of IGF2-P4 in high grade glioma demonstrated by quantitative RT-PCR performed on banked frozen glioma samples (Figure 2); and of IGF2-P4 and IGF2-P3 as demonstrated by semi-quantitative RT-PCR performed on several glioma cell lines (Figure 3).

We have previously reported that in some of the glioblastomas, expression of H19 RNA was observed in neoplastic cells as well as in proliferating microvasculature and was also associated with high immunoreactivity for p53 [46]. This finding might be related to the aforementioned link between p53 and H19 [24]. There also appears to be difference of expression of H19 RNA between low grade (WHO grade II) diffuse gliomas (low expression) and high grade diffuse gliomas (WHO grades III and IV) as indicated by H19-ISH. However, the significance of this result as well as all other results of H19-ISH and RT-PCR should be verified in a larger cohort. Nevertheless, the high expression of H19 in metastatic carcinoma as indicated by ISH is consistent with previous data regarding metastatic carcinoma to the liver [44]. Also, H19 high expression in meningioma and medulloblastoma as indicated by ISH is consistent with previous reports [14, 15].

All therapeutic vectors tested in-vitro, showed cytotoxic effect against glioblastoma cells (Figure 4), however, the double promoter construct H19-DTA-P4-DTA exhibited superior cytotoxicity in glioblastoma cell lines, relative to each of the single promoter constructs carrying either DTA DNA sequence alone (H19-DTA or P4-DTA) (Figure 4). This was in accordance with our previous report that show superior activity of the double promoter vector in urothelial carcinoma [35]. The advantage of using a double promoter over a single one has been discussed before [35], and includes the ability to target the treatment to a larger population of neoplastic cells that might express either H19 or IGF2-P4 or both. Thus, the majority of the neoplastic cells could efficiently express the diphtheria toxin. As discussed before [47, 48] once introduced into target tissue, the plasmid vectors have several advantages over viral vectors, including: no infectivity, similar levels of expression per cell without traces of extrachromosomal elements, lack of immunogenicity, which allow repeated treatments, transfecting mainly dividing cells, long term stability, safety and lack of need of special treatments or storage requirements. Direct DNA injection is considered a reliable, reproducible, and simple technique for intra-tumoral gene transfer [49]. We have transfected the plasmids into cell lines and into the target tissue of the animal model.
as complex with the linear cationic polyethyleneimine (jetPEI) as a transfection reagent. This method was chosen based on previous studies of our group showing relatively high levels of transfection efficiency [35]. Subunit A of the diphtheria toxin (DTA), which is highly potent, was chosen as an effector molecule. When only the cDNA coding for the A-fragment is expressed, the released DTA toxin from the lysed cells will not be able to enter neighboring cells in the absence of the DTB fragment [50], insuring highly specific killing activity of targeted neoplastic cells. In previous works the cytotoxic activity of diphtheria toxin conjugated to either transferrin or EGF has shown some degree of benefit in both animal models of glioma and clinical trials [51-56] with only minimal toxicity. As indicated by H19-ISH and RT-PCR results, H19 and IGF2-P4 regulatory sequences are expected to be good candidates for specifically inducing the expression of DTA in target neoplastic cells but not in cells of normal brain. Furthermore, there appears to be an additive activity of the double promoter vector versus combination of two single promoter vectors, as was shown for urothelial carcinoma, both in vitro and in vivo [35].

A superior cytotoxic activity of the double promoter vector H19-DTA-P4-DTA against U87 glioblastoma cells was exhibited, relative to the combined activity of both single promoter constructs (H19-DTA + IGF2-P4-DTA), in a dose response manner (Figure 4C). It should be emphasized that a surprising additive anti-tumor activity of the double promoter vector H19-DTA-P4-DTA was demonstrated in glioblastoma cells, although the total amount of DNA co-transfected in cells receiving both single promoter constructs was twice than the cells transfected with the double promoter construct. Thus, H19-driven and IGF2-P4-driven DTA-encoding sequences presented on a single expression vector (H19-DTA-P4-DTA), exhibited enhanced protein synthesis inhibition activity, relative to expression vectors carrying either DTA sequence alone when tested in glioblastoma cells. Due to these in vitro results we have decided to evaluate the therapeutic potential of the double promoter toxin vector H19-DTA-P4-DTA in a heterotopic mouse model. The inhibition of tumor progression resulted exclusively from the toxic effect of the diphtheria toxin.

In conclusion, the double promoter expressing vector, expressing DTA from two different regulatory sequences, H19 and IGF2-P4, namely H19-DTA-P4-DTA showed efficacy against glioblastoma, both in vitro and in vivo in heterotopic mouse model. Several reasons support this strategy. IGF2-P4 and H19 appear to be expressed in glioblastoma cells and not in normal brain. As previously noted, there appears to be a regulatory role for IGF2 in the development of glioblastoma [22, 23, 27]. By using the double promoter expression vector DTA could be better expressed in larger number of glioblastoma cells and therefore enhance tumor inhibition activity. By selective killing of glioblastoma cells, which express H19 and/or IGF2, the treated neoplastic cells as well as the neighboring tumor cells are at least partly deprived of their IGF2 supply. By that the targeted destruction of neoplastic cells expressing IGF2 or H19, accompanied by enhanced bystander effect, may lead to at least partial inhibition of tumor growth. Thus, this proposed treatment may be applied in combination with present and less targeted therapy methods for glioblastoma, such as chemotherapy and radiotherapy. This approach should naturally be tested in appropriate intracranial orthotopic animal models and clinical trials. Clinical trials (including phase III trials) of the effectiveness of targeted diphtheria toxin were based upon intra-tumoral convection-enhanced delivery [57, 58]. As noted above, some of these trials demonstrated evidence of tumor response. However, improved delivery methods and non-invasive imaging of toxin distribution are probably necessary for better results [59]. Since our group have shown promising results regarding the efficacy of H19 and IGF2 related anti-tumoral vectors for several types of carcinoma [33-41], including in clinical trials, it appears to be reasonable to test the anti-tumoral efficacy of H19-DTA-P4-DTA as a palliative treatment of brain metastases of variable carcinomas. Since brain metastases are usually well demarcated from the surrounding brain, a direct intra-tumoral vector delivery by stereotactic procedure appears plausible, and might be as efficient as metastasectomy or radiotherapy. Obviously this assumption should be tested by intracranial orthotopic animal model and by following clinical trials.

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