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Original Article

Target gene therapy of glioma: overexpression of *BAX* gene under the control of both tissue-specific promoter and hypoxia-inducible element

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Glioma-specific transcription of tumor-killing genes has been exploited as a promising gene therapeutic modality in glioma patients. Musashi1 (Msi1) and GFAP gene promoters are both cancer-specific promoters. Optimized HIF-binding site (optHBS) sequence was newly found as efficient as EPO HREs used as enhancer in cancer gene therapy. We constructed 4optHBS-Msi1/GFAP promoters and tested their ability to mediate *BAX* expression to induce apoptosis in glioma cell lines. Our results demonstrated that 4optHBS-Msi1/GFAP promoters are apparently strong and glioma-selective promoters with potential application in targeted glioma gene therapy, and 4optHBS-Msi1/GFAP-BAXα are valuable tools for glioma gene therapy.

Keywords glioma; gene therapy; GFAP promoter; Musashi1 gene promoter; hypoxia-inducible factor-binding site

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Introduction

Glioma is a kind of common and highly malignant neoplasm of the central nervous system with poor prognosis [1]. None of the current treatments could significantly prolong the patients' life span. However, gene therapy is considered as a promising alternative [2] and glioma-specific transcription of tumor-killing genes has been exploited. To achieve glioma-specific expression, glioma-specific vectors are generally composed of promoters, enhancers, and/or 5′ UTR that are responsive to glioma-specific transcription factors [3].

GFAP is a 50-kDa intracytoplasmic filamentous protein that constitutes the cytoskeleton of the astrocyte. This protein has been proved to be the most specific marker for cells of astrocytic origin under normal and pathological conditions [4]. Musashi1 (Msi1), a neural RNA-binding protein, is an evolutionarily well-conserved marker for neural stem cells/progenitor cells. It has been elucidated that Msi1 is located in a variety of tumors, such as medulloblastoma [5], glioma [6], astrocytoma [7], retinoblastoma [8], and cervix adenoma [9], and could be used as a marker for malignant glioma. Their promoters, *GFAP* promoter (P/GFAP) and *Msi1* promoter (MsiP), could allow the expression of downstream exogenous genes [4,10]. The sequence of P/GFAP was well studied [11], but that of MsiP has not been reported.

One general characteristic of almost all solid tumors, including glioma, is under microenvironment with reduced oxygen concentrations (hypoxia) [12]. Hypoxia can result from an insufficient blood supply, partly because tumor cells grow faster than the endothelial cells that make up the blood vessels, and partly because the newly formed vessels are disorganized. The hypoxia-inducible factor (HIF) activates transcription via binding to the hypoxia-responsive elements (HREs). Recent studies have demonstrated that activation of the 4optHBS (36 bp) construct by hypoxia or HIF-1a and HIF-2a was comparable with that of the 4EPO HRE (208 bp) construct [13].

BAX is a member of the Bcl-2 family that can initiate apoptotic cell death in cell culture and in animals, and is a good candidate for the induction of specific cancer cell death in cancer gene therapies [14]. There are four types of transcript variants of *BAX* gene. *BAXα* is one of them (579 bp) [15].

Therefore, in this study, we cloned the MsiP sequence, and evaluated its specific expression in different cell lines. We also constructed 4optHBS-GFAP/Msi1 promoters and

showed that 4optHBS-Msi1/GFAP promoters are significantly strong and glioma-specific promoters with potential application in targeted glioma gene therapy. We also showed that 4optHBS-Msi1/GFAP-BAXα is valuable tools for glioma gene therapy.

Materials and Methods

Potential MsiP sequence presumption

To predict the potential core MsiP sequence, we select the 10-kb long sequence upstream human *Msi1* gene containing the first exon and 5′ UTR region. We predicted four possible sequences of MsiP named M1, M2, M3, and M4, respectively, using bioinformatic tools including Promoter2.0, Promoter scan, Promoter Prediction, and CpG Island on http://bip.weizmann.ac.il/toolbox/seq_analysis/promoters.html#databases.

Plasmid construction

M1, M2, M3, and M4 were generated via PCR from genomic DNA isolated from HeLa cells. P/GFAP was preserved in our laboratory. These sequences were cloned into pGL3-basic vector (Promega, Madison, USA) in order to evaluate their promoter activity. These modified vectors were designated as pGL3-basic-M1, pGL3-basic-M2, pGL3-basic-M3, pGL3-basic-M4, pGL3-basic-GFAP, respectively. Based on the published optHBS sequence, we designed oligonucleotides that contain four tandem repeats of the optHBS (consensus sequence 5′-TACGTGCAG-3′). These oligonucleotides were used as an enhancer by inserted into the multiple cloning sites of pGL3-basic-Msi1 and pGL3-basic-GFAP. The resulting constructs were named pGL3-basic-4optHBS-Msi1 and pGL3-basic-4optHBS-GFAP, respectively. pGL3-basic-4HRE-GFAP was preserved in our laboratory, which contains an enhancer 4HRE (consensus sequence 5′-CCACAGTGCATACG-TGGGCTCCAACAGGTCCTCTTAGATCC-3′) located upstream of the P/GFAP. *BAXα* cDNA was obtained from the laboratory of Prof. Depei Liu. To construct 4optHBS-Msi1-BAXα, 4optHBS-GFAP-BAXα and 4HRE-GFAP-BAXα plasmids, luciferase gene (*luc*) was deleted using *Hin*dIII and *Xba*I enzymes and replaced with *BAXα* cDNA fragment flanked by *Hin*dIII and *Xba*I sites.

Cell culture

U251, BT325, HeLa, and 293T cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, USA), PC-12 cell line in RPMI 1640 (Hyclone, Logan, USA), supplemented with 10% fetal bovine serum (FBS; Hyclone). SH-SY5Y cell line was maintained in DMEM + F12 (1:1) (Invitrogen). Cultures were incubated in a humidified atmosphere containing 5% CO_2 at 37°C.

Transfection

Cells ($0.9 \times 10^5/8 \times 10^5$) were plated into 24-well dishes/60-mm dishes and grown for 24 h to reach 70−90% confluence. Multiple sets of wells/dishes in duplicate were prepared for each plasmid sample. DNA (0.2 μg) containing the reporter gene *luc* and 20 ng of pRL-TK-Renilla or 4 μg of 4HRE/4optHBS-Msi1/GFAP-BAXα plasmid were transfected using Lipofectamine 2000 (Invitrogen). The mixtures were added to the cells for 6 h, and then replaced with fresh culture media, which was continuously cultured for 42 h. Cells were then harvested and assayed for relative luciferase activity or for other experiment.

Induction of hypoxia in tumor cells

pGL3-basic-4optHBS-Msi1/GFAP or pGL3-basic-4HRE-GFAP was co-transfected with pRL-TK-Renilla, and after transfection for 18 h, one set of wells was grown under normoxic conditions and the other set was subjected to hypoxic conditions (0.3% O_2) by Hypoxia Management System (Coy Laboratory Products Inc., Grass Lake, USA) for 30 h.

Western blot analysis

Cells were lysed in lysis buffer (25 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 5 mM EDTA, 2 mM Na_3VO_4, leupeptin, Pefa-block, 1% Triton X-100, and 0.5% Nonidet P-40). The lysates were centrifuged for 10 min at 12,000 *g* at 4°C, and the supernatant was used for western blot analysis. Total proteins were separated via SDS-PAGE and then transferred onto pre-wetted polyvinylidene fluoride membranes (Millipore, Billerica, USA). The membrane was incubated with mouse anti-BAX monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, USA) or anti-β-actin antibody (Santa Cruz Biotechnology) at 4°C for 12 h and washed with TBS-T (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Tween-20). It was then incubated with horseradish peroxidase-conjugated anti-mouse IgG (Santa Cruz Biotechnology) at 37°C for 2 h, and the immune complex was visualized with the ECL System (Santa Cruz Biotechnology).

TUNEL staining assay

To detect apoptosis, cells were stained with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method using the Fluorescein *in situ* cell death detection kit (DeadEnd™ Fluorometric TUNEL System G3250#; Promega). TUNEL assays were conducted after the cells were transfected with plasmid using Lipofectamine 2000. The cells' nuclei were counterstained with Hoechst 33342 and visualized blue under fluorescent microscopy. The FITC-labeled cells undergoing apoptosis were recognized as green fluorescent nucleus.

Table 1 Dual-luciferase reporter system analysis of pGL3-basic-M1, pGL3-basic-M2, pGL3-basic-M3, and pGL3-basic-M4 promoter activity

Cell line	Relative luciferase activity ($\times 10^{-2}$)				
	pGL3-basic	M1	M2	M3	M4
U251	2.30 ± 0.26	8.17 ± 0.17*	1.84 ± 0.26	2.06 ± 0.22	1.93 ± 0.29
BT325	4.02 ± 0.34	21.5 ± 3.9*	3.41 ± 0.18	3.69 ± 0.37	3.66 ± 0.56
SH-SY5Y	2.61 ± 0.44	12.8 ± 1.2*	2.34 ± 0.20	2.33 ± 0.32	2.08 ± 0.35
HeLa	0.64 ± 0.08	38.9 ± 3.3*	0.62 ± 0.04	0.58 ± 0.04	0.57 ± 0.02
PC-12	6.03 ± 0.64	5.41 ± 0.57	5.13 ± 0.03	5.16 ± 0.69	5.41 ± 0.22
293T	1.60 ± 0.09	1.59 ± 0.18	1.54 ± 0.04	1.61 ± 0.07	1.54 ± 0.08

The results are shown as the mean ± SD from three independent experiments. *$P < 0.05$ versus pGL3-basic.

Table 2 Increase luciferase gene expression in six cell lines

Plasmid	Luciferase activity increase (folds)*					
	U251	BT325	SH-SY5Y	HeLa	PC-12	293T
pGL3-basic-Msi1	3.55 ± 0.32	5.36 ± 0.57	4.92 ± 0.51	60.35 ± 4.12	0.90 ± 0.18	0.99 ± 0.06
pGL3-basic-GFAP	6.16 ± 0.42	9.55 ± 0.58	0.86 ± 0.05	0.95 ± 0.10	0.79 ± 0.12	1.00 ± 0.05

The results are shown as the mean ± SD from three independent experiments. *Luciferase activity increase versus pGL3-basic.

Flow cytometry for DNA content

Cells were initially seeded at 5×10^6 cells in 6-well dishes. Cells were then infected with 4optHBS-Msi1/GFAP-BAXα or 4HRE-GFAP-BAXα for 48 h. Then cells were fixed and stained by PI staining buffer. Samples were then acquired using a FACSCalibur flow cytometer, and the sub-G_0/G_1 peak was measured by using CELLQuest software.

Statistical analysis

Statistical analysis was performed using SPSS 11.0 software. All values are presented as the mean ± SD. Statistical analysis was performed using Student's *t*-test and analysis of variance where appropriate. A *P*-value of <0.05 was considered statistically significant.

Results

M1 contains the core *Msi1* promoter sequence

Using the bioinformatic approaches mentioned above, we presumed four possible core *Msi1* promoter sequences: M1, M2, M3, and M4. M1 represented the sequence from 782 bp upstream to 50 bp downstream the starting point of *Msi1* gene transcription (−782 to +50 bp); M2 represented −5725 to −4900 bp; M3 represented −5272 to −5022 bp; and M4 represented −6427 bp to −4900 bp. In order to find out which sequence contained the MsiP active sequence, we cloned them into pGL3-basic vector respectively, and measured the relative luciferase activities in different cell lines. The results turned out that M1 showed high promoter activity in U251, BT325, SH-SY5Y, and HeLa cell lines ($P < 0.05$), but M2, M3, and M4 did not. In PC-12 and 293T cell lines, none of them showed promoter activities. These indicated that M1 contains the active sequence of MsiP (**Table 1**).

Msi1 and *GFAP* promoter activities were evaluated in different cell lines

We transiently transfected pGL3-basic-Msi1, and pGL3-basic-GFAP into six cell lines, respectively. Results turned out that both plasmids produced efficient luciferase expression in U251 and BT325 cell lines; only MsiP increased reporter gene expression in SH-SY5Y and HeLa cell lines; while none of them showed high activities in PC-12 and 293T cell lines (**Table 2**).

HRE and optHBS mediate reporter gene expression in glioblastoma cells under anoxic conditions

Plasmids were constructed by inserting oligonucleotide pairs containing four tandem repeats of optHBS upstream of the MsiP or P/GFAP in pGL3-basic vector (**Fig. 1**). Induction of hypoxia in tumor cells was carried out as mentioned above, pGL3-basic-Msi1 and pGL3-basic-GFAP were also transfected as control. Compared with oxic control cells, luciferase gene expression in anoxic cells increased 4.01, 2.67, and 2.44 folds from plasmids pGL3-basic-4optHBS-Msi1, pGL3-basic-4optHBS-GFAP, and pGL3-basic-4HRE-GFAP, respectively in U251 cell lines (**Table 3**). While in BT325 cell lines, the reporter

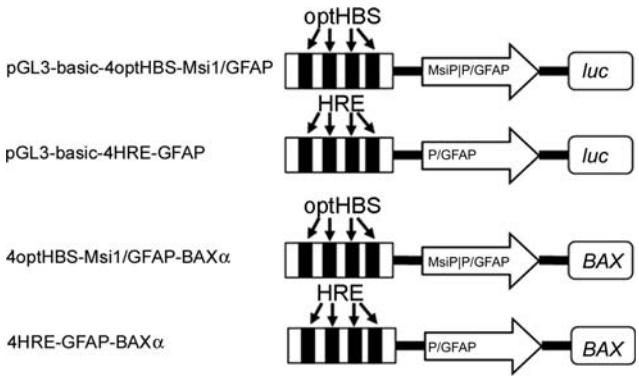

Figure 1 The pGL3-basic-4optHBS/HRE-Msi1/GFAP and 4optHBS/HRE-Msi1/GFAP-BAXα constructs In pGL3-basic-4optHBS-Msi1/GFAP and pGL3-basic-4HRE-GFAP, either four tandem repeats of optHBS or HRE were inserted into the multiple cloning sites in front of the GFAP/*Msi1* promoter in the pGL3-basic vector. In 4optHBS-Msi1/GFAP-BAXα and 4HRE-GFAP-BAXα, the luciferase gene *Iuc* was replaced with the human *BAXα* cDNA.

gene expression increased 4.96, 3.09, and 3.11 folds correspondingly (**Table 4**). These results indicated that the increased luciferase gene expression seen in the optHBS/HRE-containing plasmids was specifically mediated through optHBS/HRE.

Expression of *BAXα* in U251 and BT325 cells produced cell killing through apoptosis

We selected *BAXα* as the suicide gene. The 4optHBS-Msi1/GFAP-BAXα and HRE-GFAP-BAXα plasmid (**Fig. 1**) were transfected into U251 and BT325 cells; transfection with pGL3-basic-4HRE-GFAP was used as the control. Following transfection, cells were grown for 48 h in oxic conditions. The results of western blot analysis demonstrated that 4optHBS-Msi1/GFAP and 4HRE-GFAP promoters significantly increased the *BAXα* gene expression both in U251 and BT325 cells (**Fig. 2**). To determine whether 4optHBS-Msi1/GFAP-BAXα and 4HRE-GFAP-BAXα could be further used in glioma gene therapy, we measured apoptosis in *BAXα*-transfected U251 and BT325 cells under oxic conditions using the TUNEL assay and flow cytometry. As showed in **Figs. 3** and **4**, the expression of *BAXα* in 4optHBS-Msi1/GFAP-BAXα and 4HRE-GFAP-BAXα transfected U251 and BT325 cells caused a significant amount of apoptosis even under oxic conditions.

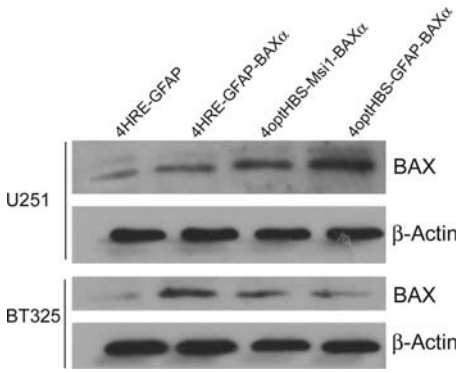

Figure 2 Western blot analysis of the expression of *BAX* in U251 and BT325 cells Overexpression of *BAX* was detected in U251 and BT325 cells, when 48 h after transfection with 4optHBS-GFAP-BAXα, 4optHBS-Msi1-BAXα, or 4HRE-GFAP-BAXα, comparing with pGL3-basic-4HRE-GFAP. β-actin was used as a control.

Table 3 Summary of independent transfection experiments in U251

U251	Relative luciferase activity ($\times 10^{-1}$)				
	Msi1[a]	GFAP[a]	4optHBS-Msi1[b]	4optHBS-GFAP[b]	4HRE-GFAP[b]
Oxic	3.29 ± 0.12	4.53 ± 0.30	3.54 ± 0.41	4.81 ± 0.11	5.17 ± 0.22
Anoxic	3.62 ± 0.56	3.85 ± 0.84	14.21 ± 2.24	12.85 ± 2.23	12.61 ± 0.22

[a]The results are shown as the mean ± SD from two independent experiments. [b]The results are shown as the mean ± SD from three independent experiments.

Table 4 Summary of independent transfection experiments in BT325 cells

BT325	Relative luciferase activity ($\times 10^{-1}$)				
	Msi1[a]	GFAP[a]	4optHBS-Msi1[b]	4optHBS-GFAP[b]	4HRE-GFAP[b]
Oxic	1.85 ± 0.21	2.80 ± 0.26	2.69 ± 0.56	2.45 ± 0.31	2.35 ± 0.12
Anoxic	1.82 ± 0.18	2.20 ± 0.25	13.34 ± 1.4	7.56 ± 0.78	7.30 ± 1.56

[a]The results are shown as the mean ± SD from two independent experiments. [b]The results are shown as the mean ± SD from three independent experiments.

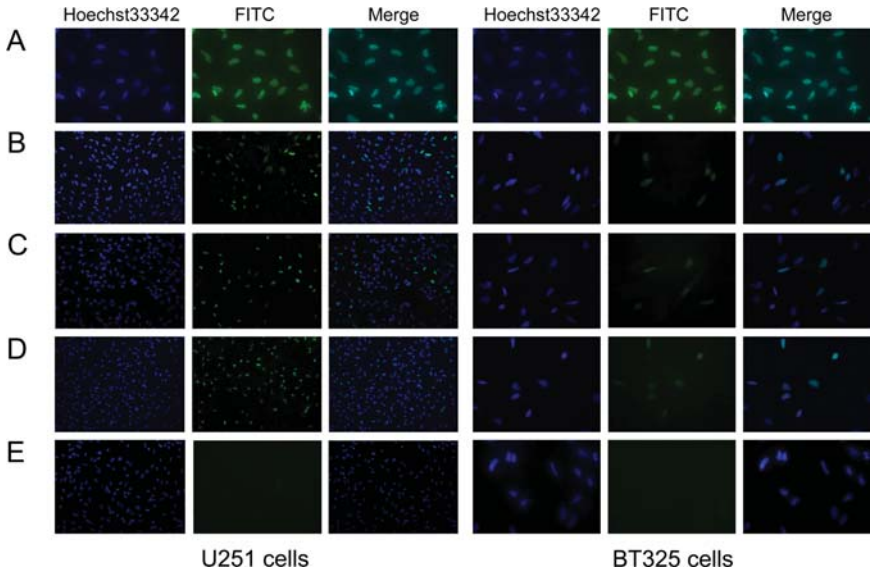

Figure 3 TUNEL stain results show that 4optHBS-GFAP-BAXα, 4optHBS-Msi1-BAXα, 4HRE-GFAP-BAXα can cause apoptosis in U251 and BT325 cell lines comparing with pGL3-basic-4HRE-GFAP (A) DNase I positive control; (B) 4optHBS-GFAP-BAXα; (C) 4optHBS-Msi1-BAXα; (D) 4HRE-GFAP-BAXα; and (E) pGL3-basic-4HRE-GFAP. In U251 cells, magnification, 100× for (A), 400× for (B–E). In BT325 cells, magnification 400× for (A–E).

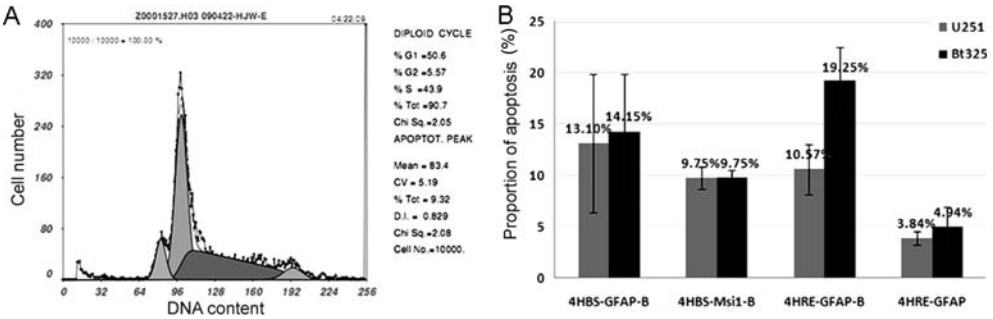

Figure 4 Flow cytometry detects rate of apoptosis in U251 and BT325 cells (A) One of the representative FCM histogram images (4optHBS-GFAP-BAXα in BT325). (B) Flow cytometry detects rate of apoptosis in U251 and BT325, when 48 h after transfection with 4optHBS-GFAP-BAXα, 4optHBS-Msi1-BAXα, or 4HRE-GFAP-BAXα, comparing with pGL3-basic-4HRE-GFAP.

Discussion

Glioma is with poor prognosis in adults. Even with aggressive multimodal therapy, average survival is a little more than 1 year, and less than 10% survival more than 2 years [1]. The absence of an effective treatment and the extremely poor prognosis of this disease necessitate new therapeutic options such as gene therapy [2].

One approach of gene therapy is to deliver a therapeutic gene under the control of glioma-specific promoter. These glioma-specific promoters allow the therapeutic gene to be delivered *in situ* and selectively target brain tumor cells while sparing the adjacent normal brain tissue [3]. Seo *et al*. [16] induced the cancer cell-specific death via MMP2 promoter-dependent *BAX* expression. In our research, we construct 4optHBS-Msi1/GFAP promoters using 4optHBS as an enhancer to improve the efficiency of regulatory element and increase the cancer specificity.

A number of glioma-specific/cancer-specific promoters have been reported to be used in glioma gene therapy, such as that of human telomerase reverse transcriptase [17], survivin [18], matrix metalloproteinase-2 (MMP-2) [16], GFAP [19], and Msi1 [10]. GFAP promoter has been used in glioma gene therapy in a wide range [4]. Msi1 has recently been shown to be a versatile marker of a variety of malignant tumors, such as medulloblastoma, glioma, and cervix adenoma. So, in the study, in order to determine the cancer specificity of the promoters we chose: (i) glioblastoma cell lines: U251 and BT325; (ii) neuroblastoma cell line: SH-SY5Y; (iii) adenocarcinoma cell line: HeLa; as our screening cell lines. PC-12 cell can differentiate into neuron cell under the induction of nerve growth factor, so we

selected this cell line as neuron model cell line to insurance the safety of glioma gene therapy. Also we chose 293T as negative control cell line. Our results proved that GFAP promoter was glioma specific; *Msi1* promoter was cancer-specific because it showed activity not only in glioblastoma cell lines (U251 and BT325), but also in neuroblastoma cell line (SH-SY5Y) and adenocarcinoma cell line (HeLa). These were in accordance with the protein expression in different cancers, which strongly suggested that we found the core sequence of the *Msi1* promoter. And our results also showed that *Msi1* promoter was less efficient than GFAP promoter in glioblastoma cell lines, but had shorter length (830 bp vs 1830 bp), which mean that both might be useful for future development of therapeutic application.

Recent evidences suggested that the enhancer element optHBSs could be useful to target hypoxic cells in gliomas [13]. Our results strongly indicated that 4optHBS-Msi1 and 4optHBS-GFAP promoters were more abundantly expressed in glioma cells than in normal cells under hypoxia; activation of the 4opt HBS construct was comparable to that of the 4EPO HRE construct as reported; in addition, 4opt HBS is much shorter in length (36 vs. 208 bp). But compared with oxic cells, the luciferase gene expression in anoxic cells was increased by 2.44–4.96 folds from respective plasmids (**Table 2**), which was not as effective as reported before [13]. These differences may reflect that HIF-1 activity varies among cell lines, although we have not yet tested this possibility.

Theoretically, 4optHBS-Msi1/GFAP DNA sequence can selectively induce gene expression in glioma cells both under regulation of Msi1/GFAP promoters and hypoxic microenvironment in solid tumors. Recently, glioma-specific promoters were often employed to retarget the cancer cells in oncolytic virus strategy [18,19]. Our study may provide a new choice for this strategy and improve the outcome in abstracto.

A variety of genes that demonstrated direct antitumor effects could be the therapeutic genes such as those encoding proapoptotic proteins p53, E1A, p202, PEA3, BAX, Bik, and prodrug metabolizing enzymes, namely thymidine kinase and cytosine deaminase [3]. In our experiment, we chose *BAX* suicide gene. We have shown that BAX can be used to trigger cellular apoptosis in U251 and BT325 cells even under oxic condition when regulated by 4optHBS-Msi1/GFAP (**Fig. 3** and **Table 3**). Our results proved the possibility that the 4optHBS-Msi1/GFAP-BAXα system might have a viable therapeutic application. It should be noted that the extent of cell killing by BAX must be underestimated in our experimental system because only 50–70% of the cells were transfected with the *BAX* construct and the cell killing was under oxic condition. Therefore, BAX may be more effective in killing cells than we have determined.

As a whole, this study revealed the possible core *Msi1* promoter. Our data proved that the 4optHBS-Msi1/GFAP promoters are strong and glioma-selective promoters with potential application in targeted glioma gene therapy. Results also showed that 4optHBS-Msi1/GFAP-BAXα can be utilized to cure cancers via the induction of cancer cell-specific death. Further studies will be carried out regarding the apoptotic effects of 4optHBS-Msi1/GFAP-BAXα under hypoxic condition delivered by rAAVs; the transfer of 4optHBS-Msi1/GFAP-BAXα *in vivo* studies with glioma transplanted nude mice.

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