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RESEARCH

Combined Antitumor Effect of Ad-bFGF-siRNA and Ad-Vpr on the Growth of Xenograft Glioma in Nude Mouse Model

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Abstract Basic fibroblast growth factor (bFGF) has been demonstrated to correlate with glioma grade and clinical outcome and has established its possible usefulness as a target for glioma therapy. Vpr has been described as an antitumor agent and displays a potent antitumor nature. Here, we try to investigate whether a combined treatment with bFGF-siRNA and Vpr gene would have a enhanced effectiveness on glioma in vitro and in vivo.After treatments with only Ad-bFGF-siRNA, only Ad-Vpr, and a combination of both, we assessed the changes in cell proliferation, cell cycle, and apoptosis in vitro by the methods of MTT, PI and FITC-AnnexinV double staining, respevtively. In addition, we also evaluated the combined effect of bFGF-siRNA and Vpr gene therapy on glioma in vivo using xenograft glioma models in nude mice. Combined Ad-bFGF-siRNA and Ad-Vpr treatment was more better successful in inhibiting cell proliferation in comparison with treatments of either Ad-bFGF-siRNA or Ad-Vpr alone. Treatment of Ad-Vpr alone or a treatment of a combination of Ad-bFGF-siRNA and Ad-Vpr induced the G2/M cell cycle arrest and apoptosis; however, combined treatment was more effective than the Ad-Vpr treatment alone. Although each single treatment can slow the growth of xenograft glioma, the combined treatment with Ad-bFGF-siRNA and Ad-Vpr was better than either the Ad-bFGF-siRNA or Ad-Vpr treatment alone. Our results suggest that the combination therapy with bFGF-siRNA and Vpr gene can achieve a enhanced activity of anti-glioma, supporting the idea that the combination of these two antitumor agents could open new perspectives in glioma therapy.

Keywords siRNA · bFGF · Vpr · Adenoviral vector

Introduction

Malignant gliomas are the most common primary intrinsic tumors of the central nervous system in adults and are generally very invasive and have a poor prognosis. Despite aggressive treatment in gliomas using surgical resection, radiation therapy, and chemotherapy, clinical recurrence or progression is nearly universal and the average life expectancy after diagnosis is usually less than 1 year for glioblastoma multiforme(GBM, WHO grade 4),the most common grade of malignant glioma [1, 2]. Available salvage therapies following progression are ineffective. Therefore, novel therapies are necessary for these devastating tumors to improve the prognosis.

Basic fibroblast growth factor (bFGF), a secreted multifunctional protein growth factor, is a potential mitogenic and angiogenic protein for glial cells and endothelial cells. Its abnormal expression by neoplastic astrocytes

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stimulated the growth of astrocytoma cells in an autocrine fashion. In contrast, the inhibition of the expression or receptor binding of bFGF has been shown to inhibit glioma growth in vitro and in vivo [3], thus establishing bFGF as a possibly useful target for gliomas therapy. In this study, to establish a new glioma therapy system based on the blockade of the human bFGF gene, we adopted RNA interference strategies. RNA interference(RNAi) is an endogenous gene-silencing mechanism that involves double-stranded, RNA-mediated sequence specific mRNA degradation; currently, there is an intense research effort aimed at developing RNAi into therapeutics against various diseases such as viral infections, neurodegenerative disorders, and cancers [4].

Resistance to apoptosis is one of the hallmarks of tumor, and as with many other tumors, malignant gliomas share this common essential characteristic of anti-apoptosis. Therefore, inducing apoptosis of glioma cells is a promising strategy for glioma gene therapy. Novel agents should provide an efficient capability for tumor cells to overcome resistance to apoptosis. One such novel agent that fulfills these requirements is the viral protein R (Vpr), a 14 kD HIV-1 accessory protein. Earlier studies have shown that it has multiple functions, including induction of G2/M cell cycle arrest and apoptosis in addition to its accessory and regulatory functions as a protein of HIV-1 [5–7]. In fact, it has been demonstrated that Vpr renders antiproliferative effects in various types of tumors [8–12]. These results support the hypothesis that Vpr could be used as an antitumor agent in glioma.

The major aim of this study is to develop a combined treatment involving a molecular target-based therapeutic and a new antitumor gene therapy in order to develop a new strategy for malignant glioma treatment.

Materials and Methods

Adenoviral Vector Construction

HIV-1 Vpr gene sequence was synthesized and cloned into plasmid vector pUC57. The sense (5′-CGAACTGGGCAG TATAAACTT-3′) and antisense (5′-GTTTATACTGCC CAGTTCGTT-3′) bFGF-siRNA sequences were cloned into plasmid vector pGenesil-1. The Vpr or bFGF-siRNA expression cassette were individually excised from the plasmid clone, pUC57-Vpr and pGenesil-1-bFGF-siRNA, with endonuclease XhoI-NheI or EcRI-HindIII, and then ligated into linearized adenoviral shuttle vector, pGStrack-CMV, to construct pGStrack-CMV-Vpr or pGStrack-CMV-bFGF-siRNA. The pGStrack-CMV-Vpr or pGStrack-CMV-bFGF-siRNA were co-transfected with the pAd vector backbone into DH5α bacteria for the recombinant generation of Ad-Vpr and Ad-bFGF-siRNA, which were further amplified in HEK293 cells. Viral particles were purified by cesium chloride density gradient centrifugation.

Cell Culture and Adenovirus Infection

The human glioma cell line U251 were grown in Dulbcco's modified Eagle medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 U/ml of penicillin, 100 μg/ml of streptomycin in a humidified atmosphere containing 5% CO2 at 37°C. All media and serum were purchased from Gibicol(Grand Island, NY,USA).

1×10^5 glioma cells U251 in serum-free DMEM were infected with Ad-bFGF-siRNA , Ad-Vpr at the 100 MOI, respectively, or combined infection with Ad-bFGF-siRNA and Ad-Vpr at the 100 MOI(Ad-bFGF-siRNA 50 MOI and Ad-Vpr 50 MOI). Infection with an adenovirus designed to express green fluorescent protein (Ad-GFP) served as a mock control(100 MOI). The virus-containing medium was removed 8 h later and replaced with fresh DMEM medium containing 10% FBS. Twenty-four hours after infection, cell proliferation was measured using MTT assay as described previously [13].

Cell Cycle and Apoptosis Analysis

U251 cells were seeded at a density of 1×10^6 on 60-mm dishes in DMEM medium. Cells were infected with Ad-GFP, Ad-bFGF-siRNA, and Ad-Vpr at 100 MOI or combined infection with Ad-bFGF-siRNA and Ad-Vpr at 100 MOI(Ad-bFGF-siRNA 50 MOI and Ad-Vpr 50 MOI) for 8 h, and then the old medium was removed and fresh medium was added to cells. After 72 h the cells were harvested, fixed in 70% ethanol, and stained with 40 μg/ml PI for cell cycle progression analysis or stained with FITC-Annexin V for apoptosis analysis. At least 30,000 cells were analyzed per sample using a FACScaliber machine (BD, San Jose, CA, USA).

In Vivo Tumor Xenograft Models

In gene therapy experiments, to establish glioma xenografts in mice, 1×10^6 glioma cells in 100 μl PBS were injected subcutaneously (s.c.) in the right flank of three 5-week-old nude mice. When tumors reached 20–30 mm^2, they were harvested, pooled, and segmented into approximate 2 mm^3 xenografts which were immediately implanted into flank of 30 female nude mice. When the mean tumor diameter reached 5–6 mm, the mice were randomly separated into five groups with six animals per group: a control group, mock group, Ad-bFGF-siRNA group, Ad-Vpr group, and combined Ad-bFGF-siRNA and Ad-Vpr group. Four groups were individually injected into tumor with 5×10^8

plaque-forming units (PFU) of Ad-GFP, Ad-bFGF-siRNA, Ad-Vpr or combined Ad-bFGF-siRNA and Ad-Vpr virus dilution in 15 μl PBS four times with 6-day intervals. The control group was injected with 15 μl PBS. All animals were then maintained to allow further growth of glioma for an additional 2 weeks. Subcutaneous tumor volume was determined by external measurements every 4 days with vernier calipers and calculated as Volume=0.5×(length×width^2). Here, we applied the endpoint. At 42 days, the animals were sacrificed and tumors were immediately fixed for TUNEL analysis as Chen described [14].

Statistical Analysis

All data were analyzed using SPSS 13.0 software. The significance differences were tested either by one-way analysis of variance (ANOVA) or by a two-sided Student t-test. A *P*-value less 0.05 was considered significant.

Results

Combination Treatment of Ad-bFGF-siRNA and Ad-Vpr Inhibits Cell Proliferation, Cell Cycle Progression, and Induces Apoptosis of Glioma Cell U251 In Vitro

Treatment with Ad-GFP did not show any change in cell proliferation (Fig. 1a), while treatment with Ad-bFGF-siRNA or Ad-Vpr alone inhibited cell proliferation compared to the control and Ad-GFP treatment. Combined Ad-bFGF-siRNA and Ad-Vpr treatment showed better effect on inhibition of cell proliferation compared with either Ad-bFGF-siRNA alone or Ad-Vpr treatment alone (Fig. 1a).

Although treatment with only Ad-bFGF-siRNA inhibited U251 cells proliferation in MMT assay and induced slight apoptosis of cells, it did not affect cell cycle progression. While treatment with only Ad-Vpr or both Ad-bFGF-siRNA and Ad-Vpr induced the G2/M cell cycle arrest and apoptosis, cells showed more apoptosis in the combined treatment group than the single Ad-Vpr treatment group (Fig. 1b, c). Combined treatment with Ad-bFGF-siRNA and Ad-Vpr was more effective than single Ad-Vpr treatment. After the glioma U251 cells were infected with Ad-Vpr, an increased number of apoptotic cells were observed compared to the Ad-bFGF-siRNA treatment group. However, a higher apoptotic frequency was observed in cells infected with combined treatment of Ad-bFGF-siRNA and Ad-Vpr gene (Fig. 1c).

These results suggest that Ad-bFGF-siRNA and Ad-Vpr gene co-infected in glioma cells synergistically reduced the proliferation and enhanced the apoptosis, thus a combination of these could be used as novel agents for glioma treatment.

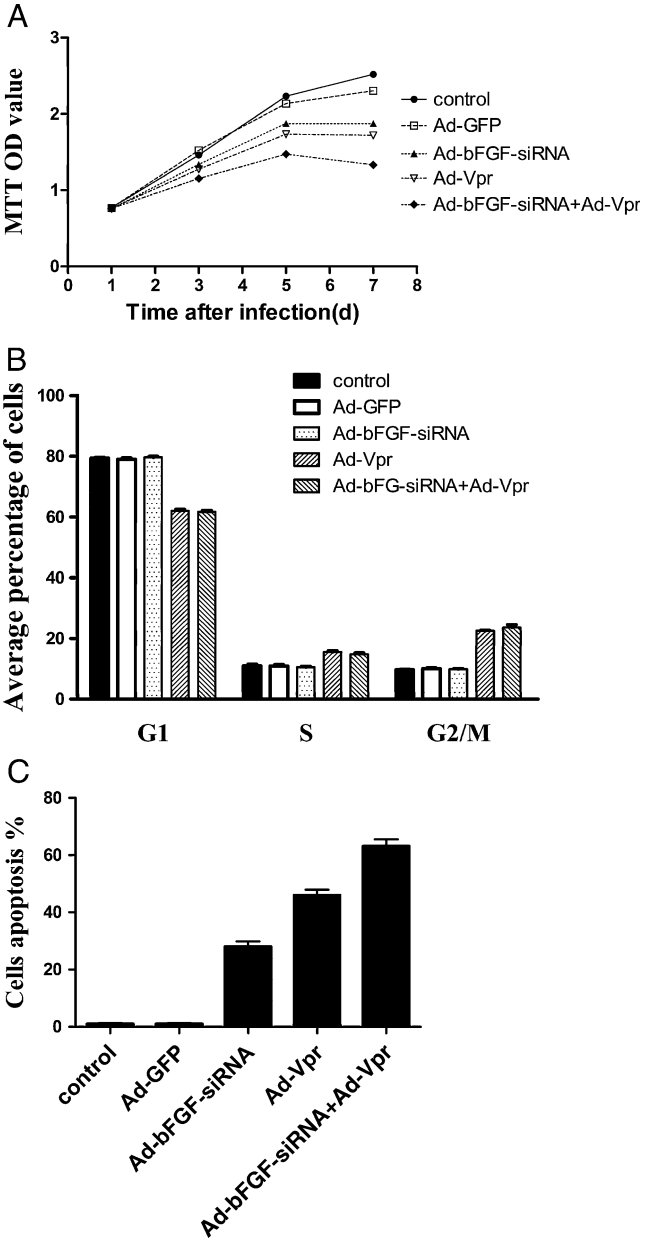

Fig. 1 Effect of combined treatment with Ad-bFGF-siRNA and Ad-Vpr gene on glioma cells proliferation,cell cycle and apoptosis *in vitro*. An equal number of glioma cells were plated and treated at a MOI of 100 with the indicated virus or PBS. The total MOI of the combination of Ad-bFGF-siRNA and Ad-Vpr (1:1) was 100. **a** MTT assay analyzed glioma cells proliferation during seven consecutive days after Ad infection. **b** Average percentage of cells at each cell cycle stage for different treatment at 72 h. **c** Percentage of cell apoptosis in each group at 72 h. The data represent the mean of three independent experiments

Combined Treatment of Ad-bFGF-siRNA and Ad-Vpr Inhibit Xenograft Glioma Growth in Nude Mice

In order to test the potential utility of combined bFGF-siRNA and Vpr gene therapy in the treatment of glioma, pre-established xnograft glioma were treated with only Ad-

bFGF-siRNA, only Ad-Vpr, or a combined treatment of both. The tumor volumes as measured for each group are shown in Fig. 2a. In this study, we applied the endpoint measurement. On day 42, when the mice were sacrificed, the tumor volumes in each group were 1900±245 mm^3(control group), 1816±236 mm^3 (mock group), 1314±229 mm^3(Ad-bFGF-siRNA group), 1243±232 mm^3(Ad-Vpr group), and 1053±220 mm^3 (Ad-bFGF-siRNA plus Ad-Vpr group). The size of xenograft glioma was reduced upon treatment with only Ad-bFGF-siTNA or only Ad-Vpr ($P<0.05$,$P<0.05$, respectively) relative to tumors treated with mock group (Fig. 2a). Importantly, combined treatment with Ad-bFGF-siRNA and Ad-Vpr significantly inhibited xenograft glioma growth compared to the mock group ($P<0.01$), and combined treatment was better than either Ad-bFGF-siRNA or Ad-Vpr treatment alone group at the same total dose of virus (Fig. 2a). In TUNEL analysis, there is scarely any apoptosis in the mock group and a small quantity of a diffused distribution of apoptosis in the only Ad-bFGF-siRNA or Ad-Vpr treatment groups, while there was

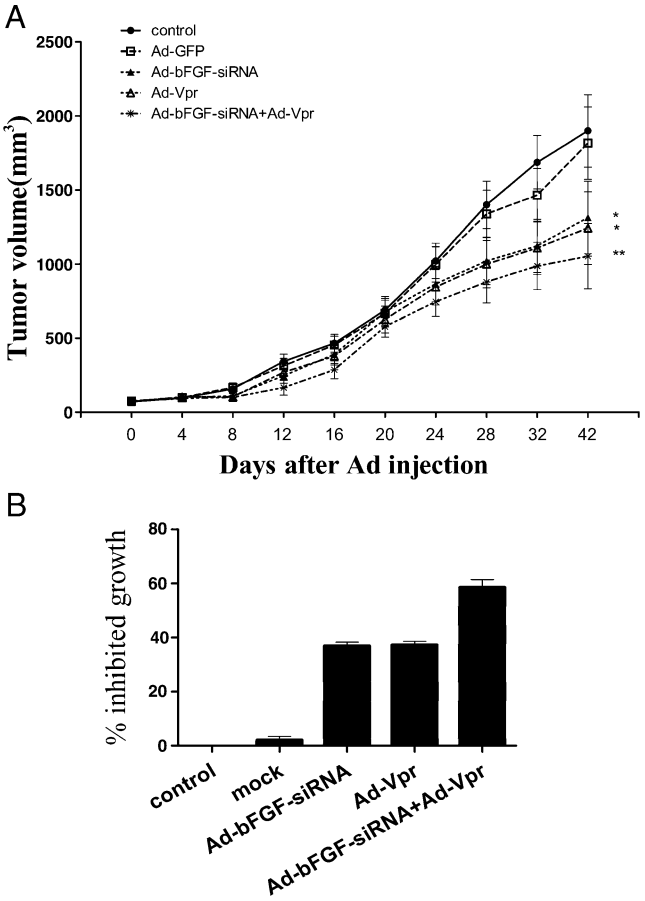

Fig. 2 Enhanced inhibition activity of intratumoral injection with Ad-bFGF-siRNA and Ad-Vpr on xenograft glioma cell growth *in vivo*. **a** Tumor volume measured in each group ($n=6$); * $P<0.05$, ** $P<0.01$ compared with control. **b** The percetange of inhibited growth in each group($n=6$)

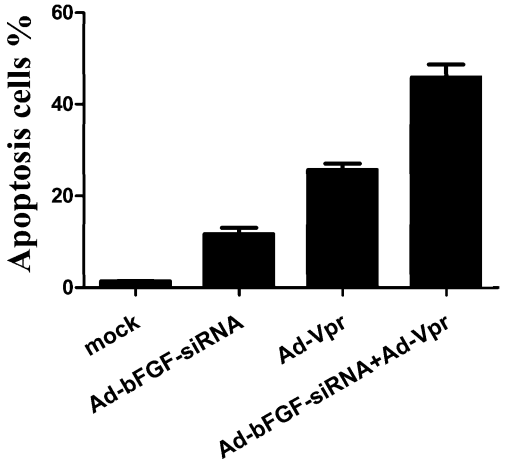

Fig. 3 TUNEL detection of cells apoptosis in xenograft glioma tissue from different treatment groups

singnificantly increased percentage of apoptosis in combined treatment with Ad-bFGF-siRNA and Ad-Vpr (Fig. 3). Accordingly, these results provide further support for the use of the combination bFGF-siRNA and Vpr gene in glioma gene therapy.

Discussions

As with other cancers, malignant gliomas share the common essential characteristics, which are unlimited proliferation and avoiding apoptosis [15, 16]. Several reports indicated a role of bFGF in tumor cell proliferation. The level of bFGF is correlated with glioma grade and clinical outcome [16–18]. Previous studies have shown that inhibition of bFGF production from glioma by antibodies or antisense sequences could be as a strategy to inhibit glioma proliferation and metastasis [19]. More recently, RNA interference has been used as molecular tool to knock-down gene expression and mounting encouraging results were obtained in several different tumor models when bFGF was targeted either by employing siRNA oligonucleotides or by vector mediated RNAi. Our previous research results also showed that the transduction of glioma cells with bFGF-siRNA is able to inhibit proliferation of the glioma cell line U251 [20]. Several investigators using different tumor models have demonstrated that Vpr induces apoptosis in tumor cell lines following G2/M arrest and retards tumor cell growth in vitro and in vivo tumor models in mice [8, 9]. This evidence from various tumors indicated that Vpr has biological activity against tumors both in vivo and in vitro models.

As mention above, both bFGF-siRNA and Vpr have potent antitumor activaties in vitro as well as in vivo. Thus, combined gene therapy with Ad-bFGF-siRNA and Ad-Vpr

could offer therapeutic advances through the additive effect induced by these two antitumor agents. Here, we evaluated whether combined Ad-bFGF-siRNA and Ad-Vpr gene may elicit a enhanced antitumor activity in xenograft glioma mouse model. So far, there is no research report about a successful combined application of bFGF siRNA and Vpr gene to inhibit glioma growth. In this study, we demonstrated that co-transfection with Ad-bFGF-siRNA and Ad-Vpr led to the significant inhibition of glioma cell growth in vitro and in the xenograft model as compared with single agent treatment. In contrast, we observed that there is only slight reduction of xenograft glioma volume in the mice treated by Ad-bFGF-siRNA alone or Ad-Vpr alone, suggesting that the anititumor effect of combined Ad-bFGF-siRNA and Ad-Vpr are better than either Ad-bFGF-siRNA alone or Ad-Vpr alone at the same dose. These results indicated that combined treatment system involving bFGF-siRNA and Vpr gene is a valid therapeutic for glioma. A enhanced effect could be explained by additive antitumor effect of antiproliferation for bFGF-siRNA and apoptosis induced by Vpr.

In the present study, we have first shown the potent antitumor effect of adenovirus mediated gene therapy of combined bFGF-siRNA and Vpr gene for glioma in nude mouse model. In this therapeutic system, we effectively used adenovirus as the delivery vector for bFGF siRNAs and Vpr gene in vitro and in vivo. There was no significant effect on the viability of the cells infected with Ad-GFP as determined by the MMT assay, thereby indicating that the decrease in proliferation were not due to the cytotoxicity of virus. At present, adenovirus is still an attractive vector to deliver gene products for the treatment of tumor because of its high infectivity, which allows for direct vector injection in the clinic, and avoids additional manipulations in vitro as required for other vector systems such as the retrovirus. The most encouraging finding of this study is that most of the mice that received the combined injections of Ad-bFGF-siRNA and Ad-Vpr could inhibit the growth of glioma, indicating that this combined gene therapy system gives a promising approach for glioma treatment.

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References

1. Hutterer M, Knyazev P, Abate A, Reschke M, Maier H, Stefanova N, Knyazeva T, Barbieri V, Reindl M, Muigg A, Kostron H, Stockhammer G, Ullrich A (2008) Axl and growth arrest-specific gene 6 are frequently overexpressed in human gliomas and predict poor prognosis in patients with glioblastoma multiforme. Clin Cancer Res 14(1):130–138
2. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352(10):987–996
3. Auguste P, Gursel DB, Lemiere S, Reimers D, Cuevas P, Carceller F, Di Santo JP, Bikfalvi A (2001) Inhibition of fibroblast growth factor/fibroblast growth factor receptor activity in glioma cells impedes tumor growth by both angiogenesis-dependent and -independent mechanisms. Cancer Res 61(4):1717–1726
4. Ryther RC, Flynt AS, Phillips JA 3rd, Patton JG (2005) siRNA therapeutics: big potential from small RNAs. Gene Ther 12(1):5–11
5. Muthumani K, Hwang DS, Desai BM, Zhang D, Dayes N, Green DR, Weiner DB (2002) HIV-1 Vpr induces apoptosis through caspase 9 in T cells and peripheral blood mononuclear cells. J Biol Chem 277(40):37820–37831
6. Muthumani K, Zhang D, Hwang DS, Kudchodkar S, Dayes NS, Desai BM, Malik AS, Yang JS, Chattergoon MA, Maguire HC Jr, Weiner DB (2002) Adenovirus encoding HIV-1 Vpr activates caspase 9 and induces apoptotic cell death in both p53 positive and negative human tumor cell lines. Oncogene 21(30):4613–4625
7. McCray AN, Ugen KE, Heller R (2007) Enhancement of anti-melanoma activity of a plasmid expressing HIV-1 Vpr delivered through in vivo electroporation. Cancer Biol Ther 6(8):1269–1275
8. McCray AN, Ugen KE, Muthumani K, Kim JJ, Weiner DB, Heller R (2006) Complete regression of established subcutaneous B16 murine melanoma tumors after delivery of an HIV-1 Vpr-expressing plasmid by in vivo electroporation. Mol Ther 14 (5):647–655
9. Muthumani K, Choo AY, Hwang DS, Ugen KE, Weiner DB (2004) HIV-1 Vpr: enhancing sensitivity of tumors to apoptosis. Curr Drug Deliv 1(4):335–344
10. Muthumani K, Lambert VM, Sardesai NY, Kim JJ, Heller R, Weiner DB, Ugen KE (2009) Analysis of the potential for HIV-1 Vpr as an anti-cancer agent. Curr HIV Res 7(2):144–152
11. Muthumani K, Lambert VM, Shanmugam M, Thieu KP, Choo AY, Chung JC, Satishchandran A, Kim JJ, Weiner DB, Ugen KE (2009) Anti-tumor activity mediated by protein and peptide transduction of HIV viral protein R (Vpr). Cancer Biol Ther 8 (2):180–187
12. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100(1):57–70
13. Jordan JP, Hand CM, Markowitz RS, Black P (1992) Test for chemotherapeutic sensitivity of cerebral gliomas: use of colorimetric MTT assay. J Neurooncol 14(1):19–35
14. Chen B, Timiryasova TM, Andres ML, Kajioka EH, Dutta-Roy R, Gridley DS, Fodor I (2000) Evaluation of combined vaccinia virus-mediated antitumor gene therapy with p53, IL-2, and IL-12 in a glioma model. Cancer Gene Ther 7(11):1437–1447
15. Fukui S, Nawashiro H, Otani N, Ooigawa H, Nomura N, Yano A, Miyazawa T, Ohnuki A, Tsuzuki N, Katoh H, Ishihara S, Shima K (2003) Nuclear accumulation of basic fibroblast growth factor in human astrocytic tumors. Cancer 97(12):3061–3067
16. Baguma-Nibasheka M, Li AW, Murphy PR (2007) The fibroblast growth factor-2 antisense gene inhibits nuclear accumulation of FGF-2 and delays cell cycle progression in C6 glioma cells. Mol Cell Endocrinol 267(1–2):127–136
17. Aoki T, Kato S, Fox JC, Okamoto K, Sakata K, Morimatsu M, Shigemori M (2002) Inhibition of autocrine fibroblast growth factor signaling by the adenovirus-mediated expression of an antisense transgene or a dominant negative receptor in human glioma cells in vitro. Int J Oncol 21(3):629–636

18. Takahashi JA, Fukumoto M, Kozai Y, Ito N, Oda Y, Kikuchi H, Hatanaka M (1991) Inhibition of cell growth and tumorigenesis of human glioblastoma cells by a neutralizing antibody against human basic fibroblast growth factor. FEBS Lett 288(1–2):65–71
19. Marek L, Ware KE, Fritzsche A, Hercule P, Helton WR, Smith JE, McDermott LA, Coldren CD, Nemenoff RA, Merrick DT, Helfrich BA, Bunn PA Jr, Heasley LE (2009) Fibroblast growth factor (FGF) and FGF receptor-mediated autocrine signaling in non-small-cell lung cancer cells. Mol Pharmacol 75(1):196–207
20. Wang SJ, Wang JH, Zhang YW, Xu XN, Liu HS (2008) Effects of small interfering RNA targeting basic fibroblast growth factor on proliferation and apoptosis of glioma cell line U251. Ai Zheng 27 (9):905–909