Gene Therapy for High-Grade Glioma

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Abstract

High-grade glioma is the most frequently occurring primary brain tumor and is associated with a poor prognosis. Current treatment regimens have had only a modest effect on the progressive course despite recent advances in surgery, radiotherapy, and chemotherapy. Gene therapy for brain tumors represents a novel and promising therapeutic approach and has been investigated clinically for the last two decades. The strategies of gene therapy include suicide gene therapy, immune gene therapy, oncolytic viral therapy, tumor suppressor gene therapy, and antisense therapy. Here, we review gene therapy approaches considering the clinical results, limitations, and future directions.

Key words: glioma, gene therapy, clinical trial

Introduction

Worldwide, glioma is the most common and deadly type of primary brain tumor.³²⁾ Current treatment regimens, including surgery followed by radiotherapy and chemotherapy, have slightly improved the clinical outcomes of glioma patients.⁶⁷⁾ However, these improvements are inadequate, and long-term control of the disease is rarely achieved. To combat this formidable neoplasm, advances in understanding of the function and control of genes and their expressions have paved the way for the development of gene therapy in the last two decades. Glioma is a good target because no effective therapy is available and, unlike other solid tumors, rarely metastasizes outside the central nervous system (CNS). Research in gene therapy has been moving forward to translational approaches, and the clinical feasibility has been investigated since 1992.¹²⁾ The latest information regarding worldwide gene therapy clinical trials is available on the website at www.wiley.co.uk/genetherapy/clinical/. Currently, the total number of the trials exceeds 1500, with more than 60% of them focusing on cancer gene therapy. Gene therapy clinical trials targeted at brain tumors include over 30 protocols and more than 400 enrolled patients worldwide (Table 1).

Vectors in Gene Therapy

Safe, efficient, and specific delivery of genes to the target cell is an important requirement in gene therapy. Various viral and non-viral vectors have been engineered and used for gene transfer, both ex-

perimentally and clinically. Viruses have been used as vehicles to carry exogenous genes into human cells as they bind to their hosts and introduce their genetic material into the host cell as part of their replication cycle. Viruses used for glioma gene therapy can be grouped into 2 categories: replication-incompetent viruses and replication-competent viruses. In the former, the vector is derived from a virus from which all or most of its genome has been removed, so minimizing the toxicity and retaining the gene delivery efficiency. In the latter, only select viral genes are deleted or mutated so that viruses can replicate in and lyse tumor cells selectively (i.e., oncolytic viral therapy). Non-viral methods present certain advantages over viral methods, such as simple large-scale production and low host immunogenicity, but low levels of transfection and expression of the gene are a disadvantage of non-viral methods. Clinical trials for anti-glioma gene therapy mainly use adenoviruses (AdVs), retroviruses (RVs), and non-viral vectors for gene delivery (Figs. 1 and 2).

I. AdV

AdV is a non-enveloped double-stranded deoxyribonucleic acid (DNA) virus. Most serotypes use the Coxsackie and adenovirus receptor for main attachment and α_v integrins for internalization, resulting in broad tissue tropism.^{4,81)} The AdV genome is not dependent on host DNA polymerases, so AdV can infect both dividing and non-dividing cells, with the potential for application to neurological diseases. AdV can be manipulated relatively easily and can be produced at high titers, and its safety record in human populations has been excellent.⁷⁶⁾ The

Table 1 Clinical gene therapy trials for glioma*

Phase	Gene(s)	Vector	Institution	Country
I	antisense IGF-1	lipofection	Case Western Reserve University	U.S.A.
I	CMV pp65-LAMP	RNA transfer	Duke University	U.S.A.
I	CMV pp65-LAMP	RNA transfer	Duke University	U.S.A.
I	GM-CSF	naked/plasmid DNA	Massachusetts General Hospital	U.S.A.
III	HSV-TK	retrovirus	University of Florida	U.S.A.
III	HSV-TK	retrovirus	Martin-Luther Universitat	Germany
II	HSV-TK	retrovirus	University of Florida	U.S.A.
I/II	HSV-TK	adenovirus	University of Kuopio	Finland
I/II	HSV-TK	lipofection	University of Koln	Germany
I/II	HSV-TK	retrovirus	Oncology Clinical Research Novartis Pharma	Switzerland
I/II	HSV-TK	retrovirus	University Canton Hospital	Switzerland
I/II	HSV-TK	retrovirus	Universidad Autonoma de Madrid	Spain
I/II	HSV-TK	retrovirus	CERVI, Hospital Pitie-Salpetriere	France
I/II	HSV-TK	retrovirus	Academic Hospital Groningen	The Netherlands
I	HSV-TK	adenovirus	Mt. Sinai Medical Center	U.S.A.
I	HSV-TK	adenovirus	The Ohio State University	U.S.A.
I	HSV-TK	adenovirus	Dana-Farber Cancer Institute	U.S.A.
I	HSV-TK	retrovirus	Beatson Oncology Centre	U.K.
I	HSV-TK	retrovirus	University of Iowa Hospital	U.S.A.
I	HSV-TK	retrovirus	Heinrich-Heine-University	Germany
I	HSV-TK	retrovirus	Massachusetts General Hospital	U.S.A.
I	IFN- β	adenovirus	University of Pennsylvania	U.S.A.
I	IFN- β	lipofection	Nagoya University	Japan
I/II	IL-12	Semliki forest virus		U.K.
I	IL-13 α 2-specific scFvFc- ζ TCR	naked/plasmid DNA	City of Hope National Medical Center	U.S.A.
I	IL-2	retrovirus	Sidney Kimmel Cancer Center	U.S.A.
I	IL-2		Academic Hospital Groningen	The Netherlands
I	IL-4	retrovirus	University of Pittsburgh	U.S.A.
I	MGMT	lentivirus	Case Western Reserve University	U.S.A.
I	MGMT	retrovirus	University of Cincinnati College of Medicine	U.S.A.
I	MGMT	retrovirus	Fred Hutchinson Cancer Research Center, University of Washington	U.S.A.
III	no transgene	herpes simplex virus	Queen Elizabeth Hospital	U.K.
II	no transgene	herpes simplex virus	Beatson Oncology Centre	U.K.
I/II	no transgene	adenovirus	The University of Texas	U.S.A.
I/II	no transgene	herpes simplex virus	University of Alabama	U.S.A.
I	no transgene	herpes simplex virus	Beatson Oncology Centre	U.K.
I	no transgene	herpes simplex virus	University of Alabama	U.S.A.
I	no transgene	herpes simplex virus	Massachusetts General Hospital	U.S.A.
I	no transgene	poliovirus	Duke University	U.S.A.
I	p53	adenovirus	University of Texas	U.S.A.
III	TGF- β 2	naked/plasmid DNA		U.S.A.
I	TGF- β 2	naked/plasmid DNA	Maxine Dunitz Neurosurgical Institute	U.S.A.
II	tyrosinase	adenovirus	Beijing YouAn Hospital	China

*Source, Gene Therapy Clinical Trials Worldwide provided by the *Journal of Gene Medicine* [Internet]. Bogner Regis, West Sussex, U.K., John Wiley & Sons, Ltd., ©2007 [updated December 2009]. Available from: www.wiley.co.uk/genetherapy/clinical/. CMV pp65-LAMP: cytomegalovirus pp65-lysosomal-associated membrane protein, DNA: deoxyribonucleic acid, GM-CSF: granulocyte-macrophage colony stimulating factor, HSV-TK: herpes simplex virus-thymidine kinase, IFN: interferon, IGF: insulin-like growth factor, IL: interleukin, MGMT: O⁶-methylguanine deoxyribonucleic acid methyltransferase, RNA: ribonucleic acid, TGF: transforming growth factor.

AdV genome persists in the cell nucleus predominantly as an extrachromosomal episome and is absent in the progeny of transduced cells, resulting in transient gene expression and the need for repeated dosage. However, the viral capsid and viral DNA cargo are thought to provoke an immune response, which limits its clinical usefulness.²⁴⁾ For example, in 1999, Jesse Gelsinger who suffered from

ornithine transcarbamylase deficiency, an X-linked genetic disease of the liver, was injected with adenoviruses carrying a corrected gene to test the safety of the procedure. He died 4 days later, apparently having suffered a massive immune response triggered by the use of AdV. Since then, work using AdV vectors has focused on more genetically attenuated versions of the virus.

Fig. 1 Vectors used in clinical trials for anti-glioma gene therapy. Retrovirus and adenovirus as viral vectors and liposomes as a non-viral vector are mainly used for gene delivery.

II. RV

RV is an enveloped single-stranded ribonucleic acid (RNA) virus. Several characteristics of the RV vector are attractive for gene therapy. RV can be manipulated relatively easily with a packaging or producer cell in which the viral gag, pol, and env proteins are expressed in *trans* from separate helper constructs. RV can accomplish efficient gene transfer in various target cell types, with the capacity to integrate the gene into the host genome along with long-term expression.^{30,85} However, RV vectors can transfect only dividing cells, because of the requirement of host DNA polymerase for completing the reverse transcription stage.^{58,69} Severe combined immune deficiency patients who received gene therapy with γ -RV vectors developed leukemia.⁹ Integrating new DNA into a chromosome carries an inherent risk of insertional oncogenesis. A small transgene capacity is also another drawback of RV.^{13,73}

III. Non-viral vectors

The non-viral technique of gene transfer is a simple and safer alternative to viral vectors. Non-viral vectors are attractive tools in gene therapy because of their low toxicity and immunogenicity and relatively simple techniques of mass production. Cationic lipid-based gene transfer was first reported in 1987.¹⁴ Cationic lipids naturally create complexes with negatively charged DNA, and their positive charge allows interactions with the negatively charged cell membranes and subsequent penetration into the cell. A series of cationic lipids have been synthesized, and cationic liposome-mediated gene transfer or lipofection represents the most commonly used non-viral gene delivery method. The

specificity of transfection is determined by the structure of cationic liposomes, especially by the hydrophobic anchor, so tumor-specific transfection has been studied by adding tumor-specific ligands. Non-viral vectors are inexpensive to produce and can be used in common drug delivery systems in safety, but the transfection efficiency must be further improved.

Gene Therapy Strategies for Glioma

Therapeutic strategies of clinical trials can be grouped into the following general categories: i) suicide gene therapy, ii) immune gene therapy, iii) oncolytic viral therapy, iv) tumor suppressor gene therapy, and v) antisense therapy.

I. Suicide gene therapy

Suicide gene therapy is the most commonly used technique in clinical trials for glioma (Table 1). Here, a “prodrug” is transferred into an activated metabolite by the enzyme expressed by the introduced gene, leading to tumor cell death.^{44,70} Induction of the “bystander effect” is one of the advantages of suicide gene therapy. The most extensively investigated suicide gene is the herpes simplex virus-1 thymidine kinase (HSV-tk) gene, which renders transduced cells sensitive to ganciclovir (GCV), acyclovir, and valaciclovir (Fig. 2). GCV is metabolized poorly by human-tk, and is thus usually non-toxic. HSV-tk monophosphorylates the nucleoside analog GCV, which is rapidly metabolized by endogenous kinases to triphosphate GCV. The triphosphate form acts as a potent inhibitor of DNA polymerase and competes with normal mammalian nucleosides for DNA replication.^{38,70} HSV-tk/GCV systems have demonstrated promising results in animal models.^{12,53} The bystander effect and enhanced sensitivity to radiation are the advantages of this technique.^{3,15,29,47,54} In a phase III clinical trial, HSV-tk gene therapy was tested using RV vectors as an adjuvant to surgical resection and radiation in 248 patients with newly diagnosed glioblastoma (GBM), who were divided into a control arm ($n = 124$) and a gene therapy arm ($n = 124$) in which RV vector-producing cells were administered into the margin of the resulting defect during craniotomy. After 4 years of follow up, survival analysis showed no difference in tumor progression and overall survival between the standard and gene therapy arms.⁵² The poor response was thought to be because of the low transduction efficiency of the RV vectors. Further studies are warranted regarding better techniques for the delivery and expression of therapeutic genes. Cytosine deaminase/5-fluorocytosine and

Fig. 2 Gene therapy strategies. For gene transfer, various viral and non-viral vectors have been engineered and have been used both experimentally and clinically. Adenovirus is a non-enveloped double-stranded deoxyribonucleic acid (DNA) virus. Most serotypes use the Coxsackie and adenovirus receptor for main attachment and α_v integrins for internalization, resulting in broad tissue tropism. The adenovirus genome is not dependent on host DNA polymerases, so adenovirus can infect both dividing and non-dividing cells, providing potential application for neurological diseases. Retrovirus is an enveloped single-stranded ribonucleic acid virus. Several characteristics of the retrovirus vector are attractive for gene therapy. Retrovirus can be manipulated relatively easily with a packaging or producer cell in which the viral gag, pol, and env proteins are expressed *in trans* from separate helper constructs. Retrovirus can accomplish efficient gene transfer in various target cell types, with the capacity to integrate the gene into the host genome along with long-term expression. Cationic liposome-mediated gene transfer or lipofection represents the most commonly used non-viral gene delivery method. Therapeutic strategies of clinical trials can be grouped mainly into the following categories: suicide gene therapy, immune gene therapy, and oncolytic viral therapy. The most extensively investigated suicide gene is the herpes simplex virus-1 thymidine kinase (HSV-tk) gene, which renders transduced cells sensitive to ganciclovir (GCV), acyclovir, and valaciclovir. GCV is metabolized poorly by human-tk, and so is usually non-toxic. HSV-tk monophosphorylates the nucleoside analog GCV, which is rapidly metabolized by endogenous kinases to triphosphate GCV. The triphosphate form acts as a potent inhibitor of DNA polymerase and competes with normal mammalian nucleosides for DNA replication. Induction of the “bystander effect” is one of the advantages of suicide gene therapy. The rationale for immune gene therapy is intratumoral secretion *in situ* by transfecting tumor cells with genes of cytokines leading to activation of immune cells such as cytotoxic T lymphocytes (CTL), dendritic cells (DC), natural killer (NK) cells, and macrophages (M ϕ) by subsequently release of interleukin (IL)-8, tumor necrosis factor (TNF)- α , and granulocyte-macrophage colony-stimulating factor (GM-CSF). Oncolytic viral therapy utilizes replication-competent viruses which ideally can infect and replicate in tumor cells. The viruses specifically lyse tumor cells and sequentially infect neighboring cells.

cytochrome P450 2B1/cyclophosphamide have been used as other materials in this approach, and are currently being investigated in preclinical studies.

II. Immune gene therapy

The aim of gene therapy strategies using immune modulation is to enhance the T-cell mediated immune response against tumors. Gliomas have evolved subtle mechanisms to evade the immune system, which pose the major problems for immune-based therapies. However, experimental studies

have recently demonstrated the susceptibility of brain tumor stem cell-like populations to the cytotoxic effect of immune cells, which has laid the foundation for developing immunotherapeutic approaches.^{6,8)} Such strategies include cytokine gene therapy, vaccination therapy, and genetically modified T-cell therapy.

Cytokine gene therapy: The rationale for cytokine gene therapy is intratumoral secretion *in situ* by transfecting tumor cells with genes of cytokines such as interleukin (IL)-2, IL-4, IL-12, in-

terferon (IFN)- γ , and IFN- β .^{5,17-19,63,86} The advantage of in situ secretion over systemic administration includes lower toxicity, high local concentrations, longer cytokine persistence, and secretion by the tumor cell itself (Fig. 2). Severe CNS toxicity has been documented in animal models when IL-12 or IFN- γ was secreted intracranially by tumor cells.⁷¹ However, gene transfer of these immunomodulating cytokines has revealed promising results, including complete tumor regression, in several preclinical studies.^{51,59,79,86} Several early phase trials are currently underway for this approach. IFN- β is a potential cytokine with multiple antitumor effects, including direct antiproliferative effects and indirect antitumor effects such as immunomodulation.^{64,65} Our group at Nagoya University, Japan, started using cytokine gene therapy in 2000. The IFN- β gene was delivered via cationic liposomes based on the following preclinical and experimental studies. In vitro experiments demonstrated that the cationic liposome-mediated human IFN- β gene transferred to cultured human glioma cells induced a cytotoxic but not a cytostatic response, even in IFN-resistant human glioma cell lines, probably by inducing apoptosis.⁵ In vivo experiments using nude mice implanted with human glioma cells intracranially or subcutaneously revealed that the local administration of cationic liposomes containing the human IFN- β gene induced apparent tumor growth reduction, natural killer (NK) cell activation, and prolonged survival.^{43,83} In addition, a similar growth-inhibitory effect was also observed in a syngeneic intracranial mouse glioma model treated with the liposome-mediated murine IFN- β gene. This gene therapy system induced specific cytotoxic T-cell immunity against mouse glioma and NK cells.^{45,46} Based on these observations, a phase I clinical trial of IFN- β gene therapy was performed in 5 patients with recurrent malignant glioma.⁸⁴ This was a two-stage trial in which the initial treatment comprised tumor removal and injection of liposomes containing the human IFN- β gene into the margin of the resulting defect, and subsequent delivery of subsequent injections via an implanted catheter. The clinical toxicity was found to be minimal. At 10 weeks after treatment initiation, 2 patients showed more than 50% reduction in tumor size while others had stable disease. The median survival was longer in the treated subjects than in the matched historical controls from our institution. After the gene therapy, significant changes were observed in the histology and gene expression related to the immunoresponse, apoptosis, and neovascularization.⁷⁸ This study provides the foundation for a phase II trial of IFN- β gene therapy. Very recently, a phase I clinical trial (a dose-escalat-

ing cohort) of stereotactic injection of an IFN- β -expressing AdV vector was reported in 11 patients with malignant glioma. Direct injection of the vector into the tumor and the surrounding normal brain areas after surgical tumor removal was feasible. A reproducible increase in tumor cell apoptosis was observed after the treatment.¹¹

Vaccination therapy: A number of experimental and preclinical studies have reported strong immune responses to the tumor with the vaccination approach, including gene-modified tumor cell vaccines and RNA-loaded dendritic cell (DC) vaccines. The efficacy of autologous glioma cell vaccines secreting IL-4 has been reported.⁴⁸ DCs are the most potent antigen-presenting cells in the immune system and have been previously used as vaccine vehicles. Tumor RNA-pulsed DC vaccines can induce tumor-specific immune response.⁷ There is no consensus regarding the condition of gene-modified vaccines, but supportive evidence has been accumulating.^{42,66,75}

Genetically modified T-cell therapy: Induction of tumor-specific effector T-cells is an efficient method for the eradication of bulky solid tumors, and is the final goal of all immune-gene approaches. Tumor-specific cytotoxic T-cells can be genetically engineered to express altered or totally artificial T-cell antigen receptors (TCR). The efficacy of this approach has been reported, and a clinical trial is ongoing.²⁸ Indeed, there is room for improvement in this technique, including the choice of target antigen, minimization of mispairing between transgenic and endogenous TCR chains, and elevation of the expression rate, but this strategy shows promise for improving the management of glioma.

III. Oncolytic viral therapy

Oncolytic viral therapy uses replication-competent viruses infect and lyse the target cells. Oncolytic HSV, AdV, and poliovirus (PV) have been extensively investigated and are now employed for this strategy. Some researchers have modified the oncolytic viruses with a suicide gene or tumor suppressor gene to improve the oncolytic activity.

Oncolytic HSV: HSV is an enveloped double-stranded DNA virus with natural neurotropism and the ability to replicate in dividing and nondividing cells with high efficacy and low viral ratio. In the host cell, wild-type HSV-1 may proceed to a lytic life cycle or persist as an intranuclear episome, but never integrates into the host genome. The other advantage of HSV-1 is the sensitivity to acyclovir and GCV, adding to its safety profile in human use. The major drawbacks of HSV are a high rate of pre-existing HSV immunity in the host and difficulties in

genetic manipulation due to its large genome size. The first-generation oncolytic HSV vectors are already undergoing clinical investigations, with either one or both of the attenuating mutations in the neurovirulence gene γ_1 34.5 and gene U_L 39 that encodes ribonucleotide reductase. Further, G207 is a conditionally replicative HSV, with deletion of both copies of its γ_1 34.5 gene in addition to *lacZ* insertion into the U_L 39 locus.⁴¹⁾ Preclinical studies have demonstrated that G207 decreased the growth of experimental gliomas.^{26,68)} Based on these results, three phase I trials using G207 in patients with malignant glioma were conducted and have been completed with promising results.^{1,36,37)} Another HSV mutant 1716 also lacks both copies of the γ_1 34.5 gene, but with its U_L 39 gene intact. In the initial HSV 1716 phase I trial for recurrent glioma, no dose-limiting toxicities (DLTs) were observed in the 9 enrolled patients.⁵⁵⁾ In the second phase I trial, its safety was proven again and viral replication was demonstrated in tumor.⁴⁹⁾ Both G207 and 1716 are currently under further clinical investigation. According to the website of Crusade Laboratories, the HSV 1716 technology is undergoing Europe-wide phase III clinical trials in patients with GBM.

Oncolytic AdV: Oncolytic AdVs are engineered or naturally occurring strains. ONYX-015, a conditionally replicative AdV (CRAdV), was developed by deletion in its viral protein E1B-55K, which is responsible for binding and inactivating cellular p53. Although this mutant AV was believed to replicate in and lyse p53-deficient tumor cells only, other functions of E1B-55K are responsible for its tumor selectivity. Preclinical studies with this virus have demonstrated cell lysis and impaired tumor growth in human glioma xenografts, which were artificially enhanced by radiation therapy.^{20,21)} In the initial phase I study for recurrent glioma, no DLTs were observed, even at the highest dose, but the efficacy was not determined.¹⁰⁾ More recently, other modified CRAdVs have been also undergoing investigations. Such CRAdVs are designed to replicate with dependence on glial fibrillary acidic protein or hypoxic microenvironment.^{25,50)}

PV: PV is a non-enveloped RNA virus with natural neurotropism. PV recombinants are naturally tropic for malignant gliomas due to ectopical up-regulation of the PV receptor CD155 on those tumor cells.^{23,39,62)} The neuropathogenicity can be attenuated by mutations within the internal ribosome entry site (IRES) element in the 5' untranslated region of its genome.²²⁾ Exchanging the IRES element of PV with that of human rhinovirus type 2 severely attenuates propagation in normal neuronal cells while retaining excellent lytic growth in malignant glioma

cells. This mutant, named PV-RIPO, showed significant biologic effects in preclinical studies.^{23,39)} It is expected that PV-RIPO will soon be applied in a clinical setting.

IV. Tumor suppressor gene therapy

The rationale for this approach is to use a gene to encode a tumor-suppressor protein in glioma cells that is mutated or absent. *TP53* is a commonly studied suppressor gene, whose mutations have been reported in 30–60% of malignant gliomas.^{35,77)} Cellular and animal studies have demonstrated that the replacement of wild-type p53 in tumor cells induced rapid cell death even in cells with the intact functional gene.^{34,57)} The strategy of restoring normal p53 expression in glioma cells has been evaluated in early-phase clinical trials. In 2003, a group of researchers at the MD Anderson Medical Center administered AdV vectors carrying wild-type *TP53* to 15 recurrent glioma patients by direct intratumoral injection followed by tumor resection as a phase I clinical trial. Minimal toxicity occurred, but no inspiring efficacy was reported. The relatively low transfection efficacy of AdV vectors, coupled with the lack of strong bystander effects, is considered to limit the potential applications for treating glioma patients unless more efficient vectors are developed.

V. Antisense therapy

RNA interference is a highly conserved mechanism found in both plant and animal cells that demonstrates a form of transcriptional inhibition.¹⁶⁾ Antisense oligonucleotides, antigen oligonucleotides, and short interfering RNAs (siRNA) are used for RNA interference. Antisense oligonucleotides bind to a specific RNA sequence and inhibit gene expression at the translational level. There have been several clinical trials using antisense oligonucleotides specific for insulin-like growth factor-1 (IGF-1) or transforming growth factor β 2 (TGF- β 2). Activation of the IGF signaling pathway is involved in tumor growth and proliferation of gliomas.^{2,56)} A phase I clinical trial is underway at the Case Western Reserve University using antisense IGF-1. The TGF- β 2 isoform is specifically overexpressed in malignant gliomas, suppressing the immune system.^{80,82)} Phase I/II clinical trials to evaluate the efficacy of antisense TGF- β 2 in recurrent or refractory high-grade glioma patients have been completed, and a phase III study has already begun.^{60,74)} No clinical trials involving siRNAs are reported, but siRNAs are expected to have great potential in glioma therapy because siRNAs can inhibit a specific target safely and with relative ease. For example, the inhibition of methyl-guanine methyl-transferase with siRNA is

one of the potential approaches for overcoming the resistance to temozolomide in gliomas.

Future Directions

Each of the described strategies has its own distinct advantages and limitations inherent to the employed technology. In general, these strategies have shown safety in clinical use and encouraging results in glioma models, but have not yet demonstrated significant benefits in phase II or III clinical trials. The problems of gene delivery and efficient gene transfer remain the most significant hurdles in anti-glioma gene therapy. One of the innovative approaches is to use neural stem cells (NSCs) as gene delivery systems. NSCs possess inherent tumor tropism, and so can be used as a delivery vehicle for therapeutic genes to invasive glioma cells.³¹⁾ Anti-angiogenic gene therapy is also a promising approach, because neovascularization is one of the biggest biological features of malignant gliomas. Anti-angiogenic approaches have already received worldwide attention, with some genetic strategies demonstrating efficacious inhibition of tumor growth.^{27,40)} In addition to the improvements in each strategy, a rational combination of therapeutic strategies with different modes of action has the potential to deliver synergistic benefits. The primary direction of gene therapy has been shifting toward immune gene therapy, but this approach needs to overcome the challenges posed by immunoinhibitory networks created by cytokines and regulatory T-cells. Recently, the development of therapies specifically targeting brain tumor stem cells (BTSCs), which are resistant to the cytotoxic effects of chemotherapy and radiotherapy, has been described.^{33,61,72,87)} Gene therapy strategies may also have the potential capacity to target BTSCs. At present, anti-glioma gene therapy is not a standard therapeutic strategy, and so is not available outside clinical trials. However, we believe that translational researches from bench to bedside will establish this therapeutic tool in anti-glioma therapy. In the near future, malignant gliomas will probably be treated through the synergistic effects of a multipronged attack.

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