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Combined *HSV-TK*/*IL-2* gene therapy in patients with recurrent glioblastoma multiforme: biological and clinical results

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Following our pilot clinical study of combined *IL-2/HSV-TK* gene therapy for recurrent glioblastoma multiforme (GBM), we extended the protocol to a larger population of patients and evaluated safety, feasibility, and biological activity of treatment. A total of 12 patients received intratumor injection of retroviral vector-producing cells (RVPCs), followed by intravenous ganciclovir (GCV). Treatment was well tolerated with only minor adverse events. Transduction of tumor cells was demonstrated in tumor biopsies. A marked and persistent increase of intratumor and plasma Th1 cytokine levels was demonstrated after RVPC injection. At magnetic resonance imaging evaluation, two patients had a partial response (including a patient showing disappearance of a distant noninjected tumor mass), four had a minor response, four had stable disease, and two had progressive disease. The 6- and 12-month progression-free survival rates were 47 and 14%, respectively. The 6- and 12-month overall survival rates were 58 and 25%, respectively. In conclusion, the results of our clinical protocol of gene therapy for recurrent GBM, based on combined delivery of a suicide and a cytokine gene, demonstrate that intratumor injection of RVPCs was safe, provided effective transduction of the therapeutic genes to target tumor cells, and activated a systemic cytokine cascade, with tumor responses in 50% of cases.
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Although primary malignant brain tumors account for only 2% of all adult cancers, these neoplasms still represent a heavy social burden in terms of cancer-related disability and death. In patients with glioblastoma multiforme (GBM), the most common adult glioma, prognosis is dismal, with a median survival of about 1 year. Initial treatment consists of surgery followed by radiotherapy and, in selected cases, adjuvant chemotherapy. Notwithstanding therapeutic efforts, all malignant gliomas recur and, at recurrence, the median survival is 2–3 months.[1,2]

Gene therapy represents a promising new therapeutic option for recurrent malignant gliomas. Several clinical trials for recurrent gliomas are currently ongoing and results from the first clinical studies are now available.[3] In most cases, treatment was based on the intratumor injection of retroviral vectors or retroviral vector-producing cells (RVPCs) carrying the "suicide" gene thymidine kinase of herpes simplex virus type 1 (*HSV-TK*). Results from phase I/II studies of retroviral vector-mediated *HSV-TK* gene therapy in a total of 113 patients with recurrent GBM showed complete or partial responses in 8% of cases, and minor responses or stable disease in 6%.[3] Lack of efficacy of this approach was confirmed by a phase III clinical trial, which showed no significant benefit of gene therapy over radiotherapy in newly diagnosed patients with GBM.[4] However, a positive remark from clinical trials is the demonstration of feasibility of gene therapy for brain tumors without significant toxicity.[3]

The antitumor effect of *HSV-TK* gene transfer is mediated by ganciclovir (GCV)-induced toxicity to transduced tumor cells and to nontransduced neighbor cells (bystander effect),[5] and, at least in part, by stimulation of immune response involving tumor-infiltrating T cells and natural killer (NK) cells.[6] The immunological component of HSV-TK/GCV gene therapy was demonstrated also in GBM patients treated by direct intratumor injection of RVPCs and subsequent systemic GCV administration.[7,8] Serum samples from treated patients showed increased FasL and IL-12,[8] consistent with activation of a Th-1 response, and an immune response against retroviral vector proteins and RVPCs.[9,10] Moreover, peripheral blood mononuclear cells (PBMCs) cocultivated with autologous tumor cells secreted interferon γ (IFNγ), but immunophenotyping of PBMCs did not show a significant activation of T cells

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or NK cells during gene therapy.[8] Our gene therapy strategy for cancer, based on combined delivery of a cytokine gene (human interleukin-2, *IL-2*) together with *HSV-TK*, aims at amplifying this antitumor immune response.[11–13] In preclinical experimental models, it demonstrated not only efficient killing of transduced cancer cells, but also growth inhibition of distant nontransduced tumor masses.[13] Moreover, our clinical experience with *IL-2*/*HSV-TK* gene therapy in a pilot study in four patients with recurrent GBM showed the presence of intense cellular infiltration in tumor biopsies obtained after injection of vector-producing cells, represented mainly by T helper/inducer lymphocytes and activated cytotoxic T cells and macrophages.[14] Treatment was safe and devoid of toxicity. Tumor necrosis around injection site and/or significant tumor regression was documented in all cases.[14] Following this preliminary experience, we evaluated this therapeutic modality in a larger group of patients with recurrent GBM. We report here the results of our clinical protocol of retroviral vector-mediated *IL-2*/*HSV-TK* gene therapy for recurrent GBM. The primary end point of the study was to determine the safety of treatment; secondary end points were to assess antitumor efficacy and systemic response to gene therapy.

Patients and methods

Clinical protocol and study design

Experimental and clinical procedures of this protocol of gene therapy for recurrent GBM[14] were based on those already approved by RAC/FDA.[15] Primary end points of the study were assessment of safety of intratumor combined delivery of the *HSV-TK* gene and human *IL-2* gene by RVPCs and demonstration of *in vivo* transduction of target tumor cells; secondary end points were clinical, neurological, and radiological evaluation of treatment efficacy. The clinical protocol was approved by the Ethical Committees of the University of Padova and of Vicenza City Hospital. All patients gave informed consent in accordance with the Vicenza Hospital Institutional Review Board using Declaration of Helsinki guidelines.

Eligibility criteria

Patients ≥ 18 and < 75 years of age, with a Karnofsky Performance Score (KPS) ≥ 70, histological evidence of GBM, with clinical and/or radiological evidence of tumor progression after surgical treatment, radiotherapy, and conventional chemotherapy, were enrolled in this study. Exclusion criteria were severe impairment of renal (serum creatinine > 1.5 mg/dl or creatinine clearance < 80 ml/min/m^2), liver (ALT and alkaline phosphotransferase levels $>$ two times normal ranges), and bone marrow function (granulocyte count < 1000/mm^3 and platelet count $< 100{,}000$/mm^3). Moreover, patients were excluded if they received radiotherapy during the 4 weeks before study entry, had an active uncontrolled bacterial, viral, or fungal infection, positive HIV test, severe and worsening neurological impairment, pregnancy, severe systemic diseases, KPS < 70, or if they were not able to understand the investigative nature of the gene therapy study and give their informed consent.

Treatment procedures

All patients with recurrent GBM were treated by intratumor injection of LIL-2-TK RVPCs.

Two different types of surgical procedures were adopted, according to the characteristics of the tumor recurrence.

(1) A stereotactic technique[16] was used in patients harboring tumor recurrences unsuitable to surgical removal (patients no. 1, 2, 3, 5, 7, 8, 11). After head frame fixation and complete neuroradiological work-up, 2–6 injection sites were defined and their coordinates calculated. By standard procedures, in local anesthesia, through a 5 mm burr hole, a 2 mm bore needle was advanced in sequence to the predetermined tumor targets, where multiple stereotactic injections were performed. The total injected volume was 2–6 ml, containing the highest tolerable number of RVPCs (about 3×10^8–1×10^9 RVPCs).
(2) An open surgical technique was adopted for patients harboring tumor recurrences deemed suitable to microsurgical removal (patients no. 4, 6, 9, 12). In general anesthesia, patients underwent craniotomy with bulk resection of the tumor recurrence. In these cases, RVPCs were injected, under direct visual control, into the tumor-infiltrated surgical cavity walls and in any accessible residual tumor.

At 8 days after RVPC implantation, patients were treated by intravenous infusion of GCV (Cymevene; Recordati, Milano, Italy) at a dose of 5 mg/kg body weight over 1 hour, twice daily for 14 days. In patients no. 1, 2, 3, who expressed written consent, the site of RVPC implantation was biopsied immediately before GCV treatment to ascertain transduction efficiency *in vivo* and the presence of immune-inflammatory response.

Retroviral vector and vector-producing cells

The RVPCs were derived from a single clone of the PA317 packaging cell line (derived from a mouse embryonic fibroblast clone, ATCC CRL 9078), obtained by transfection with the pLIL-2-TK retroviral vector and selection for G418 resistance.[14] The LIL-2-TK retroviral vector, which is derived from the Moloney murine leukemia virus, expresses two therapeutic genes, human *IL-2* and *HSV-TK*, that are transcribed from the 5′ viral long terminal repeat and separated by an IRES (internal ribosome entry site of encephalomyocarditis virus) sequence, and the antibiotic resistance gene neomycin phosphotransferase (*neo*) transcribed from an internal simian virus 40 (SV40) early promoter. Construction and preclinical application of the LIL-2-TK vector have been previously reported.[11] The LIL-2-TK-transfected PA317 packaging cell clone used in this study had a titer $> 1 \times 10^6$ CFU/ml. Producer cells were grown in

Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Milano, Italy) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37°C with 5% CO_2. RVPCs for injection were grown free of microbial contamination in a biosafety level 3 GMP facility and tested for sterility, viability, vector titer, and for the presence of replication-competent retroviral particles (RCR), as reported.[14]

Assessment of safety

Clinical safety. Patients underwent daily clinical evaluation (including general physical exam, neurological exam, and KPS score determination) and standard laboratory analyses of blood, urine, stools, and cerebro-spinal fluid (CSF) during hospitalization and at follow-up visits, scheduled at 15-day intervals for 1 month and monthly thereafter. Laboratory investigations included hematological testing (complete blood count, differential blood count, platelet count, coagulative parameters), biochemistry (levels of standard electrolytes, calcium, magnesium, phosphate, blood urea nitrogen, creatinine, glucose, ALT, AST, total protein, albumin, alkaline phosphatase, bilirubin, total cholesterol, triglycerides, creatinine phosphokinase, lactate dehydrogenase, and uric acid), and standard urinalysis. Rate and severity of side effects and adverse events possibly related to gene therapy were recorded and scored as Grade 1 (mild), Grade 2 (moderate), Grade 3 (severe), and Grade 4 (life threatening) according to the Common Toxicity Criteria published by NCI (http://ctep.cancer.gov/forms/CTCAEv3.pdf). Each adverse event was categorized as related to treatment under study (RVPCs, GCV), disease progression, or concomitant disorders.

Biological safety. Biosafety monitoring consisted of assays for the presence of RCR and/or retroviral sequences in biological samples, including blood, urine, and stool specimens, and, when possible, CSF and tumor biopsy samples. Biological samples were collected before treatment, weekly during hospitalization and monthly during follow-up.

Detection of RCR was performed at day 7 after RVPC injection and weekly during hospitalization by reverse transcriptase assay[17] and provirus mobilization assay.[18]

The presence of vector DNA sequence and specific transcripts was assessed by a sensitive quantitative real-time direct and reverse PCR method, using oligonucleotide primers and probes specific for *HSV-1 TK, hIL-2*, *neo*, *gag*, and human and murine *GAPDH* genes. The following forward and reverse primers and probes were utilized for detection: *HSV-TK* (forward, 5′ CCA ACG GCG ACC TGT ACA A 3′; reverse, 5′ CAT CCC GGA GGT AAG TTG CA 3′; probe, FAM-CTG GGC CTT GGA CGT CTT GGC C-TAMRA); *hIL-2* (forward, 5′ CCA GGA TGC TCA CAT TTA AGT TTT AC 3′; reverse, 5′ GAG GTT TGA GTT CTT CTT CTA GAC ACT GA 3′; probe, FAM-TGC CCA AGA AGG CCA CAG AAC TGA A-TAMRA); *neo* (forward, 5′ ACT GAA GCG GGA AGG GAC TGG 3′; reverse, 5′ AGA AGC CGA TAG AAG GCG ATG 3′); *gag* (forward, 5′ CTT CCT AGA GAG ACT TAA GG 3′, 5′ GTT GGG ACC TCC TTC GTT CTC 3′); human *GAPDH* (forward, 5′ GAA GGT GAA GGT CGG AGT C 3′; reverse, 5′ GAA GAT GGT GAT GGG ATT TC 3′; probe, FAM-CAA GCT TCC CGT TCT CAG CC-TAMRA); murine *GAPDH* (forward, 5′ ACT CCA CTC ACG GCA AAT TC 3′; reverse, 5′ TCT CCA TGG TGG TGA AGA CA 3′).

Assessment of tumor response

Efficacy of treatment was assessed on the basis of clinical and radiological evaluation. Follow-up radiological evaluation was performed by T1-weighted magnetic resonance imaging (MRI) with gadolinium contrast enhancement at days 5, 15, 21, 35, 60, and monthly thereafter. The minimum tumor size after treatment was used to define response. A complete, partial, or minor response required that the reduction of tumor size occurred on at least two consecutive scans separated by at least 1 month, and that the patient was being treated with the same, or lower, dose of steroids than the dose at baseline (immediately pretreatment) MRI. A complete response was defined as a complete disappearance of the tumor mass at imaging evaluation; a partial response was defined as a reduction of $>50\%$ in the volume of enhancement in a contrast-enhanced MRI relative to baseline MRI; minor response was defined as a decrease of 25–50% of the tumor volume at enhanced MRI; stable disease was defined as tumor size ranging from less than 25% to an increase of less than 50% of the tumor volume at enhanced MRI relative to baseline MRI; tumor progression was defined as an increase of 50% or more of the tumor volume at enhanced MRI, or the development of a new lesion noncontiguous (more than 4 cm away) with the treated lesion.[19] When MRI evidence of progression was unavailable, the time of death or time of clinical deterioration was recorded as the time of disease progression.

Evaluation of tumor cell transduction

Whenever possible, before GCV treatment, a biopsy of the tumor at the site of injection of RVPCs was performed in order to assess transduction efficiency and therapeutic gene expression. Transduction efficiency in tumor samples was evaluated in DNA purified from tumor samples by real-time quantitative PCR by using oligonucleotide primers and probes as reported above. Therapeutic gene expression was evaluated in RNA purified from tumor samples by real-time quantitative RT-PCR, using primers specific for *HSV-TK* and *hIL-2*, as reported above.

Assessment of the immune response to treatment

Immune-inflammatory activity inside the injected tumors was measured by quantitative real-time RT-PCR using RNA extracted from tumor biopsies obtained at the site of RVPC injection, before GCV treatment. In particular, mRNA levels of *TNFα, IFNγ, IL-2, IL-1β*, and *IL-10* were

analyzed by using the following oligonucleotide primers and probes: *TNFα* (forward, 5′ CCC AGG GAC CTC TCT CTA ATC 3′; reverse, 5′ ATG GGC TAC AGG CTT GTC ACT 3′; probe, FAM-TGG CCC AGG CAG TCA GAT CAT C-TAMRA); *IFNγ* (forward, 5′ AGC GGA TAA TGG AAC TCT TTT CTT AG 3′; reverse, 5′ AAG TTT GAA GTA AAA GGA GAC AAT TTG G 3′; probe, FAM-TCT GTC ACT CTC CTC TTT CCA ATT CTT CAA AAT G-TAMRA); *IL-1β* (forward, 5′ ACA GAT GAA GTG CTC CTT CCA 3′; reverse, 5′ GTC GGA GAT TCG TAG CTG GAT 3′; probe, FAM-CTC TGC CCT CTG GAT GGC GG-TAMRA); *IL-10* (forward, 5′ ACG GCG CTG TCA TCG ATT 3′; reverse, 5′ TTG GAG CTT ATT AAA GGC ATT CTT C 3′; probe, FAM-CTT CCC TGT GAA AAC AAG AGC AAG GCC-TAMRA); *IL-2*, as reported above.

Systemic immune response was assessed by measuring plasma IL-2, IL-6, TNFα, and IFNγ levels during gene therapy. Blood samples for cytokine evaluation were taken at baseline, weekly during hospitalization, and monthly during follow-up. Plasma cytokines were detected by using commercial ELISA kits (BioSource, Europe SA, Nivelles, Belgium) according to the manufacturer's instructions.

Statistical analysis

Data are presented as median and range. Progression-free survival and overall survival were calculated by the Kaplan–Meyer method and were defined as the time from the first injection of RVPCs to tumor progression or death, respectively.

Results

Study population and gene therapy

From January 1996 to October 2002, 12 patients with recurrent GBM, all fulfilling the inclusion criteria, were referred to the Neurosurgery Division of the Vicenza City Hospital and enrolled in the clinical protocol of gene therapy with HSV-TK/hIL-2. Four patients (patients no. 1–4 in Table 1), who were treated in the period from 1996 to 1997, were also described in a previous report.[14] The other eight patients (patients no. 5–12 in Table 1) were treated in the period from November 2000 to October 2002. The demographic and clinical characteristics of patients at the time of enrollment are summarized in Table 1. There were five female and seven male patients, median age 50 years, range 28–69 years, with tumor mass size ranging from 20 to 180 ml, median 90 ml.

At first diagnosis, all patients underwent microsurgical removal of primary GBM and subsequent fractionated radiotherapy (11 patients), chemotherapy (six patients), or radioimmunotherapy (two patients). Two patients underwent repeated microsurgery for relapse, before undergoing gene therapy. In all patients, diagnosis of GBM was confirmed by histological examination. Median time interval between the first surgical intervention and genetic treatment was 13 months, range 7–96 months.

Table 1 Clinical features of patient population

Patient no.	Age	Sex	KPS score	Interval from diagnosis to gene therapy (months)	No. of previous resections	Radiotherapy (Gy)	Immunotherapy	Chemotherapy	Location of GBM recurrence	Volume of GBM recurrence (ml)
1	22	M	70	10	1 MR	FRT, sterotactic Curiether.	–	+	Bilateral, corpus callosum	150
2	50	M	70	23	2 MR	FRT	–	–	Temporal left	180
3	58	F	90	9	1 MR	FRT	–	–	Bilateral, corpus callosum, occipital p.	80
4	50	F	70	15	1 MR	FRT	+	+	Frontal–temporal left (cystic)	60
5	20	F	70	19	1 MR	FRT	+	+	Frontal–temporal right, corpus callosum	120
6	54	F	90	7	1 MR	FRT	–	+	Frontal–temporal right	40
7	60	M	80	7	2 MR	FRT, IORT	–	–	Frontal left	110
8	46	M	90	96	1 MR	Radiosurgery	–	+	Frontal–temporal left (cystic)	110
9	69	F	90	7	1 MR	FRT	–	–	Parietal–occipital left	40
10	28	M	90	11	1 MR	FRT	–	+	Corpus callosum, temporal left	20
11	58	M	70	20	1 MR	—	–	–	Temporal–parietal left	100
12	35	M	90	17	1 MR	FRT	–	–	Parietal–occipital left	90

MR, microsurgical removal; FRT, fractionated radiotherapy; KPS, Karnofsky Performance Score; IORT, intra-operative radiotherapy.

Eight patients were treated with computerized tomography (CT) or MRI-guided stereotactic intratumor injection of RVPCs in a total volume of 2–6 ml; four patients (patients no. 4, 6, 9, 12) were injected with packaging cells into the residual tumor walls after microsurgical removal of tumor relapse.

Injections were distributed in 2–6 different areas of the tumor, in order to improve transduction of target cells. The median number of injected RVPCs was 5×10^8, range 2×10^8–1×10^9. Four patients (patients no. 2, 6, 9, 10) received two cycles of gene therapy, two of whom (patients no. 6, 9) underwent open surgical resection of the tumor recurrence and injection of RVPCs into the tumor walls. GCV was administered in all patients i.v. at doses of 5 mg/kg, twice daily, starting on day 8 after injection of RVPCs, for 14 days.

Adverse events

After gene therapy, patients were monitored for the occurrence of adverse events and for efficacy. No patients were lost at follow-up observation.

Treatment was generally well tolerated, with minor adverse events occurring in eight out of 12 (67%) patients (Table 2). All toxicities were graded 1 and 2, and none required medical intervention. No complications were observed both in patients receiving stereotactic injection of RVPCs and in those undergoing microsurgical removal of tumor masses and RVPCs injection, nor severe side effects that could be attributed to RVPCs. MRI evaluation following RVPCs administration did not show hemorrhage or edema at the site of injection. The most frequent side effect was transient increase of transaminase levels, observed in five patients during GCV treatment, which was mild in all but one case (patient no. 2). This patient showed a progressive asymptomatic increase of transaminase levels in the course of the second cycle of genetic treatment, which was attributed to GCV toxicity. Markers of viral hepatitis were negative and liver ultrasonography showed inhomogeneous hyperechoic liver structure. Two patients showed transient mild leukocytosis and one mild leukopenia, which could possibly be attributed to RVPC injection or GCV treatment, whereas no patients showed abnormalities of coagulative parameters or renal function tests. Neurologic events, possibly related to gene therapy, occurred in one patient (patient no. 5) with a large 120 ml recurrence involving the fronto-temporal cortex and corpus callosum, who had fever and seizure in the first week after RVPC injection. The patient eventually died after 1 month of progressive disease. In the other patients, neurologic deterioration and impairment of vital signs observed during follow-up seemed to be related to disease progression, as shown by radiological evaluation, rather than to gene therapy.

Biosafety monitoring and evaluation of retroviral vector dissemination

No evidence of RCR was found in any of the tumor biopsies and biological samples obtained from all patients. PCR analysis of retroviral vector or RVPC sequences in biological samples demonstrated the presence of proviral DNA and RVPCs (i.e. positive results from *HSV-TK*, *IL-2*, *neo, gag,* and mouse *GAPDH* PCR amplification) in PBMCs from three patients (patients no. 2, 6, 12) at 3–20 days from RVPC inoculation. In patient 2, the PCR signal decreased following GCV treatment, to rise again to the original level after GCV discontinuation. No vector sequences were detected in urine and stool samples.

Efficacy

Results of the gene therapy trial are summarized in Table 2. None of the patients achieved a complete response, two had a partial response, four had a minor response, four had stable disease, and two had progressive disease.

Of the two patients who showed a $>50\%$ reduction of mass size, the first patient (patient no. 3), who had an 80 ml bilateral mass in the corpus callosum with dysphasia and hemiparesis, underwent stereotactic injection of 2×10^8 RVPCs in a total volume of 2 ml, distributed in four targets. After treatment, the patient experienced a mild clinical improvement associated with regression of the tumor mass to 40 ml, as demonstrated by MRI performed at 1 month after completion of GCV treatment and as shown in our previous report.[14] Duration of response was 6 months. The patient died of pulmonary embolism 11 months after RVPC implantation. The second patient (patient no. 10), who achieved partial response, was admitted because of a recurrent 20 ml GBM in the corpus callosum and appearance of a second small (4 ml) lesion in the left temporal lobe, notwithstanding fractionated radiotherapy received the year before. Neurological evaluation demonstrated mild dysphasia and right hemiparesis. The patient underwent stereotactic injection of 6×10^8 RVPCs in 6 ml volume into the corpus callosum lesion. Puncture of the superficial, critically located, small temporal lesion was deemed too dangerous because of the close proximity to middle cerebral artery vessels and consequently discarded. Treatment led to substantial amelioration of neurological signs. Previous corticosteroid medication could be reduced. MRI follow-up evaluation showed progressive regression not only of the injected mass, but also of the temporal lesion. At 6-month follow-up, radiological assessment showed a regression to 3 ml of the injected mass and disappearance of the temporal tumor. A further decrease of tumor size was demonstrated at MRI performed 9 months after gene therapy (Fig 1). However, the patient subsequently showed tumor relapse and died 13 months after gene therapy.

Necrosis, appearing as a markedly hypointense area of about 2 ml at the site of injection, was documented in three patients with minor response. Inflammation at the site of injection was documented at MRI as a contrast-enhanced ring feature surrounding the injected area in another patient with minor response (Fig 2). Patients who achieved minor response had large GBM

Table 2 Gene therapy and outcome in 12 patients with recurrent GBM

Patient no.	Surgery	RVPC volume (ml (cell x 10^8))	No. of targets	KPS day 28	Adverse events (grade)	MRI evaluation	Tumor response	Time to progression (months)	Survival from gene therapy (months)	Cause of death
1	—	6 (1.5)	3	Unchanged	Increase of transaminase levels (1)	Local necrosis (2 ml)	MR	4	5	Disease progression
2	—	3 (2)	2	Unchanged	Dysphasia (1), confusion (1), emiparesis (2), increase of transaminase levels (1)	Local necrosis (2 ml)	MR	8	12	Disease progression
	—	3 (2.5)	3	Clinical improvement	Increase of transaminase levels (2), leukopenia (1)					
3	—	2 (2)	2	Clinical improvement	Dysphasia (1), left emiparesis (2)	Reduction of tumor volume (40 ml)	PR	11	11	Pulmonary embolism
4	—	3 (3)	Reservoir and intracystic catheter	Unchanged	—	Unchanged	SD	13	16	Disease progression
5	—	3 (2)	1	50	Fever (1), seizure (2), increase of transaminase (1)	Unchanged	PD	1	1	Disease progression
6	MR	6 (2)	5	Unchanged	Facial paresis (1)	Inflammation	MR	4	8	Disease progression
	MR	5 (4)	5		—					
7	—	3 (8)	1	Unchanged	Leukocytosis (1), increase of transaminase (1)	Unchanged	SD	3	3	Disease progression
8	—	6 (4)	2	Unchanged	—	Unchanged	SD	2	2	Disease progression
9	MR	6 (6)	6	70	—	Progression	PD	5	11	Disease progression
	MR	6 (5.5)	6		—					
10	—	6 (6)	2	Clinical improvement	—	Tumor mass reduction	PR	9	13	Disease progression
11	—	6 (10)	3	Unchanged	Increase of transaminase (1)	Local necrosis (2 ml)	MR	2	2	Pulmonary embolism
12	MR	6 (5.5)	6	Clinical improvement	Leukocytosis (1)	Unchanged	SD	16	29	Disease progression

MR, microsurgical removal; PR, partial response; MR minor response; SD, stable disease; PD, progressive disease.

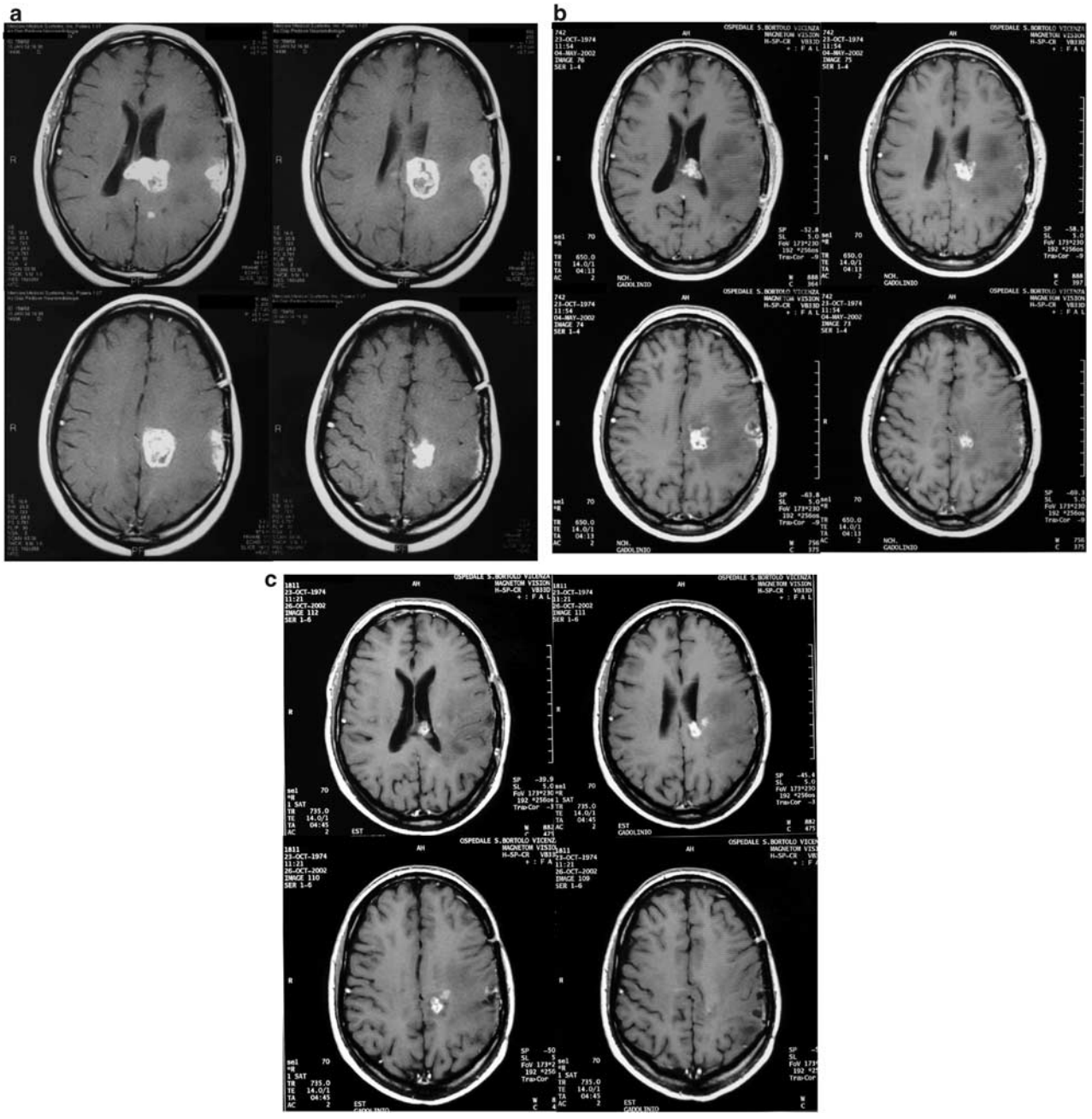

Figure 1 Serial T1-weighted postgadolinium MRI of patient no. 10. (**a**) Initial MRI demonstrated GBM recurrences in the corpus callosum and in the left temporal lobe. (**b**) The MRI performed 2 months after RVPC injection into the lesion in the corpus callosum shows significant reduction of both the injected lesion and the distant nontreated lesion in the temporal lobe. (**c**) The MRI performed 7 months after gene therapy showed a further decrease of the volume of both lesions.

relapses, injected with a total number of RVPCs ranging from 1.5×10^8 to 1×10^9. Of these patients, one underwent tumor debulking before RVPC implantation, and two received a second genetic treatment when disease progressed.

Of four patients with stable disease as best response, one (patient no. 12), who underwent surgical removal of a 90 ml parieto-occipital tumor mass before gene therapy, had a long-term stabilization of his disease (16 months) and neurological condition (dysphasia and mild facial paresis, KPS > 90). In this patient, PET showed decreased metabolic activity in the cerebral region involved by the neoplasm (Fig 3). Since pretreatment PET was not performed, it cannot be ascertained if this feature was a result of the genetic treatment. The patient died because of disease progression 29 months after gene therapy. The other patients died because of disease progression at 2, 3, and 13 months from treatment.

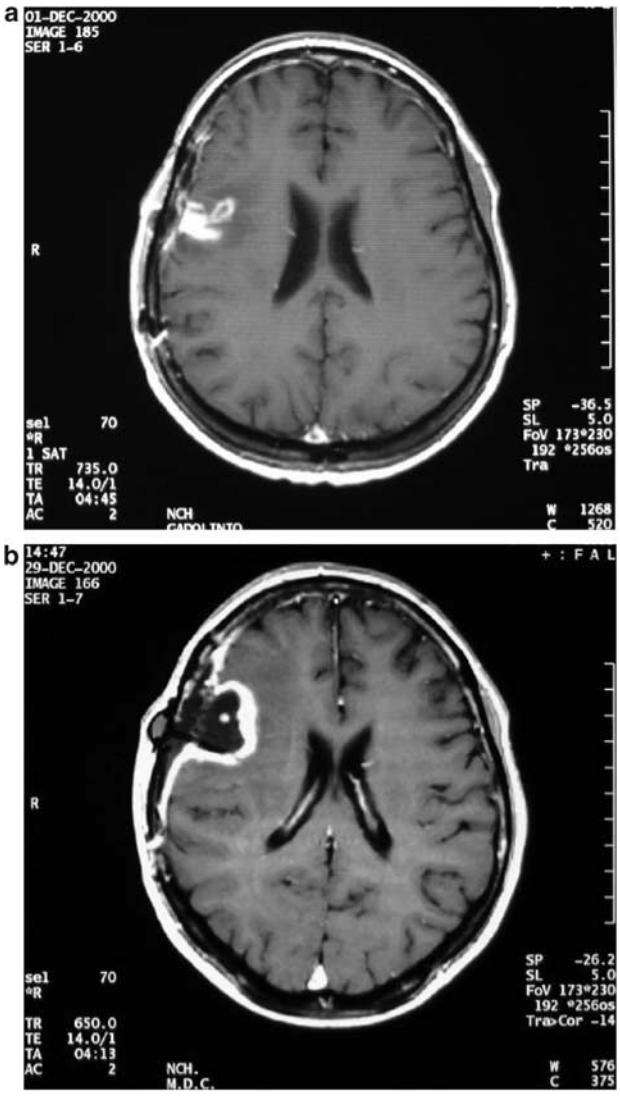

Figure 2 Serial imaging of patient 6. (**a**) MRI showing GBM relapse. (**b**) MRI, performed after open craniotomy and RVPC injection, showing a contrast-enhanced ring surrounding the injected area.

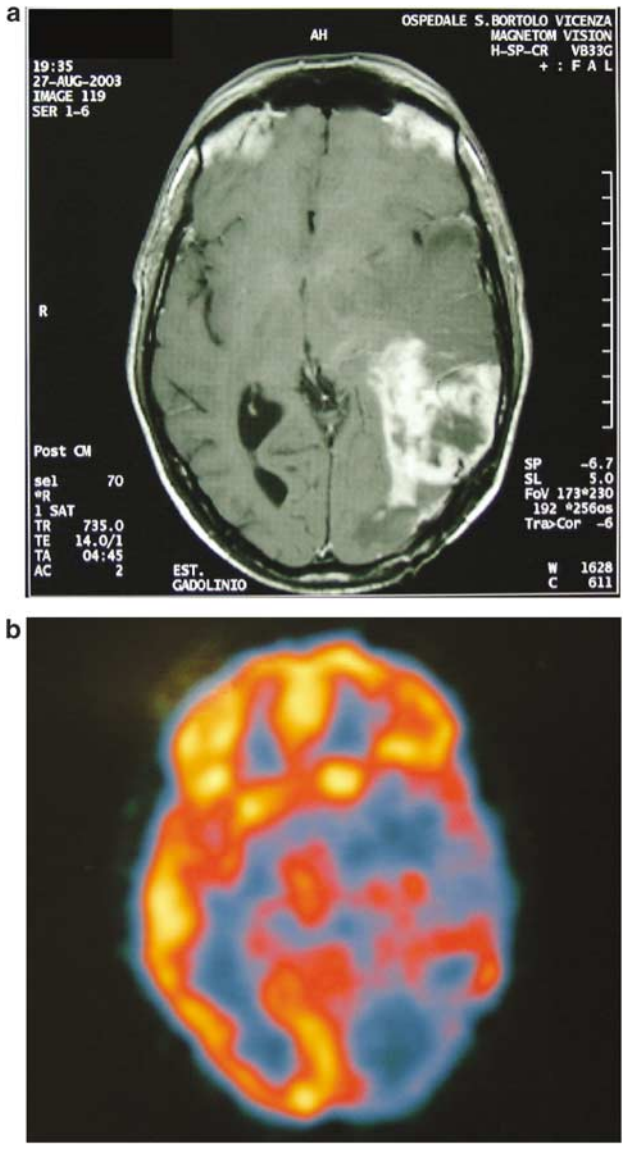

Figure 3 MRI and PET imaging of patient 12. (**a**) MRI showing a parieto-occipital GBM relapse after surgical resection and gene therapy. (**b**) PET image showing a decreased metabolic activity of the cerebral region involved by the neoplasm.

Patients with progressive disease were case no. 5, whose clinical course is reported in the above section, and case no. 9 with a 40 ml lesion involving the left parieto-occipital cortex, who underwent microsurgical removal of the mass followed by RVPCs implantation and, because of disease progression, after 5 months underwent re-operation and second gene treatment. He eventually died of his disease at 18 months from the first cycle of gene therapy.

Clinical outcome and survival analysis

A total of 10 patients died of progressive disease at 1–29 months from gene therapy; two patients died because of pulmonary embolism at 11 and 2 months from genetic treatment. Median progression free survival was 4.5 months (range 1–16) and median overall survival was 7.5 months (range 1–29 months). Kaplan–Mayer analysis showed that 47 and 25% of patients were free of progression at 6 and 12 months from the beginning of treatment, respectively, and that 58 and 33% of patients were alive at 6 and 12 months from the beginning of treatment, respectively (Fig 4).

Tumor transduction and local immune-inflammatory response

Tumor biopsies, performed at the site of RVPC injection and before GCV therapy, were available in three cases (patients no. 1, 2, 3). In these tumor samples, PCR analysis demonstrated the presence of retroviral vector DNA, and RT-PCR analysis showed expression of the therapeutic gene *HSV-TK*. RT-PCR analysis also

demonstrated high levels of *TNFα, IFNγ, IL-1β*, *IL-2*, and *IL-10* mRNA, suggesting the presence of an immune-inflammatory infiltrate (Table 3). Molecular analysis of transgene expression by *in situ* hybridization and characterization of cell infiltrates were reported in our previous study.[14]

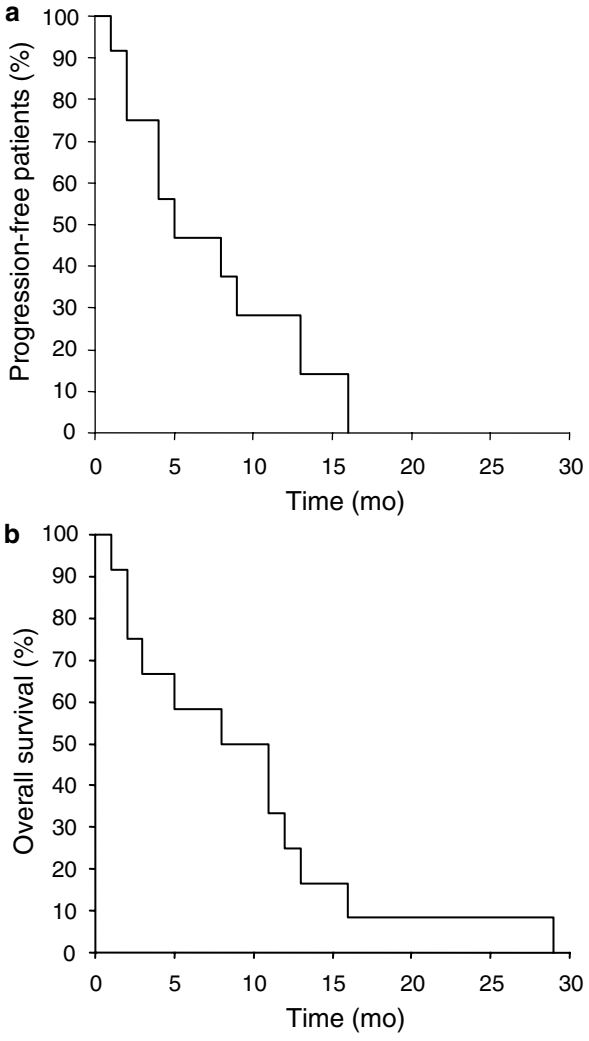

Figure 4 Kaplan–Meyer plots showing (**a**) progression-free survival and (**b**) overall survival rates.

Systemic cytokine response

Analysis of plasma cytokine response after gene therapy demonstrated a marked increase of circulating IFNγ, TNFα, IL-2, and IL-10 in the period ranging from immediately after RVPC injection to about 1–2 months after gene therapy. Interestingly, patients who achieved tumor response showed a more pronounced and persistent elevation of plasma cytokine levels. In particular, IFNγ, IL-2, and TNFα production seemed to be associated with tumor regression and long-term response. At variance, IL-6 levels did not generally change following RVPC injection, but showed a marked increase with disease progression (Fig 5). The extent of cytokine response did not seem to be related to the number of injected packaging cells nor to the volume of tumor recurrence.

Discussion

This study represents the first clinical protocol of gene therapy for recurrent GBM, based on the combined delivery of a suicide gene (*HSV-TK*) and a cytokine gene (*IL-2*). Moreover, to the best of our knowledge, it also represents the first report of combined suicide and immunomodulating gene therapy for cancer. The results of our experience show that injection of RVPCs is safe, results in transduction of the therapeutic genes to target tumor cells, and activates a cytokine response, with tumor responses in 50% of cases.

The main finding of this study is the safety of intratumor administration of a total dose of up to 1×10^9 RVPCs. Adverse events were mild, and mainly related to GCV administration, such as transient mild elevation of liver function tests. Neurological adverse events were generally related to disease progression. Safety of treatment is noteworthy, considering that

Table 3 Analysis of vector sequences and expression of therapeutic and cytokine genes in stereotactic biopsies obtained from patients with recurrent GBM after RVPC implantation and before GCV administration[a]

	Patient 1	Patient 2	Patient 3
Vector DNA sequences (DNA copies/μg DNA)			
HSV-TK DNA	210±64	376±87	135±37
IL-2 DNA	315±120	448±109	422±130
neo DNA	177±91	391±102	92±46
Gene expression (mRNA copies/μg total RNA)			
HSV-TK mRNA	11,075±636	14,711±824	7746±450
IL-2 mRNA	14,744±1008	9372±870	9938±1285
IFNγ mRNA	22,378±1671	17,940±2337	28,090±3440
TNFα mRNA	10,489±652	13,080±1552	13,455±1088
IL-10 mRNA	380±54	724±68	216±97
IL-1β mRNA	17±10	32±9	11±4

[a]Analyses were performed by quantitative direct and RT-PCR. Data represent mean±SD of triplicate tests.

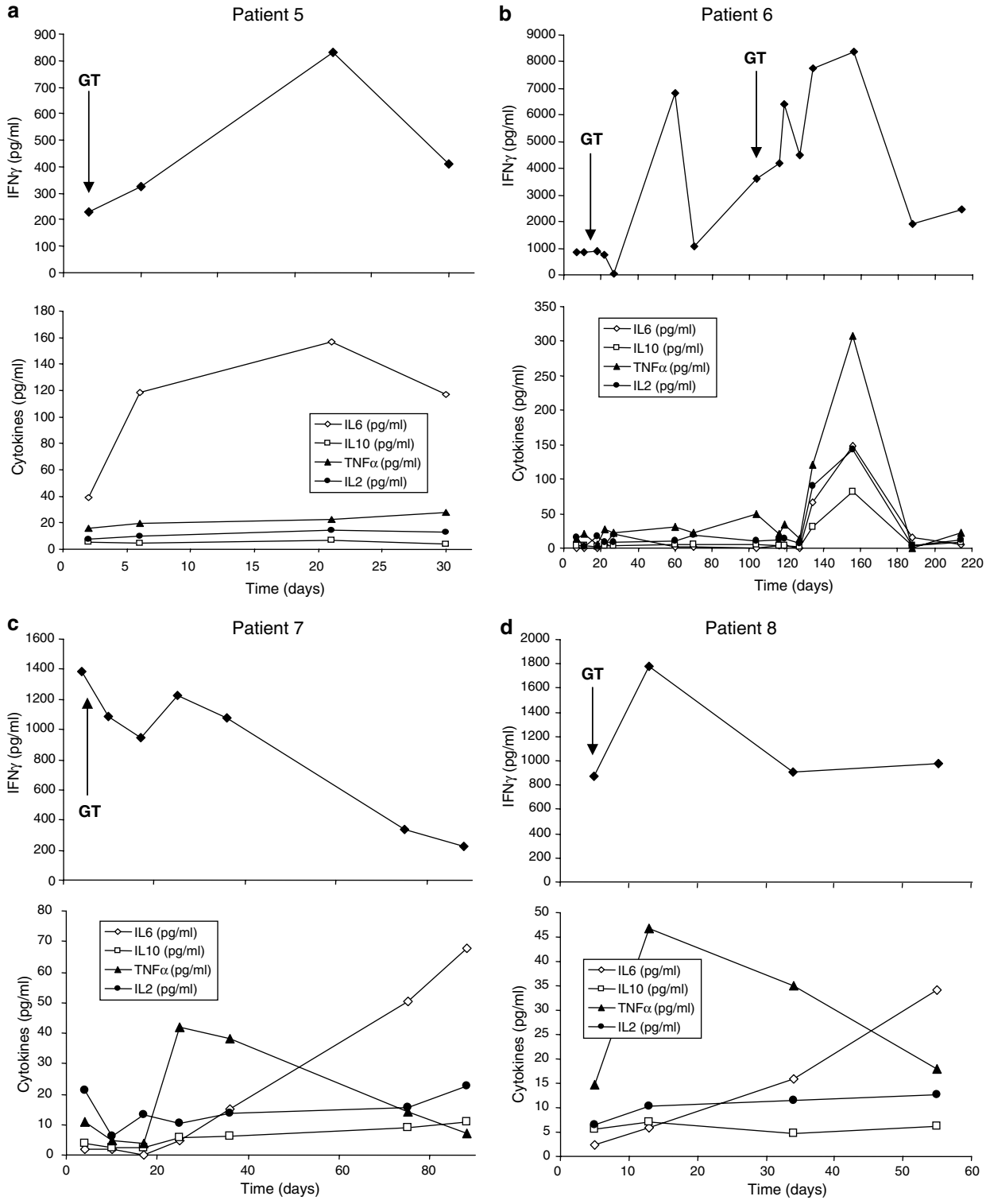

Figure 5 Cytokine profile in serum of GBM patients treated with gene therapy. Cytokine levels were measured by ELISA.

IL-2/*HSV-TK* delivery by intratumor injection of xenogenic RVPC elicited a marked local inflammation in the CNS. However, a remark should be made about the death by pulmonary embolism of two patients 2 and 11 months after gene therapy. Although cancer patients, and in particular those with primary gliomas, are at increased risk of pulmonary embolism,[20] the systemic activation of cytokine cascades after gene therapy could have

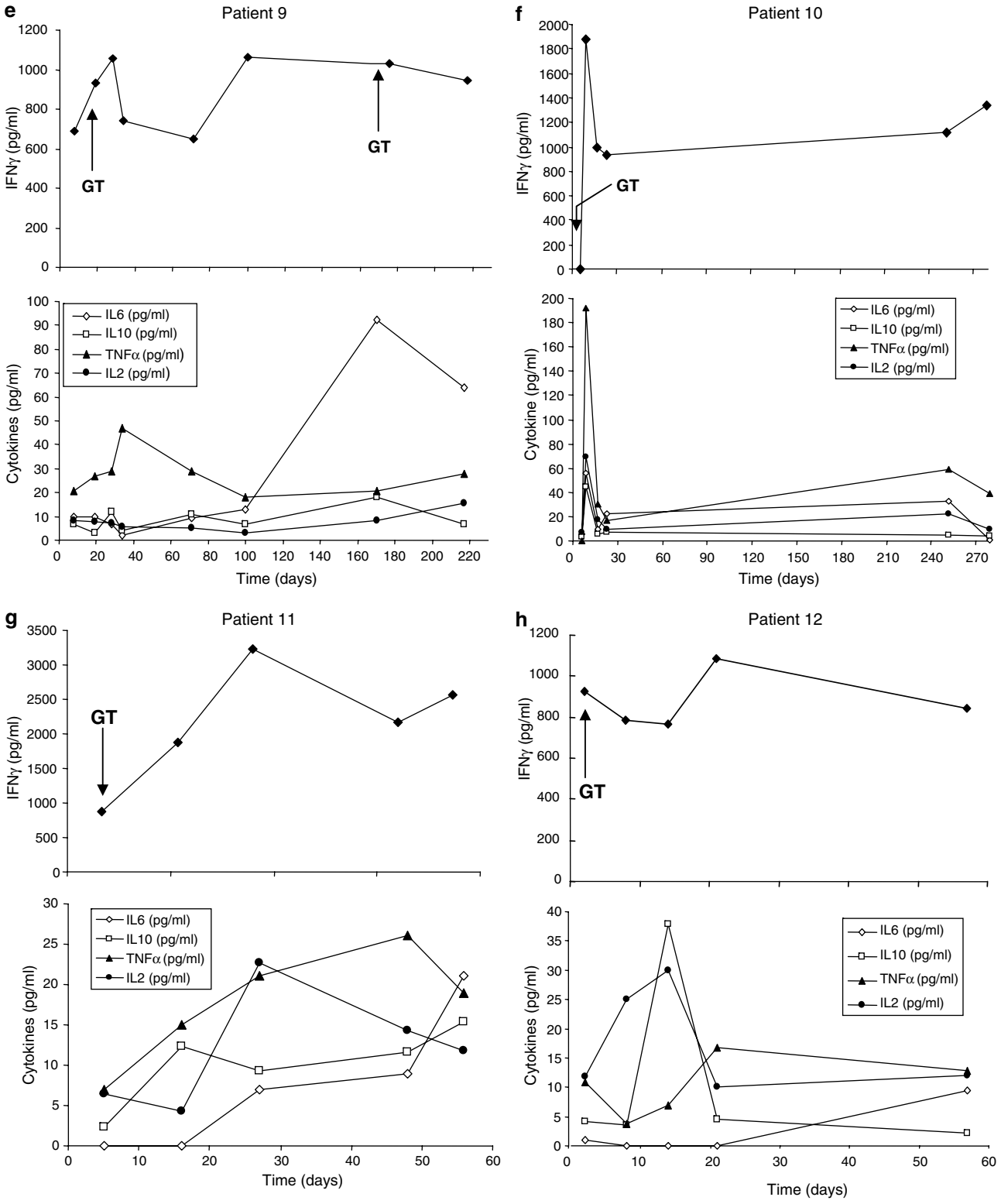

Figure 5 (Continued)

contributed to the occurrence of the thromboembolic events.[21] It is interesting to note that both patients who died of pulmonary embolism had a marked increase of plasma cytokine levels after gene therapy, which was associated with long-term tumor size reduction and clinical response in one of the patients.

Another biosafety concern of the use of retroviral vectors is represented by the risk of emergence of

RCR, which could lead to active retroviral infection. Screening for RCR in our patients yielded negative results, consistent with data from about 400 patients from the literature, treated with similar gene therapy protocols.[4,10,22]

The clinical use of retroviral vectors has been recently questioned after three cases of B-cell leukemia that occurred in children with X-SCID as a consequence of insertional mutagenesis by retroviral vectors.[23,24] The risk of a second malignancy, even if never demonstrated so far in cancer gene therapy clinical trials,[24] exists also for cancer patients and should always be weighted with the potential benefits of gene therapy, especially in patients with highly lethal cancers, such as GBM. Vector DNA sequences have been detected in PBMCs from patients with brain tumors, treated by intracerebral administration of RVPCs,[4,10,22,25,26] including three of our patients. Tumor debulking before RVPC injection could have favored spread of vector and packaging cells into the bloodstream, as demonstrated in two of our patients. The presence of a suicide gene in transduced cells would, however, allow to eliminate any transformed cell by GCV treatment *in vivo*. Screening for retroviral vector insertion sites in the DNA obtained from PBMCs of our patients with positive PCR results is currently ongoing. The risk of transducing circulating stem cells however seems quite low.

Although the nature of this study did not allow to draw conclusions regarding the efficacy of treatment, we could document two cases of partial response that might be ascribed to gene therapy. Moreover, in four cases, MRI investigation demonstrated local tumor necrosis and/or inflammation surrounding the injection site. In other four cases, genetic treatment was followed by stabilization of disease, even though progression finally occurred. Median progression-free survival and overall survival were 4.5 and 7.5 months, respectively, and 25% of patients were alive at 12 months of follow-up. These results seem to compare favorably to those reported on a large systematic analysis on 1415 high-grade glioma recurrences aggressively treated in 40 trials of chemotherapy, radiation therapy, and surgery, strategies already exploited in our patients.[1,2]

Regarding the surgical procedure employed (stereotactic *versus* open surgery), advantages of the stereotactic technique are the reduced surgical trauma and the accurate treatment planning. Moreover, the availability of precise spatial information allows an accurate measurement of the range of therapeutic effect.[16] Its principal shortcomings are the lack of a debulking effect with no immediate improvement on mass effect. Injection after surgical removal allows an accurate hemostasis control (under microscopic vision) and reduces the risk of injection into ventricles and CSF spaces. Moreover, the number of injection sites and the volume of injected material can be increased. Using visual control alone, planning of injections is forcibly less accurate, even when targeted to evident residual tumor.

In most series published in the literature, RVPCs were injected into the surgical cavity margin after tumor debulking. We preferred a stereotactic approach with direct injection of RVPCs into the tumor mass for our suicide/cytokine gene therapy, hypothesizing that intratumor production of IL-2 combined with xenoantigen expression (murine packaging cells, HSV-TK and neomycin phosphotransferase) and with the expression of tumor antigens likely associated with GBM could increase specific and nonspecific antitumor immune responses.[27,28] A gene therapy protocol based on intratumor injection of RVPCs was reported by Ram et al,[9] who observed tumor responses in four out of 13 evaluable patients with recurrent GBM. All patients who responded had small tumor recurrences of less than 1.5 ml. Moreover, tumor responses were limited to the site of RVPC injection, whereas untreated tumor masses progressed rapidly.[9] In our series, all patients had much larger tumor masses, ranging in size from 20 to 180 ml. However, we observed tumor regression also in patients with large masses, and the extent of tumor response was unrelated to tumor size. Moreover, we observed a complete regression of a distant 4 ml untreated distant brain lesion, besides reduction of the tumor mass injected with RVPCs. It is conceivable that this result, which has never been observed in other clinical trials of gene therapy with *HSV-TK*, was due to the immune-inflammatory response elicited by RVPC injection and IL-2 production. In fact, this patient showed a marked increase of circulating Th1 cytokine levels, especially IFNγ and TNFα, immediately after RVPC injection and a persistent cytokine hypersecretion during follow-up observation. Unfortunately, a tumor sample was not available to assess the presence of a specific antitumor T-cell response *in vitro*. A similar profile of plasma cytokine levels was observed in all our patients treated with gene therapy. In particular, patients showing tumor response to treatment had a marked increase of TNFα and IFNγ levels. Examination of tumor biopsies performed at the site of RVPCs injection showed expression of transgenes and production of Th-1 cytokines. Moreover, as extensively documented in our previous report,[14] tumor samples showed an immune-inflammatory cell infiltrate of polymorphonucleates, activated lymphocytes and macrophages, which was conceivably recruited by IL-2, together with xenoantigens from RVPCs and retroviral proteins. As reported above, however, major limitation of our results is the lack of data from ELISPOT or other techniques to demonstrate a specific antitumor T-cell-mediated immune response. The systemic and local cytokine response of our patients during gene therapy seemed more pronounced than the response observed in patients treated by tumor resection followed by injection of RVPCs expressing the HSV-TK gene alone.[8] In this study, tumor samples obtained after gene therapy showed no significant increase of intratumor lymphocyte infiltrates[8] nor patients showed any improvement of survival,[4] although serum IL-12 and FasL levels were higher than in the control group of patients and a cell-mediated immune response against RVPC was demonstrated.[8]

As an alternative to intratumor injection of RVPCs, adenoviral vectors have been more recently investigated for gene therapy of malignant gliomas.[29,30,31] Intratumor

injection of adenoviral vectors at doses up to 1×10^{10} PFU has been demonstrated to be associated with minimal toxicity and to determine transduction of tumor cells located within a short distance of the injection site.[29,30] A randomized, controlled study of adenovirus-mediated *HSV-TK* gene therapy in patients with operable primary or recurrent malignant gliomas demonstrated a significant increase of survival time of treated patients with respect to control patients receiving standard treatment.[31] It is not possible to compare the results of this study with our results due to differences in patient selection (the adenoviral vector study included a large proportion of patients with primary tumors, including anaplastic astrocytomas) and in treatment modality (all patients underwent tumor resection). A previous report by the same authors,[32] who compared safety and efficacy of adenovirus- *versus* RVPC-mediated *HSV-TK* gene therapy for patients with malignant glioma, showed that both treatments were well tolerated, but that the mean survival for the adenoviral vector-treated group was superior to that for patients treated with RVPCs. Although further studies are needed to evaluate central nervous system toxicity of adenoviral vectors,[30] these vectors have some advantages for clinical use, since they can be produced at higher titer than retroviral vectors and can efficiently transduce a variety of cells, including human malignant gliomas. The immunogenicity of these vectors and the presence of antiadenoviral antibodies in most patients may hamper the efficacy of treatment and prevent repeated administrations, even if vector immunogenicity could be exploited as an adjuvant to stimulate antitumor immune-inflammatory responses.

In conclusion, our study, which represents the first clinical trial of combined cytokine/suicide gene therapy for cancer, demonstrates, in a larger population of patients with recurrent GBM, that injection of RVPCs is safe, results in transduction of the therapeutic genes to target tumor cells, and activates a systemic and local cytokine response, which could account for tumor regression and long-term control of tumor growth.

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