ON-01, an engineered recombinant oncolytic herpes simplex virus type-1, in recurrent glioma: a single-arm, phase 1/2 study

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Abstract

Background: The prognosis of patients with recurrent WHO grade 4 glioma is poor, particularly in glioblastoma (GBM), which has a median survival of approximately 6 months and no effective treatment options. We evaluated the short-term (28-day) safety and efficacy of ON-01, an engineered recombinant oncolytic herpes simplex virus type-1, in patients with recurrent WHO grade 4 glioma.

Methods: In this single-arm, phase 1/2 clinical trial, eligible patients received intratumoral injections of ON-01 under stereotactic guidance. The primary endpoint was to assess the short-term safety profile of ON-01 treatment. Secondary endpoints included progression-free survival (PFS), overall survival (OS), and the 2-year OS rate. An exploratory objective was to identify tumor-related biomarkers predictive of treatment efficacy.

Results: Of the 30 patients treated with ON-01, 13 (43.3%) were male, and the median age was 50.0 years (range, 22–75). A total of 36 grade 1, 12 grade 2, and 2 grade 3 adverse events were reported. Among all treated patients, the median OS was 12.0 months (95% CI, 10.1–13.9), median PFS was 3.0 months (95% CI, 1.7–4.3), and 2-year OS rate was 27.7% (95% CI, 12.6%–45.0%). Seven patients with recurrent multifocal gliomas demonstrated regression of non-injection site lesions following ON-01 therapy. Furthermore, patients with elevated expression of herpesvirus entry mediator exhibited significantly prolonged survival (p=0.015).

Conclusions: Intratumoral infusion of ON-01 appeared safe and demonstrated efficacy in patients with recurrent malignant glioma, with no evidence of neurotoxicity. The therapeutic response to ON-01 may be associated with HVEM expression levels.

Keywords: recurrent glioma; oncolytic virotherapy; herpes simplex virus; single-arm trial

Key Points

1. Intratumoral infusion of ON-01 confirmed the absence of neurovirulent potential.
2. ON-01 exhibited preliminary therapeutic efficacy in patients with recurrent WHO grade 4 glioma.
3. ON-01 enables targeted intracellular chemotherapy in GBM by converting 5-FC to 5-FU.

Importance of the Study

This study evaluates ON-01, a genetically engineered oncolytic herpes simplex virus type-1 (HSV-1) designed to enhance tumor lysis and enable intracellular chemotherapy in patients with recurrent WHO grade 4 malignant gliomas. The results demonstrate a favorable short-term safety profile, with manageable adverse events and no significant virus-related neurotoxicity. ON-01 also exhibited preliminary therapeutic efficacy, with a median overall survival of 12.0 months and measurable responses, particularly in smaller tumors. Its dual mechanism—oncolytic virotherapy combined with prodrug activation—distinguishes ON-01 from existing therapies. These findings support ON-01 as a promising candidate for the treatment of recurrent WHO grade 4 glioma and provide important insights into the clinical development of oncolytic virus-based strategies for malignant gliomas. Larger, controlled trials are warranted to confirm its therapeutic potential and to further assess its impact on survival and treatment options for patients with inoperable or multifocal disease.

Introduction

Despite the establishment of standardized treatment protocols, the median survival time for newly diagnosed patients with WHO grade 4 malignant gliomas remains <15 months.[1,2] Nearly all patients experience recurrence, with median survival after recurrence typically about 6 months[3,4] due to the lack of effective interventions.[5] The highly immunosuppressive tumor microenvironment and limited immune cell infiltration of glioblastoma (GBM) hinder the efficacy of immune checkpoint

inhibitors.[6,7] Therefore, patients with recurrent GBM urgently require more effective therapies to improve prognosis.

Oncolytic virotherapy is an emerging approach that selectively infects and destroys tumor cells while stimulating an antitumor immune response[8]. Multiple clinical trials[9-12] are currently evaluating its efficacy and safety in recurrent gliomas. HSV-1 oncolytic viruses, such as G47Δ,[13,14] CAN-3110,[15] and G207,[16] have demonstrated potent direct tumor-killing effects. Their large genome allows genetic engineering to improve both safety and therapeutic efficacy.

ON-01 is an innovative recombinant oncolytic HSV-1 engineered through deletion of the neurotoxic gene *ICP34.5*[17] and the immune evasion gene ICP47[18], combined with insertion of the *Escherichia coli* cytosine deaminase (CD) gene to enhance therapeutic activity.[19,20] Unlike G207 and G47Δ, ON-01 retains the *ICP6* gene,[10,21,22] ensuring effective replication. T-VEC, another *ICP6*-retained oncolytic HSV-1, is FDA-approved for treating malignant melanoma, supporting the safety of this approach.[23,24] ON-01 enters tumor cells via the herpesvirus entry mediator (HVEM),[25,26] directly lysing tumor cells while enhancing antitumor immunity. Additionally, the CD enzyme converts the non-toxic prodrug 5-fluorocytosine (5-FC) into the cytotoxic chemotherapy agent 5-fluorouracil (5-FU), enabling targeted intracellular chemotherapy for solid malignancies.

Here, we report the first single-arm, phase 1/2 clinical trial of ON-01 involving 30 patients with recurrent WHO grade 4 glioma. This study assessed short-term safety and provided an initial evaluation of efficacy.

Materials and Methods

Study design and participants

From October 2018 to August 2022, adult patients with recurrent WHO grade 4 gliomas—including IDH-wildtype GBM and IDH-mutant astrocytoma—were enrolled at the Department of Neurosurgery, Beijing Tiantan Hospital, and Beijing Electric Power Hospital. Tumor diameters ranged from 1 to 5 cm, with the largest lesion measured in patients with multifocal disease. Diagnoses were based on the 2021 5th edition of the World Health Organization Classification of Tumors of the Central Nervous System (WHO CNS 5). In patients with an initial diagnosis of WHO grade 4 glioma, atypical MRI enhancement prompted the use of PET-CT to differentiate tumor recurrence from radiation necrosis.

This single-arm, phase 1/2 clinical trial was designed to evaluate the short-term (28 days) safety and efficacy of ON-01 in patients with recurrent WHO grade 4 malignant gliomas. Written informed consent was obtained from all participants prior to enrollment, and the study protocol was approved by the Ethics Committee of Beijing Tiantan Hospital. The trial is registered with the Chinese Clinical Trial Registry (ChiCTR; registration number ChiCTR1900022570). A detailed experimental protocol is available in the supplementary materials.

Sample Size Justification

The sample size was determined based on the 6-month progression-free survival (PFS6)

rate (PMID: 17108063). Assuming a PFS6 rate of 15% under the null hypothesis (H_0), based on historical controls for recurrent high-grade glioma, the study was designed to detect a clinically meaningful improvement to a target rate of 40% under the alternative hypothesis (H_1). With a one-sided significance level of $\alpha = 0.05$, enrolling 30 patients provides over 85% power using an exact binomial test, accounting for a potential dropout rate of approximately 15%. Further details are provided in the experimental protocol.

Inclusion and exclusion criteria

Eligible participants were adults aged 18–75 years who were able to provide voluntary written informed consent. Patients were required to have supratentorial high-grade glioma confirmed by intraoperative frozen pathology, along with radiologic evidence of tumor recurrence. The recurrent enhancing lesion was required to measure $\geqslant$1 cm and <5 cm on MRI and/or PET-CT. In addition, patients needed to have a Karnofsky Performance Status (KPS) score greater than 60. Key exclusion criteria included inability to provide informed consent, pregnancy or lactation, and participation in another clinical trial within the previous 30 days. Patients with a history of encephalitis, multiple sclerosis, other central nervous system infections, or active oral herpetic lesions were also excluded. A complete list of inclusion and exclusion criteria can be found in the experimental protocol provided in the supplementary materials.

Procedures

All injections were performed under stereotactic guidance. The planned trajectory targeted the tumor core while avoiding eloquent cortical and subcortical regions, major vessels, the ventricular system, and any prior surgical cavity. A stereotactic biopsy was first performed to confirm the nature of the enhancing lesion. After the biopsy was completed, the biopsy needle was withdrawn, and the injection cannula was introduced along the same trajectory and to the same depth. ON-01 delivery was conducted using a custom-designed injection device (Supplementary Figure 3) developed by our team. First, 1 mL of viral suspension was slowly loaded into the device's internal tubing (total internal volume approximately 3 mL). The syringe within the injection device (Supplementary Figure 3B) was then filled with 3–5 mL of normal saline, and the injection device was connected to the flow-control module. The flow-control device (Supplementary Figure 3C) was set to a rate of 3 mL/h to gradually advance the fluid column. When the air within the tubing had been nearly fully evacuated, the system was connected to the cannula, and viral infusion into the target site was initiated. At the end of the infusion, the flow-control device was stopped, and the cannula was left in place for 15 minutes to reduce potential backflow. The cannula was then withdrawn slowly, and the scalp was closed with a single suture. A CT scan was performed 6–8 hours postoperatively to exclude hematoma. Beginning on postoperative day 1, patients received a 20-day course of oral 5-FC (100 mg/kg/day in four divided doses). No repeat intratumoral ON-01 injections were administered to the same lesion in any patient.

All patients were monitored for adverse events (AEs) for 28 days after ON-01 administration. AEs were recorded and graded according to the National Cancer

Institute Common Terminology Criteria for Adverse Events (CTCAE), version 5.0. Serious AEs (SAEs) were reported to the Ethics Committee of Beijing Tiantan Hospital within 24 hours, and emergency measures were implemented immediately to ensure patient safety. Treatment interruption was performed as necessary.

Tumor response assessment

The study was originally designed to assess tumor response using RECIST criteria. For this revision, all imaging data were re-evaluated using the RANO criteria, which are more appropriate for high-grade gliomas. The final response results reported in the manuscript are based on the RANO criteria. Brain MRI was performed at baseline, every 1 month within a half-year, every 2 months thereafter, and at any time if clinical progression was suspected. Tumor response was assessed by the investigators, including a senior neurosurgeon and a neuroradiologist. The imaging and response evaluations were independently reviewed by five central experts who were not involved in the study. There was 100% agreement among the five independent central reviewers (Supplementary Figure 4). After two months or longer of oncolytic virus therapy, response criteria (CR, PR, SD, PD) were used to evaluate outcomes, and these results were included in the Results section (Table 1 and Supplementary Table 1). Suspected pseudo progression was monitored through subsequent MRI scan monthly and if necessary, PET-CT were performed to distinguish pseudo progression through monitoring metabolic activity levels of local lesions.

Anti-edema management

Postoperative anti-edema therapy was administered according to clinical symptoms and radiographic findings. Regimens included mannitol (250 mL twice daily for 3–10 days), dexamethasone (10 mg once daily for 3–10 days), and bevacizumab (2.5 mg/kg, 1 time, administered selectively to patients with severe cerebral edema). The detailed use of these agents is summarized in Supplementary Table 4.

Immunohistochemistry

Immunohistochemical analysis was performed on biopsy samples to evaluate HVEM expression. Samples with HVEM positivity <10% were classified as low expression, whereas those with positivity ≥10% were classified as high expression. HVEM expression for all available samples is shown in Supplementary Figure 1 and Supplementary Figure 2.

TMB, MSI, POLE/D1 mutation

Genomic DNA was extracted from frozen tumor samples and assessed for quantity and integrity using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and 1% agarose gel electrophoresis. Approximately 3 μg of DNA was fragmented to 150–220 bp using a Covaris sonicator, purified, end-repaired, and ligated with Agilent adapters (SureSelect Human All Exon v6, Agilent Technologies, USA). Libraries were PCR-amplified, hybridized with custom probes, washed, eluted, and sequenced on an IDNBSEQ-T7 platform to generate 150 bp paired-end reads. Whole-

exome sequencing and analysis were performed by OE Biotech Co., Ltd. (Shanghai, China). The somatic mutations including somatic single nucleotide variants (SNVs) and somatic INDELs were screened out using MuTect2. Variants with an alternate allele depth <10 or a variant allele frequency (VAF) <0.05 were excluded. Tumor mutational burden (TMB) was calculated based on the remaining somatic mutations. TMB = Number of nonsynonymous somatic mutations in the area of coding sequence (CDS)/Length of CDS. The hg19 CDS length used was 34.3944 Mb. Filtered somatic variants in VCF format were converted to MAF format, from which mutations in the POLE or POLD1 genes were extracted and defined as POLE/POLD1 mutations. Microsatellite instability (MSI) status was assessed using MSIsensor2, based on the pre-trained machine learning model models_b37_HumanG1Kv37.

Outcomes

The primary endpoint of this study was to evaluate the short-term (28-day) safety profile of ON-01 treatment. Secondary endpoints included progression-free survival (PFS), overall survival (OS), and the 2-year OS rate. An exploratory objective was to identify tumor-related biomarkers predictive of efficacy.

Statistical analysis

All statistical analyses were conducted using SPSS (version 17.0). OS was defined as the time from ON-01 injection to death or last follow-up. Continuous variables were expressed as mean (SD) for normally distributed data, and categorical variables were

presented as percentages. Survival outcomes were estimated with the Kaplan–Meier method, and group differences were assessed with the log-rank test. All *P* values were two-sided, with $P < 0.05$ considered statistically significant.

Results

Patient characteristics

A total of 30 adult patients were enrolled to receive ON-01 treatment (Figure 1). The study population included 13 males (43.3%) and 17 females (56.7%), with a median age of 50.0 years (range, 22–75 years). Among them, 13 patients (43.3%) presented with single lesions and 17 patients (56.7%) with multifocal lesions. The inclusion criteria were expanded to allow patients with larger tumor diameters, ranging from 1 to 5 cm. The baseline characteristics of the patients are summarized in Table 1, and detailed information for each patient is provided in Supplementary Table 1. Of all patients, 6 underwent surgical resection due to tumor progression; 7 received temozolomide chemotherapy; 1 was treated with nivolumab; and 1 underwent Gamma Knife radiosurgery. Six patients underwent a second oncolytic virus injection. For Patients 1, 5, and 15, the second injection targeted recurrent lesions, whereas for Patients 13, 14, and 27, it targeted newly developed lesions. Injection sites varied according to lesion location, and surgical access was selected to ensure accurate targeting of each lesion (Supplementary Table 7). In the tumor tissues available for analysis, 8 patients (26.7%) showed HVEM expression >10%. MGMT promoter methylation was observed in 11 patients (36.7%), while 18 patients (60.0%) were

unmethylated. Regarding tumor mutational burden (TMB), 14 patients (46.7%) exhibited high TMB (≥10 mut/Mb), and 15 patients (50.0%) had low TMB (<10 mut/Mb). For microsatellite instability (MSI) status, no patient was MSI-high, whereas 29 patients (96.7%) were microsatellite stable (MSS). Analysis of POLE/D1 status revealed that 3 patients (10.0%) carried mutations, and 26 patients (86.7%) were wild type.

Safety

The safety profile of ON-01 intratumoral injection is summarized in Table 2. Treatment-related AEs were predominantly mild to moderate, with the most common being hyponatremia (33.3%), hypokalemia (33.3%), fever (20.0%), and anemia (20.0%). Grade 3 AEs occurred in two patients (6.67%): one case of pyramidal tract syndrome and one case of hyponatremia, both of which resolved with appropriate management. The patient with hyponatremia achieved electrolyte normalization before discharge, while the patient with pyramidal tract syndrome, who had recurrence in the right frontal lobe and basal ganglia, presented with left upper limb spasticity and muscle weakness but showed marked improvement within one week. No grade 4 or 5 AEs were observed during the study period. These findings indicate that ON-01 intratumoral injection has a favorable short-term (28-day) safety profile, with all AEs being clinically manageable and no severe or life-threatening complications reported.

Clinical and imaging outcomes

The median PFS among patients treated with ON-01 was 3.0 months (95% CI, 1.7–4.3) (Figure 1A), and the median OS was 12.0 months (95% CI, 10.1–13.9) (Figure 2B). The 2-year OS rate was 27.7% (95% CI, 12.6%–45.0%) (Figure 2B). Among the patients treated with ON-01, 2 patients (6.7%) achieved a complete response (CR), 6 patients (20.0%) achieved a partial response (PR), 13 patients (43.3%) had stable disease (SD), and 9 patients (30.0%) experienced progressive disease (PD). In a representative case (patient #3), a left thalamic glioma recurred 7 months after resection and was treated with ON-01 injection. Serial T1-weighted MRI demonstrated progressive shrinkage of the recurrent lesion, with near-complete resolution observed at the 18-month follow-up (Figure 3A, red arrow). However, a new lesion distant from the injection site appeared at 32 months (blue arrow), ultimately leading to patient death. Notably, 7 of 17 patients (41.2%) with multifocal lesions exhibited partial regression at both injected and non-injected sites within 2 months of ON-01 therapy. Representative examples of three typical cases are presented in Figure 3B. These findings suggest that ON-01 exerts both local oncolytic effects and potential systemic antitumor activity.

Subgroup survival analyses

To evaluate the efficacy of ON-01 across different patient populations, we conducted stratified analyses based on clinical characteristics, demographic factors, and treatment response profiles to identify potential subgroups with differential outcomes (Figure 4 and Supplementary Table 2). Recurrent grade 4 glioma with high HVEM expression demonstrated greater sensitivity to ON-01 therapy (p=0.015, 30.5 [26.7-34.3] vs. 10.0

309 [7.3-12.7] months), and therapeutic efficacy was higher in tumors measuring 1 to 3 cm
310 in diameter (p=0.007, 18.5 [7.8-29.2] vs. 8.0 [3.7-12.3] months). No statistically
311 significant difference in efficacy was observed in IDH1 status (p=0.759), MGMT
312 promotor status (p=0.185), TMB (p=0.549) and POLE/D1 status (p=0.889).
313
314 **Discussion**
315 Oncolytic viruses have emerged as a novel therapeutic approach for malignant tumors
316 following CAR-T therapy[27] and targeted agents[28], characterized by their dual
317 mechanisms of tumor lysis and immune activation. In addition to these properties, ON-
318 01, the oncolytic virus we developed, also demonstrates the ability to induce
319 intracellular chemotherapy.[19]
320 This single-arm, phase 1/2 clinical trial evaluated the safety and efficacy of ON-01
321 in 30 patients with recurrent WHO grade 4 malignant gliomas. The results demonstrated
322 a favorable short-term (28-day) safety profile with excellent patient tolerance. Most
323 patients experienced only grade 1 AEs, and no significant virus-related neurotoxicity
324 was observed. Among those treated with ON-01, the trial revealed a median OS of 12.0
325 months following recurrence. At the time of this manuscript submission, two patients
326 (Patients 17 and 19) had achieved remarkable long-term survival, exceeding 60 months
327 after ON-01 injection. Both were IDH1 wild-type and MGMT-methylated, and their
328 detailed characteristics are provided in Supplementary Table 6.
329 The use of adjunctive anti-edema agents, particularly corticosteroids, mannitol, and
330 bevacizumab, may confound both radiographic and clinical outcome assessments in

patients with recurrent malignant gliomas. Bevacizumab, in particular, has been associated with rapid reductions in contrast enhancement on MRI, raising the possibility of a "pseudo-response." In our cohort, 29 patients (96.7%) received corticosteroids, 24 (80.0%) received mannitol, and 8 (26.7%) received bevacizumab after surgery and ON-01 injection. Bevacizumab was administered selectively to patients with severe cerebral edema or disease progression at a dose of 2.5 mg/kg (1 time). To address this potential confounder, we examined the relationship between the administration of these agents and OS but found no statistically significant associations (mannitol, p=0.820; dexamethasone, p=0.890; bevacizumab, p=0.240). Furthermore, to minimize the risk of misinterpreting pseudo-response or pseudo-progression, we performed monthly follow-up MRI scans to continuously assess tumor size. Although these findings suggest that adjunctive therapies did not significantly influence survival outcomes in this study, we acknowledge that their use, particularly bevacizumab, may still complicate radiographic interpretation and represents an important limitation in evaluating therapeutic response.

Among all patients in this study, only Patient 19 was suspected to have pseudo-progression. In this patient, the oncolytic lesion (left temporal) showed a slight increase in size within 4 months after injection. After dexamethasone administration, the lesion gradually shrank and nearly resolved completely. Although the incidence was low, this case highlights the importance of careful imaging follow-up and timely intervention to differentiate true tumor progression from treatment-related effects.

In ON-01, the neurotoxic *ICP34.5* and immune evasion *ICP47* genes were deleted.

These genetic modifications resulted in significantly reduced neurotoxicity and enhanced immune-mediated viral clearance, with no virus-associated neurotoxicity observed among the 30 treated patients. The most common AEs included electrolyte disturbances and anemia, which appeared to be primarily related to cancer metabolism rather than viral therapy and were effectively managed with standard clinical interventions, and no long-term complications were reported. Other transient adverse reactions, including flushing (10%), fever (20%), thrombocytopenia (6.7%), and leukopenia (3.3%), were successfully resolved with symptomatic treatment. Neurological complications, including delirium and seizures (3.3% each), were associated with tumor recurrence and stereotactic surgical procedures[29] and resolved following appropriate therapeutic interventions. Grade 3 AEs were uncommon (6.7%) and consisted of one case of pyramidal tract signs and one case of hyponatremia, both likely procedure-related. These findings suggest that ON-01 represents a promising therapeutic option for recurrent malignant gliomas, with a safety profile characterized by the absence of significant virus-related neurotoxicity and manageable treatment-associated AEs.

The design of ON-01 was specifically tailored to address the aggressive nature and limited treatment options for malignant gliomas. Unlike conventional approaches, such as those used in G47Δ and CAN-311 modifications, ON-01 retains the *ICP6* gene to enhance viral replication efficiency within tumor cells. Additionally, incorporation of a *CD* gene enables the enzymatic conversion of the prodrug 5-FC into the cytotoxic agent 5-FU, disrupting tumor DNA synthesis. This innovative design facilitates synergistic

oncolysis through intracellular chemotherapy. Preclinical investigations further revealed that HSV-1-mediated downregulation of dihydropyrimidine dehydrogenase (DPD) sustains therapeutic concentrations of 5-FU within tumor cells, prolonging its cytotoxic effects. This unique combination of enhanced viral replication, prodrug activation, and metabolic modulation distinguishes ON-01 from other oncolytic virus platforms and may contribute to its potential therapeutic efficacy against recurrent Grade 4 malignant glioma (IDH-wt and IDH-mutated).

The randomized, open-label phase 2/3 trial of Tocagen 511[30] reported a median OS of 11.1 months, in which patients underwent tumor resection followed by Toca 511. In our non-randomized, open-label phase 1/2 trial, we employed a distinct approach with direct intratumoral injection of ON-01 and no surgical resection at the beginning of enrollment, which resulted in a median OS of 12.0 months. The fundamental distinction between ON-01 and Toca 511 lies in their viral vector characteristics. ON-01, based on an HSV-1 backbone, demonstrates inherent oncolytic activity, as evidenced by in vitro experiments: even without the *CD* gene, the parental oHSV-1 vector achieves an IC50 of 0.6 MOI against U87 glioma cells.[20] In contrast, Toca 511 is a nonlytic retroviral replicating vector. The vector of Tocagen 511 without the *CD* gene (AC3-GFP) shows negligible direct cytotoxic effects on U87 cells.[31] Preclinical investigations further revealed that HSV-1-mediated downregulation of DPD sustains therapeutic concentrations of 5-FU within tumor cells, prolonging its cytotoxic effects.[19] This combination of enhanced viral replication, prodrug activation, and metabolic modulation distinguishes ON-01 from other oncolytic virus platforms and supports its

potential success in future phase 2/3 clinical trials.

In this study, we employed stereotactic techniques to administer a single injection of 10^8 pfu of ON-01, utilizing a relatively low dose and frequency without repeated injections into the same recurrent lesion. Even at this low dosage, ON-01 demonstrated significant antitumor efficacy, with some patients experiencing substantial tumor volume reduction or complete tumor disappearance. As a novel aspect, this study is the first to include patients with multifocal recurrent gliomas. The results revealed that ON-01 not only exhibited therapeutic effects at the injection site but also induced tumor shrinkage or disappearance in distant, non-injected lesions. Regarding enrollment criteria, we expanded the tumor diameter range to 1–5 cm; however, survival analysis indicated that ON-01 was particularly effective for small tumors measuring 1–3 cm, with a median survival of 20 months. This finding suggests that oncolytic virotherapy may be especially suitable for patients with small, functionally located, inoperable tumors, offering a new therapeutic option for this population.

IDH serves as a crucial molecular marker for predicting the prognosis of patients with CNS WHO grade 4 glioma.[32] Typically, patients with IDH-mutant astrocytoma exhibit significantly better prognosis than those with IDH-wildtype GBM from the time of initial diagnosis. However, in our study of recurrent patients treated with ON-01, no significant difference in OS was observed between IDH-mutant and wildtype (p=0.759; 12.0 [6.6–17.4] vs. 12.5 [9.1–15.9] months). These findings are consistent with previous reports indicating that IDH1 mutation status does not significantly impact survival outcomes in patients with recurrent high-grade glioma.[33] Similarly, other

oncolytic virus clinical trials have also shown that IDH1 status is not associated with OS.[14,34] These findings indicate that IDH mutation is associated with longer survival from the time of diagnosis, but no difference is observed after tumor recurrence.

Through further subgroup analysis, we identified another key prognostic indicator—HVEM. The data demonstrated that HVEM-positive patients had significantly longer survival, a phenomenon potentially related to the mechanism of action of oncolytic viruses. Specifically, as the primary entry receptor for HSV-1, high expression of HVEM in tumor tissues facilitates more efficient viral entry into tumor cells. In contrast, IDH mutation status does not influence the ability of oncolytic viruses to enter tumor cells, thus failing to result in significant differences in patient survival. This important finding suggests that HVEM may serve as a clinically valuable biological marker for future ON-01 therapy, particularly in guiding HSV-1-based oncolytic viral treatment strategies.

This study enrolled patients with multifocal intracranial tumors to investigate the effects of oncolytic virus therapy. The results demonstrated that ON-01 not only induced significant oncolytic responses at the injection sites but also led to volume reduction or even complete disappearance of tumors at distant locations. However, the study did not explore systemic and local immune responses in depth, as only preliminary foundational research was conducted. In subsequent clinical studies, we plan to incorporate analyses of immune-related indicators to further elucidate the underlying mechanisms.

Regarding the relationship between the efficacy of ON-01 and molecular

pathological IDH subtypes, there was no significant correlation. However, only five patients with IDH mutations were included, resulting in a relatively small sample size. Future studies should expand the cohort of IDH-mutant patients to further validate the reliability of this conclusion.

The 28-day monitoring period for AEs in this study may be inadequate to fully capture ON-01-related delayed toxicities or cumulative effects, particularly in long-term survivors. We will implement more comprehensive long-term safety monitoring in subsequent studies to address this limitation.

In terms of study design, as a phase 1/2 clinical trial, this study primarily focused on short-term safety assessment and preliminary efficacy observation and therefore did not adopt a randomized controlled design. This decision was based on two considerations: first, the use of stereotactic injection, an invasive procedure, made it technically challenging to implement a double-blind design; second, from an ethical perspective, administering placebo injections would not align with medical ethical principles. These limitations will be addressed in subsequent larger-scale clinical trials.

In conclusion, the results demonstrated a favorable short-term safety profile of ON-01 intratumoral injection and provides preliminary evidence of its efficacy in patients with recurrent WHO grade 4 malignant gliomas.

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Conflict of Interest

None declared.

Authorship Statement

FSL and JWZ designed the study. WZ curated the data. PWW and SF performed the formal analysis. FSL acquired funding. FSL and WZ contributed to methodology. FSL and JWZ were responsible for project administration. SQG developed the software to aid data collection. WZ, PWW, and SF contributed to data visualization and drafted the original manuscript. WFJ, SRP, MYW, XYQ, WXZ, JJG, XDS, GQY, JKW, YDL, YWW, HCL, RM, FW, QC, GSJ, and FGM were involved in data investigation. All authors reviewed, edited, and approved the final manuscript. SQG performed the statistical analysis and directly verified the underlying data in the manuscript. All authors had full access to all the data in the manuscript. FSL had final responsibility for the decision to submit for publication.

Data Availability

Access to the data will be granted upon submission of a formal application, including a study proposal, to the study's steering committee through the corresponding authors

of this Article. Data sharing is contingent upon approval by both the steering committee and the institutional review board.

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586

587 **Figure 1. Trial profile.**

588 [a]Patient No.9 was lost to follow-up in the fifth month. [b]Patient No.4 passed away due
589 to pneumonia in the fourth month and patient No.5 passed away due to a
590 cerebrovascular accident in the seventeenth month.

591

592 **Figure 2. Survival outcomes in patients treated with ON-01.**

593 (A) Progression-free survival in the population treated with ON-01. (B) Overall
594 survival (OS) in all patients treated with ON-01. Shaded areas indicate 95% CIs. Black

Crosses denote censored patients, while red Crosses denote patients alive.

Figure 3. Summary of radiological responses in patients treated with ON-01.

(A) T1-weighted MRI images at the indicated observation time points for Patient No. 3. Following surgical resection of a left thalamic glioma, the patient experienced recurrence after 7 months and subsequently received an ON-01 injection (Figure 3A, red arrow). Serial follow-up MRI examinations demonstrated progressive regression of the recurrent lesion following ON-01 therapy, with near-complete resolution observed at the 18-month follow-up. Subsequent imaging at 32 months post-treatment identified a new lesion at a site distal to the initial ON-01 injection site (Figure 3A, blue arrow), which ultimately led to patient mortality. (B) T1-weighted MRI images at the indicated observation time points for three patients with multifocal lesions. All three patients demonstrated varying degrees of regression at both injected and non-injected lesion sites within 2 months of ON-01 therapy (Figure 3B; injection sites: red arrows, non-injected lesions: yellow arrows). mo, months; wk, weeks.

Figure 4. Univariate survival analysis of patients in different groups.

(A) OS of patients with different HVEM expression levels. (B) OS of patients grouped by tumor diameter. (C) OS of patients based on detectable IDH1 status. (D) OS of patients based on MGMT promotor status. (E) OS of patients with either single or multifocal lesions. Crosses denote censored patients.

Table 1. Baseline characteristics for all patients.

Abbreviations: KPS, karnofsky performance status; IDH1, isocitrate dehydrogenase 1; HVEM, herpesvirus Entry Mediator; PD-1, programmed death receptor 1; MGMT, O^6-methylguanine-DNA methyltransferase; TMB, tumor mutational burden; MSI, microsatellite Instability; MSS, microsatellite stable; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

Table 2. Adverse Events, According to Grade, in the 30 Patients.

Table 1

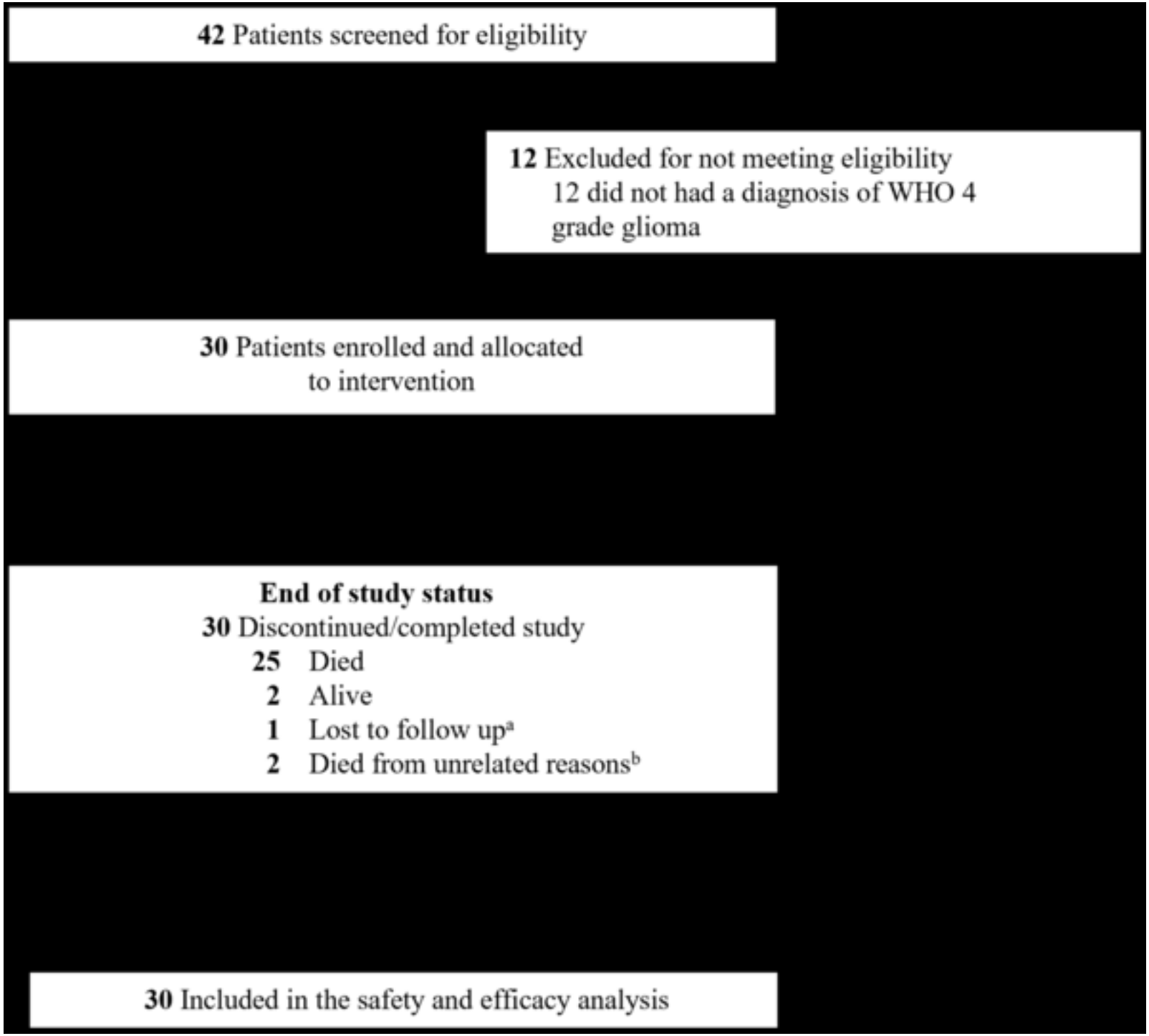
Table 1. Baseline characteristics for all patients.

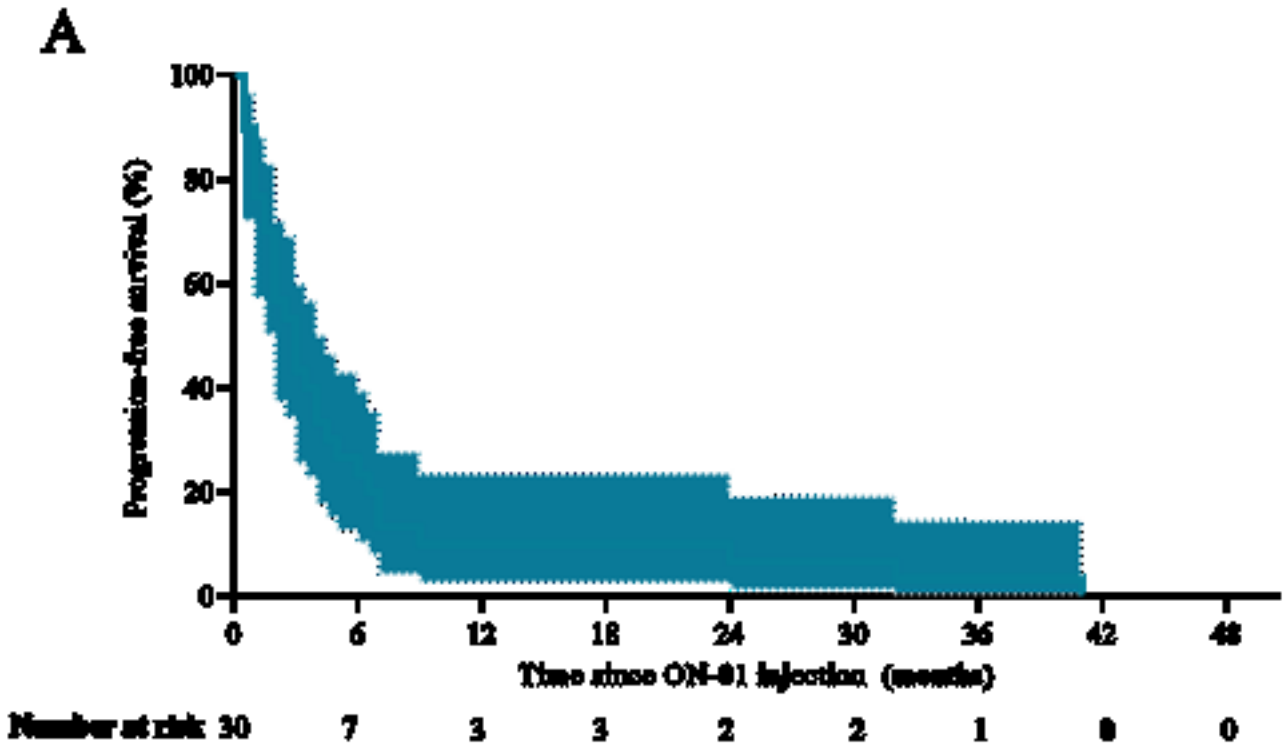
Clinical characteristics	No. of patients (%)
Gender	
Male	13 (43.3)
Female	17 (56.7)
Age at ON-01 initiation, median (range), yrs	50.0 (22–75)
KPS score, median (range)	80 (60–100)
Number of lesions	
Single	13 (43.3)
Multiple	17 (56.7)
IDH1 status	
Mutant	5 (16.7)
Wild type	24 (80.0)
N/A	1 (3.3)
HVEM expression	
Positive	8 (26.7)
Negative	19 (63.3)
N/A	3 (10.0)
MGMT promotor status	
Methylated	11 (36.7)
Unmethylated	18 (60.0)
N/A	1 (3.3)
TMB	
High (≥10 mut/Mb)	14 (46.7)
Low (<10 mut/Mb)	15 (50.0)
NA	1 (3.3)
MSI status	
MSI-high	0 (0)
MSS	29 (96.7)
NA	1 (3.3)
POLE/D1 status	
Mutant	3 (10.0)
Wild type	26 (86.7)
N/A	1 (3.3)
Response to treatment	
CR	2 (6.7)
PR	6 (20.0)
PD	9 (30.0)
SD	13 (43.3)
Retreatment of recurrence post ON-01 injection	
ON-01 re-injection	6 (20.0)
Temozolomide	7 (23.3)
Anti-PD-1 antibody	1 (3.3)
Gamma Knife	1 (3.3)

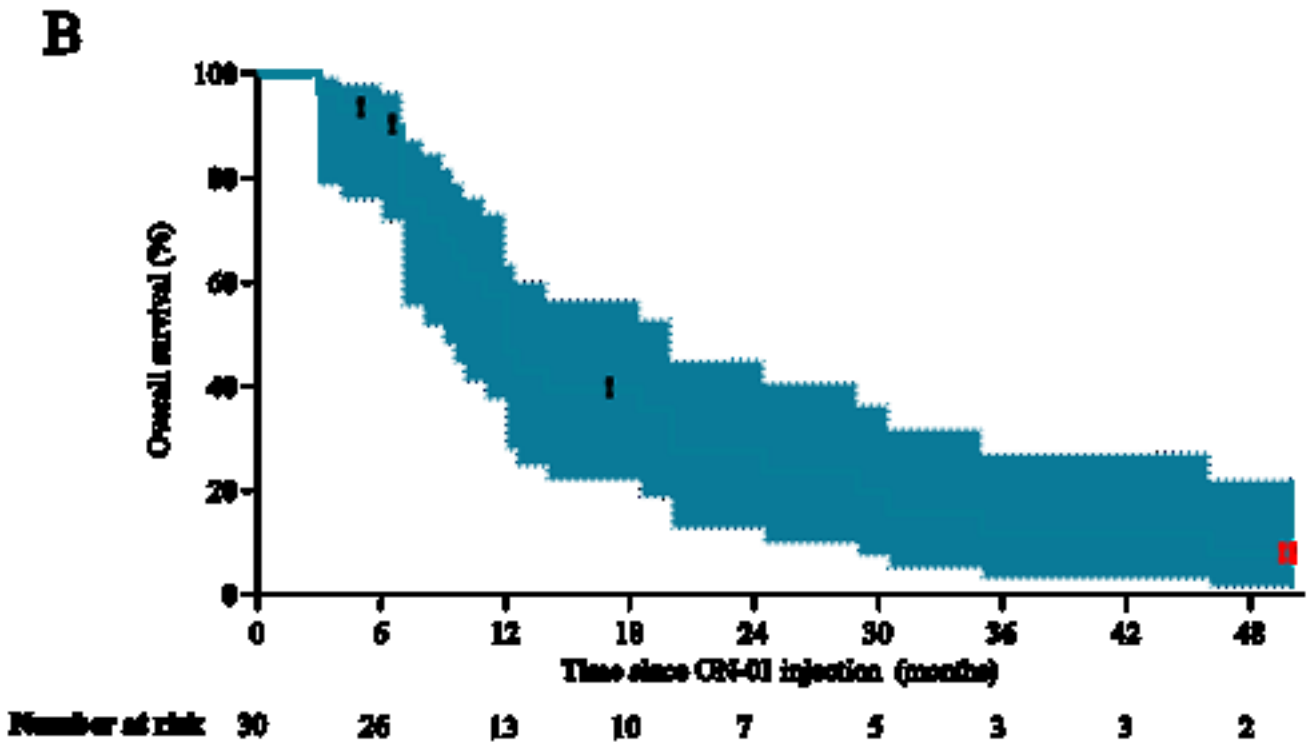
Re-operation	6 (20.0)

Table 2. Adverse Events, According to Grade, in the 30 Patients.

Adverse Event, n (%)	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5
General disorder					
Fever	5 (16.7%)	1 (3.3 %)	/	/	/
Gastrointestinal disorder					
Nausea	1 (3.3 %)	/	/	/	/
Vascular disorder					
Flushing	3 (10.0%)	/	/	/	/
Cardiac disorder					
Ventricular Arrhythmia	1 (3.3%)	/	/	/	/
Nervous system disorders					
Seizure	/	1 (3.3%)	/	/	/
Pyramidal tract syndrome	/	/	1 (3.3%)	/	/
Psychiatric disorder					
Delirium	/	2 (6.7%)	/	/	/
Blood and lymphatic system disorder					
Anemia	5 (16.7%)	1 (3.3 %)	/	/	/
White blood cell count decreased	1 (3.3 %)	/	/	/	/
Platelet count decreased	2 (6.7%)	/	/	/	/
Metabolism and nutrition disorder					
Hyponatremia	7 (23.3%)	2 (6.7%)	1 (3.3 %)	/	/
Hypokalemia	8 (26.7%)	2 (6.7%)	/	/	/
Hypocalcemia	3 (10.0%)	3 (10.0%)	/	/	/







Total No. of Patients	No. of Deaths	Median Survival (95%CI)	Survival Rate% (95% CI) 6 mo	12 mo	18 mo	24 mo	30 mo	36 mo
30	25	12.0 mo (10.1-13.9)	89.9 (71.9-96.6)	46.7 (27.8-63.6)	39.5 (21.9-56.9)	27.7 (12.6-45.0)	19.8 (7.4-36.5)	11.9 (3.0-27.2)

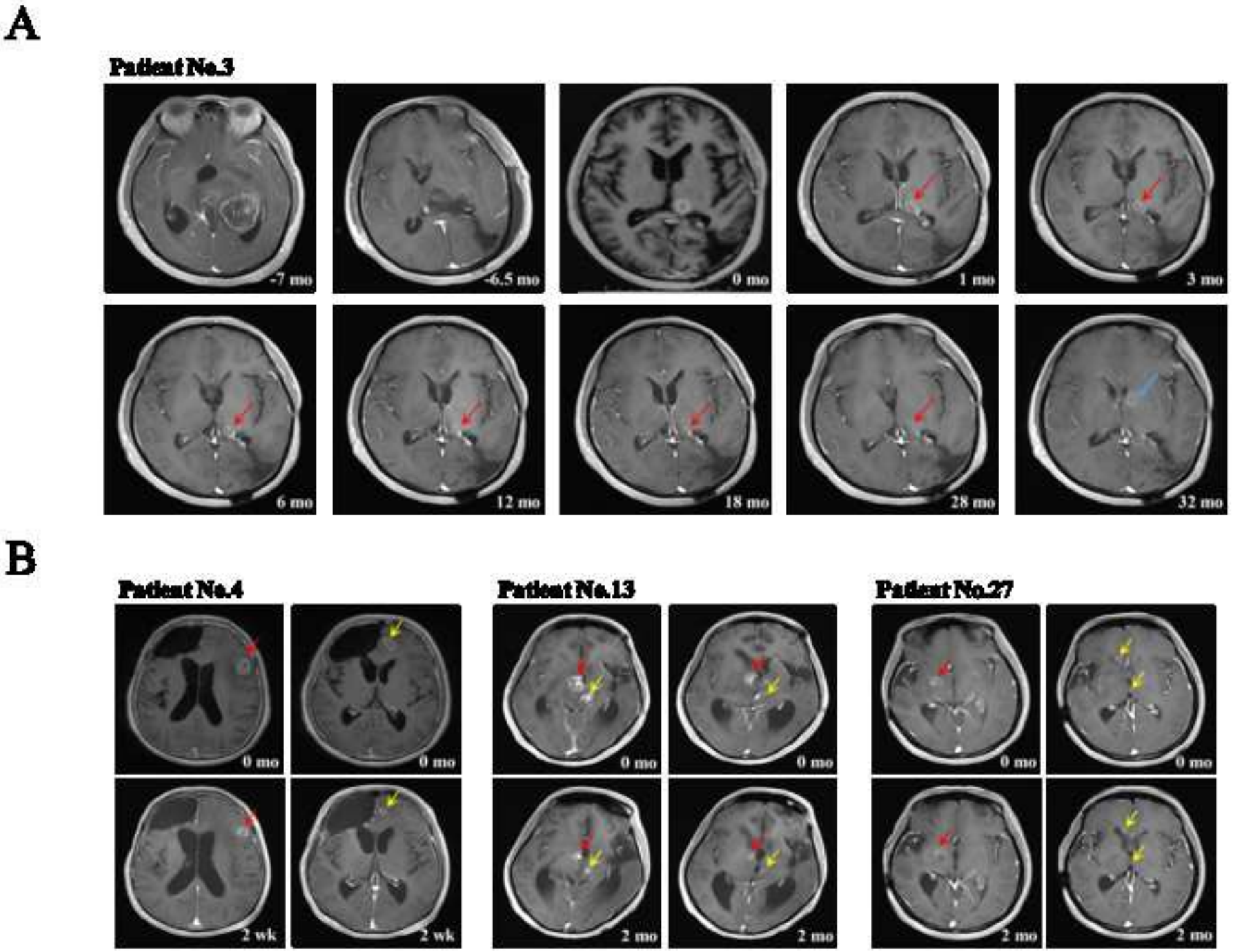


Figure 4