Neural stem cell delivery of an oncolytic adenovirus in newly diagnosed malignant glioma: a first-in-human, phase 1, dose-escalation trial

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Summary

Background Malignant glioma is the most common and lethal primary brain tumour, with dismal survival rates and no effective treatment. We examined the safety and activity of NSC-CRAd-S-pk7, an engineered oncolytic adenovirus delivered by neural stem cells (NSCs), in patients with newly diagnosed high-grade glioma.

Methods This was a first-in-human, open-label, phase 1, dose-escalation trial done to determine the maximal tolerated dose of NSC-CRAd-S-pk7, following a 3+3 design. Patients with newly diagnosed, histologically confirmed, high-grade gliomas (WHO grade III or IV) were recruited. After neurosurgical resection, NSC-CRAd-S-pk7 was injected into the walls of the resection cavity. The first patient cohort received a dose starting at $6·25×10^{10}$ viral particles administered by $5·00×10^{7}$ NSCs, the second cohort a dose of $1·25×10^{11}$ viral particles administered by $1·00×10^{8}$ NSCs, and the third cohort a dose of $1·875×10^{11}$ viral particles administered by $1·50×10^{8}$ NSCs. No further dose escalation was planned. Within 10–14 days, treatment with temozolomide and radiotherapy was initiated. Primary endpoints were safety and toxicity profile and the maximum tolerated dose for a future phase 2 trial. All analyses were done in all patients who were included in the trial and received the study treatment and were not excluded from the study. Recruitment is complete and the trial is finished. The trial is registered with ClinicalTrials.gov, NCT03072134.

Findings Between April 24, 2017, and Nov 13, 2019, 12 patients with newly diagnosed, malignant gliomas were recruited and included in the safety analysis. Histopathological evaluation identified 11 (92%) of 12 patients with glioblastoma and one (8%) of 12 patients with anaplastic astrocytoma. The median follow-up was 18 months (IQR 14–22). One patient receiving $1·50×10^{8}$ NSCs loading $1·875×10^{11}$ viral particles developed viral meningitis (grade 3) due to the inadvertent injection of NSC-CRAd-S-pk7 into the lateral ventricle. Otherwise, treatment was safe as no formal dose-limiting toxicity was reached, so $1·50×10^{8}$ NSCs loading $1·875×10^{11}$ viral particles was recommended as a phase 2 trial dose. There were no treatment-related deaths. The median progression-free survival was 9·1 months (95% CI 8·5–not reached) and median overall survival was 18·4 months (15·7–not reached).

Interpretation NSC-CRAd-S-pk7 treatment was feasible and safe. Our immunological and histopathological findings support continued investigation of NSC-CRAd-S-pk7 in a phase 2/3 clinical trial.

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Introduction

High-grade malignant gliomas are the most common and lethal CNS tumours in adults. Despite aggressive therapeutic regimens that comprise neurosurgical resection, radiotherapy, and chemotherapy, median survival time in patients with newly diagnosed glioblastoma ranges from 14 months to 21 months.[1,2] The presence of aberrant chemoresistant and radioresistant glioma stem cells within the tumour tissue contributes to relapse and poor survival outcomes,[3] whereby the median survival time upon tumour recurrence is typically 9–11 months.[4,5] As such, a dynamic approach that targets dispersed tumour cells and resistant glioma stem cells, without disrupting the delicate neural architecture in the brain, is necessary for effective treatment.[6]

Oncolytic adenoviral therapy is a promising therapeutic approach in malignant glioma owing to its direct viral oncolytic effects and its ability to elicit an immune response. It has also been proven to be safe and tolerable in clinical trials.[7,8] Nevertheless, oncolytic viruses have been shown to have poor distribution and spread through the tumour mass and had limited abilities to effectively cross the blood–brain barrier.

Neural stem cells (NSCs) are multipotent progenitor cells that originate from the developing and adult CNS.[9] Preclinical experiments have shown their inherent ability

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Research in context

Evidence before this study

On Jan 23, 2021, we searched PubMed, without any language or date restrictions, using the term: "glioma" OR "glioblastoma" AND "stem cells" OR "neural stem cells" AND "oncolytic virus" OR "oncolytic adenovirus". We found no trials that used neural stem cells (NSCs) for the delivery of an oncolytic adenovirus in patients with glioma. Previous research has described the safety and activity of oncolytic viruses in patients with recurrent gliomas; however, their tumour-targeting properties were limited by decreased dissemination and infiltration of the viruses in the tumour tissue. NSCs have been shown to effectively migrate and disseminate in the brain to target glioma cells. This finding provided the rationale for the use of NSCs to deliver an engineered oncolytic adenovirus in patients with high-grade malignant glioma.

Added value of this study

To the best of our knowledge, this is the first-in-human trial to test the neural stem cell delivery of an engineered oncolytic adenovirus. Additionally, it is distinctive among neuro-oncology and virotherapy trials, because it includes patients with newly diagnosed gliomas, rather than recurrent disease. This phase 1 trial showed that NSC-CRAd-S-pk7 was safe and tolerable in patients with high-grade malignant gliomas and immediate start of standard chemoradiotherapy was feasible without undue delay or complications. Additionally, NSC-CRAd-S-pk7 elicited an immune-mediated anti-glioma response and showed preliminary antitumour activity, especially in patients with gliomas with unmethylated *MGMT* promoters.

Implications of all the available evidence

This trial provides the foundation for a phase 2/3, controlled trial, in which survival outcomes in response to NSC-CRAd-S-pk7 can be evaluated in a larger cohort of patients. Multiple injections of NSC-CRAd-S-pk7 through an intracranial catheter or its combination with checkpoint inhibitors, or other immune modulatory therapies could increase clinical benefit.

to cross the blood–brain barrier, distribute within the tumour bed, surround the tumour border, and migrate within the brain parenchyma to target glioma cells.[10] Additionally, the tumour tropism of NSCs can be used to deliver therapeutic molecules across the blood–brain barrier.[11]

In previous work, we engineered an oncolytic adenovirus, CRAd-S-pk7, by incorporating a survivin promoter to drive expression of the E1A gene, which is essential for viral replication, and modifying the Ad5 fibre protein through the incorporation of a poly-lysine sequence (pk7).[12] These alterations enhanced viral replication and targeting of glioma cells, which improved antitumour activity and increased survival in mouse and hamster models.[12,13] Using an NSC cell line, HB1.F3.CD21, which is approved by the US Food and Drug Administration as a cell carrier for human clinical trials,[14] we were able to deliver a therapeutic payload of CRAd-S-pk7 in experimental glioblastoma mouse models.[15] This approach combined the tumour tropism of NSCs with the enhanced ability of CRAd-S-pk7 to target chemoresistant and radioresistant glioma stem cells.[16] Preclinical work showed that mice treated with NSC-CRAd-S-pk7, the engineered oncolytic virus delivered by the NSCs, had 50% longer median survival compared with mice that were treated by the oncolytic virus alone.[15] Additionally, NSCs were capable of migrating throughout the brain and delivering the therapeutic payload of CRAd-S-pk7 to targeted glioma cells.[15] These findings warranted the translation of this therapeutic approach to the clinical setting. Therefore, in this study, we aimed to determine the safety and activity of NSC-CRAd-S-pk7 in patients with newly diagnosed high-grade gliomas.

See Online for appendix

Methods

Study design and participants

We did an open label, phase 1, dose-escalation trial that followed a 3+3 design. It was primarily done at the Northwestern Memorial Hospital (Chicago, IL, USA), with a secondary site at the City of Hope National Medical Center (Duarte, CA, USA).

We enrolled patients with newly diagnosed high-grade malignant glioma (WHO grade III or IV), confirmed through clinical and radiological evaluation. Pathological confirmation of malignant glioma was made at the time of resection on frozen section by a neuropathologist before NSC-CRAd-S-pk7 injection. Diagnoses made through frozen section analysis were later confirmed through permanent section analysis. It was planned that patients would receive standard chemoradiotherapy, and their tumours had to be accessible for injection. Eligible patients were aged 18 years or older and had a Karnofsky performance scale score of 70 or more. To be included, participants also had to have adequate organ and bone marrow function within 28 days before registration, as defined by an aspartate transaminase concentration less than three times the upper limit of normal, serum creatinine less than 2 mg/dL, platelets more than 100 000 per mm³, and white blood cells more than 3000 per mm³. Further baseline evaluations comprised panels for haematology, coagulation, and serum chemistry, a urinalysis with microscopy, an ECG, replication competent retrovirus testing, and viral shedding. Eligible participants were also able to undergo a brain MRI scan (see protocol in the appendix).

Patients were excluded if the tumour invaded the ventricular system, had received previous radiotherapy or other experimental therapy, or were taking

immunosuppressive medications (other than corticosteroids) within 28 days of the surgical procedure. Patients with previous or ongoing liver disease (cirrhosis, or active hepatitis B or C virus infection) or known HIV infection were also excluded. Details of the inclusion and exclusion criteria are provided in the protocol.

City of Hope National Medical Center provided the HB1.F3.CD21 NSCs for the clinical trial. CRAd-S-pk7 was produced and loaded into HB1.F3.CD21 cells at the University of Alabama at Birmingham Vector Production Facility (Birmingham, AL, USA), in accordance with current good manufacturing practice for phase 1 investigational drugs. Regulatory approvals were obtained from the Center for Biologics Evaluation and Research of the US Food and Drug Administration and the local institutional research ethics committees (FDA IND 17365). The study was done in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. This trial was done in compliance with the Data Safety Monitoring Plan of the Robert H Lurie Comprehensive Cancer Center of Northwestern University (Chicago, IL, USA). A data safety monitoring board (DSMB) was instituted to review any complications arising from the proposed therapy before the enrolment of new patients. Additionally, the study abided by the safety reporting regulations, as set forth in the Code of Federal Regulations. All protocol amendments were approved by the trial sponsor and the DSMB. All participants provided written, informed consent.

Procedures

Patients with resectable disease received NSC-CRAd-S-pk7 injection into the tumour bed after surgical resection. Pathological confirmation of a malignant glioma had to be made at the time of surgery before injection. Freehand injections of 100 µL of NSC-CRAd-S-pk7 were done in up to ten sites in the wall of the resection cavity. The sites were at least 1 cm apart and were selected by the surgeon to avoid injections into adjacent motor or speech cortices, leakage into cerebral ventricles, or spillage into the subarachnoid space. The dose-escalation phase started with an initial three patients enrolled per dose, and a further three patients recruited after review by the DSMB. The lowest dose was based on a previous human study, in which $5{\cdot}00\times10^{7}$ NSCs were injected and were found to be safe.[14] The dose at which no more than one of the six patients had a dose-limiting toxic effect was defined as the recommended phase 2 dose. Three doses were evaluated. The first cohort (n=3) received a dose of $5{\cdot}00\times10^{7}$ NSCs loading $6{\cdot}25\times10^{10}$ viral particles, the second cohort (n=3) received a dose of $1{\cdot}00\times10^{8}$ NSCs loading $1{\cdot}25\times10^{11}$ viral particles, and the third cohort (n=6) received a dose of $1{\cdot}50\times10^{8}$ NSCs loading $1{\cdot}875\times10^{11}$ viral particles. No further dose escalation was planned. After the injections were completed, the remainder of the surgery consisted of routine wound closure. Standard radiotherapy and chemotherapy (see protocol in the appendix) began 10–14 days after surgery and NSC-CRAd-S-pk7 injection. All patients were maintained on low-dose steroids throughout the treatment regimen; however, the dose was tapered down whenever possible (appendix p 21). Long-term follow-up of general health status and any side-effects continued for life. Patients were able to withdraw from the study at any time. Patients could also be taken off the study treatment or study as a whole at the discretion of the investigator for safety, behavioural, or administrative reasons.

Disease assessment was done through medical history, MRI, blood counts, and chemistry values at screening and follow-ups. MRI assessments were done at screening and after surgery and product injection at days 2, 28, 56, and every 8 weeks thereafter. Clinical laboratory monitoring was done at screening, day 0 (the day of surgery and product injection), and at specific timepoints thereafter. Haematology and serum chemistry panels were checked at screening and days 0, 2, 3, 7, 14, 28, 42, 56, and every 8 weeks thereafter. Coagulation panels were assessed at screening and days 0, 3, 7, and 14. Blood sampling for cytokine profiling and immune studies was done at days 0, 3, 7, 14, and 28; additional samples were collected at day 56 and after day 200 for immune explorations. Urine analysis and microscopy were done at screening and at days 0 and 14. Urine or serum pregnancy test was done at screening and at day 0.

Adverse events were graded using Common Terminology Criteria for Adverse Events (version 4.03), and the association with NSC-CRAd-S-pk7 was assessed. Dose-limiting toxicity was defined as any NSC-CRAd-S-pk7-related, non-haematological adverse event of grade 3 or more occurring from surgery until the end of chemoradiotherapy. Adverse events were reported in a routine manner at scheduled times during the trial at screening and at days 0, 1, 2, 3, 7, 14, 28, and 56. The DSMB reviewed any complications arising from the therapy before the enrolment of new patients. All patients with an adverse event, regardless of its association with the study drug, were followed up until the adverse event resolved or the symptoms or signs that constituted the adverse event returned to baseline, any abnormal laboratory values returned to baseline, there was a satisfactory explanation other than the study drug for the changes observed, or death.

Quality of life was evaluated using the summary score on a patient self-report measure, Functional Assessment of Cancer Therapy—Brain component (FACT-Br) version 4.[17] Assessments were done less than 7 days from day 0, once during radiotherapy administration, 2–4 weeks after completion of radiotherapy, and every 3 months afterwards for the first year or until disease progression.

Blood collection for immunological research studies, such as lymphocytic infiltration studies and serum cytokine profiling, was done before surgery and product injection, on the day of surgery and product injection, after surgery and product injection, and after radiotherapy.

	Total evaluable (N=12)	Cohort 1 (N=3)	Cohort 2 (N=3)	Cohort 3 (N=6)
Age, years				
Mean (SD)	54 (14)	58 (10)	55 (10)	52 (18)
Median (IQR)	52 (48–65)	63 (55–64)	50 (49–58)	52 (47–65)
Sex				
Female	7 (58%)	2 (67%)	2 (67%)	3 (50%)
Male	5 (42%)	1 (33%)	1 (33%)	3 (50%)
Ethnicity				
Non-Hispanic	12 (100%)	3 (100%)	3 (100%)	6 (100%)
Hispanic	0	0	0	0
Race				
Asian	1 (8%)	0	1 (33%)	0
Black	1 (8%)	0	0	1 (17%)
White	10 (83%)	3 (100%)	2 (67%)	5 (83%)
Karnofsky Score				
70	1 (8%)	1 (33%)	0	0
80	1 (8%)	0	1 (33%)	0
90	7 (58%)	2 (67%)	2 (67%)	3 (50%)
100	3 (25%)	0	0	3 (50%)
Glioma subtype				
Glioblastoma (WHO grade IV)	11 (92%)	3 (100%)	3 (100%)	5 (83%)
Anaplastic astrocytoma (WHO grade III)	1 (8%)	0	0	1 (17%)
MGMT promoter				
Methylated	3 (25%)	1 (33%)	0	2 (33%)
Unmethylated	9 (75%)	2 (67%)	3 (100%)	4 (67%)
IDH1/2				
Mutant	2 (17%)	0	0	2 (33%)
Wild	10 (83%)	3 (100%)	3 (100%)	4 (67%)
Median follow-up, months (IQR)*	18 (14–22)	24 (21–30)	16 (14–18)	16 (12–21)
Number of surgeries				
1	6 (50%)	1 (33%)	2 (67%)	3 (50%)
2	6 (50%)	2 (67%)	1 (33%)	3 (50%)

Data are n (%) unless otherwise specified. *Follow-up time is calculated from day of surgery and NSC-CRAd-S-pk7 injection.

Table 1: **Demographic and baseline clinical characteristics in evaluable population**

Details on blood processing, flow cytometry, viral titrations, serum biomarker analysis, and enzyme-linked immunospot (ELISpot) analysis are presented in the appendix (pp 3–4).

Post-treatment surgical specimens were immunostained for adenovirus proteins and for tumour-specific proteins. Autopsy examination of the brains of four consenting patients allowed histopathological assessment of the tumour microenvironment. Samples were collected from various regions throughout the brain for evaluation (appendix p 7). A nested PCR amplification of *v-myc* DNA from brain specimens was done to check for the presence of NSCs after autopsy. Multiplex staining was done in tumor tissue samples of three patients for detection of immune cells before and after NSC-CRAd-S-pk7 injection. Further details are presented in the appendix (pp 4–6).

Outcomes

The primary endpoints were safety and toxicity profile and the maximum tolerated dose for a future phase 2 trial of NSC-CRAd-S-pk7 in patients with high-grade gliomas. The secondary endpoints were objective tumour response determined by the iRANO criteria,[18] progression-free survival (time from surgery and product injection to first confirmed disease progression as determined by the objective tumour response), overall survival (calculated from the time of surgery and product injection to death), and quality of life of patients while on treatment. Exploratory endpoints aimed to evaluate cytokine profiles and blood immune responses and to determine whether overall survival rates correlated with the extent of the immune response.

Statistical analysis

This study had a 3+3 design, so the final sample size would depend on the real-time response of patients for each dose level, whereas the maximum sample size is usually six times the number of doses. All analyses were done in the safety population (n=12), which was defined as all patients who received at least one dose of NSC-CRAd-S-pk7. Adverse events were summarised by term and grade. Participants who had the same event on more than one occasion were counted once in the event frequency. Objective response rates and two-sided Clopper-Pearson 95% CIs were estimated. To visualise the total evaluable population, Kaplan-Meier curves were plotted for both progression-free survival and overall survival. Post-hoc analyses were done to visualise subgroups on the basis of NSC-CRAd-S-pk7 dose, *IDH1/2* mutation status, and *MGMT* methylation status, and to assess neutrophils, monocytes, and lymphocytes in the blood samples of dose cohorts between days 3 and 14 using the student's paired *t* test. GraphPad Prism version 8 and R version 4.0.2 were used for statistical analyses, with survival (version 3.2–3), survminer (version 0.4.8), and GenBinomApps (version 1.1) packages.

This trial is registered with ClinicalTrials.gov, NCT03072134.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Between April 24, 2017, and Nov 13, 2019, 13 patients were screened. 12 patients were enrolled and treated with NSC-CRAd-S-pk7 according to the assigned dose (n=12; appendix p 22). One patient was excluded, because their tumour invaded the ventricular system. At the time of analysis on March 3, 2021, median follow-up was 18 months (IQR 14–22).

Histopathological evaluation identified 11 (92%) of 12 patients with glioblastoma and one (8%) with

	Grade 1	Grade 2	Grade 3
All adverse events*			
Lymphocyte count decreased	3 (25%)	3 (25%)	5 (42%)
Headache	4 (33%)	5 (42%)	1 (8%)
Hypoalbuminaemia	7 (58%)	3 (25%)	0
Anaemia	7 (58%)	2 (17%)	0
Fatigue	7 (58%)	2 (17%)	0
Nausea	8 (67%)	0	0
Hypertension	1 (8%)	2 (17%)	5 (42%)
Hyperglycaemia	1 (8%)	5 (42%)	1 (8%)
Hyponatraemia	4 (33%)	0	3 (25%)
Alopecia	5 (42%)	2 (17%)	0
Constipation	4 (33%)	2 (17%)	0
Aspartate aminotransferase increased	6 (50%)	0	0
Platelet count decreased	5 (42%)	1 (8%)	0
Sinus bradycardia	5 (42%)	0	0
Sinus tachycardia	5 (42%)	0	0
Alanine aminotransferase increased	5 (42%)	0	0
Muscle weakness	0	1 (8%)	4 (33%)
Vomiting	4 (33%)	0	0
Fever	3 (25%)	1 (8%)	0
Thromboembolic event	1 (8%)	2 (17%)	1 (8%)
Hypocalcaemia	3 (25%)	0	0
Hypophosphataemia	0	3 (25%)	0
Insomnia	1 (8%)	2 (17%)	0
Rash maculo-papular	3 (25%)	0	0
Hypotension	3 (25%)	0	0
NSC-CRAd-S-pk7-related adverse events			
Meningitis†	0	0	1 (8%)
Subdural fluid collection‡	0	1 (8%)	0

Data are n (%). Two grade 4 events, encephalopathy and cerebral oedema, were reported in one patient. *Adverse events occurring in more than 2 patients are presented. †Probably related. ‡Possibly related.

Table 2: **Adverse events in evaluable patients (N=12)**

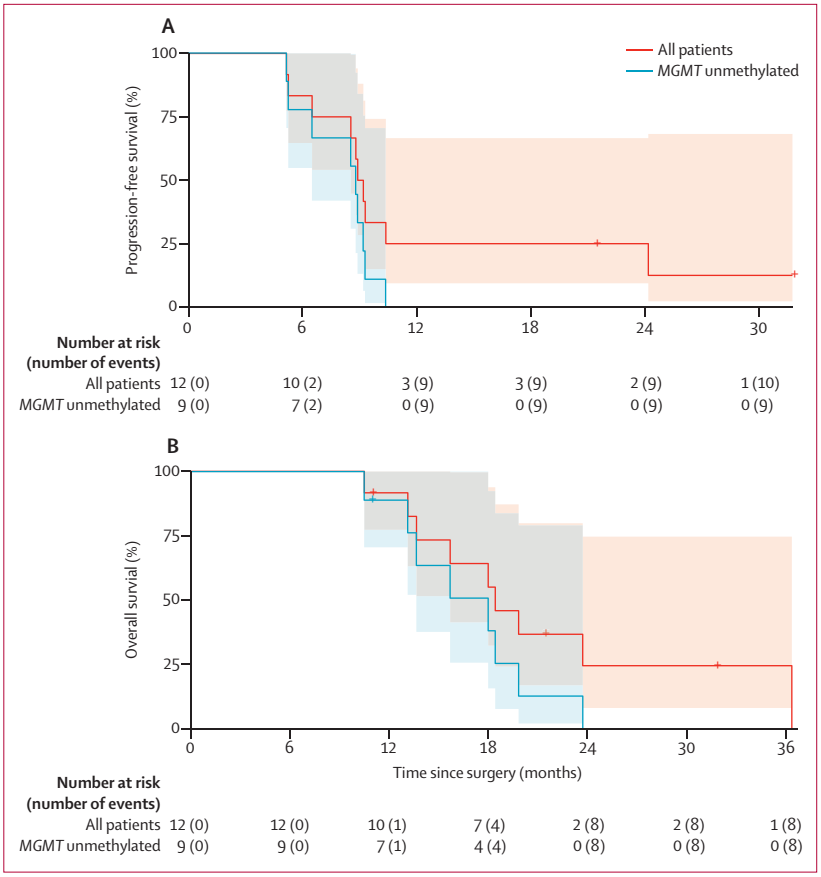


Figure 1: **Survival outcomes in patients treated with NSC-CRAd-S-pk7**
(A) Progression-free survival in all evaluable patients and in the subset of patients with high-grade gliomas containing an unmethylated *MGMT* promoter. (B) Overall survival in all evaluable patients and in the subset of patients with high-grade gliomas containing an unmethylated *MGMT* promoter. Shaded areas represent 95% CIs. Crosses denote censored patients.

anaplastic astrocytoma (table 1). Two (17%) of 12 tumours harboured an *IDH1* mutation. The *MGMT* gene promoter was methylated in three (25%) of 12 patients, including the two *IDH1*-mutated tumours.

One (17%) of six patients taking the third dose ($1{\cdot}50\times10^{8}$ NSCs loading $1{\cdot}875\times10^{11}$ viral particles) developed a grade 2 subdural fluid collection 22 days after surgery and product injection that was deemed possibly related to NSC-CRAd-S-pk7 administration. Another patient (17%) of the six taking the third dose developed meningitis (grade 3) due to the inadvertent injection of NSC-CRAd-S-pk7 into the ventricle. Cerebrospinal fluid trickled into the open ventricle, collection and subsequent analysis of which was consistent with viral meningitis. After hospitalisation, the patient fully recovered. Subsequently, three additional patients were enrolled at the same dose without major toxicity and complications. This was the highest prespecified dose, a formal dose limiting toxicity was not observed, and $1{\cdot}50\times10^{8}$ NSCs loading $1{\cdot}875\times10^{11}$ viral particles was recommended for phase 2. Details on trial dosage are provided in the appendix (p 8).

Most treatment-emergent adverse events were not related to NSC-CRAd-S-pk7 and all were commonly observed toxicities of subsequent chemotherapy and radiotherapy (table 2). The most common grade 3 adverse events were decreased lymphocyte count (five [42%] of 12 patients), hypertension (five [42%]), and muscle weakness (four [33%]). A full record of reported adverse events is presented in the appendix (pp 9–13). Five severe adverse events were reported, including a thromboembolic event, encephalopathy, cerebral oedema, muscle weakness, and meningitis. Only viral meningitis was probably related to NSC-CRAd-S-pk7 (appendix p 14). All patients recovered fully from their adverse events, and there were no dropouts or deaths due to an adverse event.

After resection, residual evaluable tumour was present in nine (75%) of 12 patients. Assessment of best response showed that one (8%) of 12 patients had a partial response, one (8%) of 12 patients had pseudo-

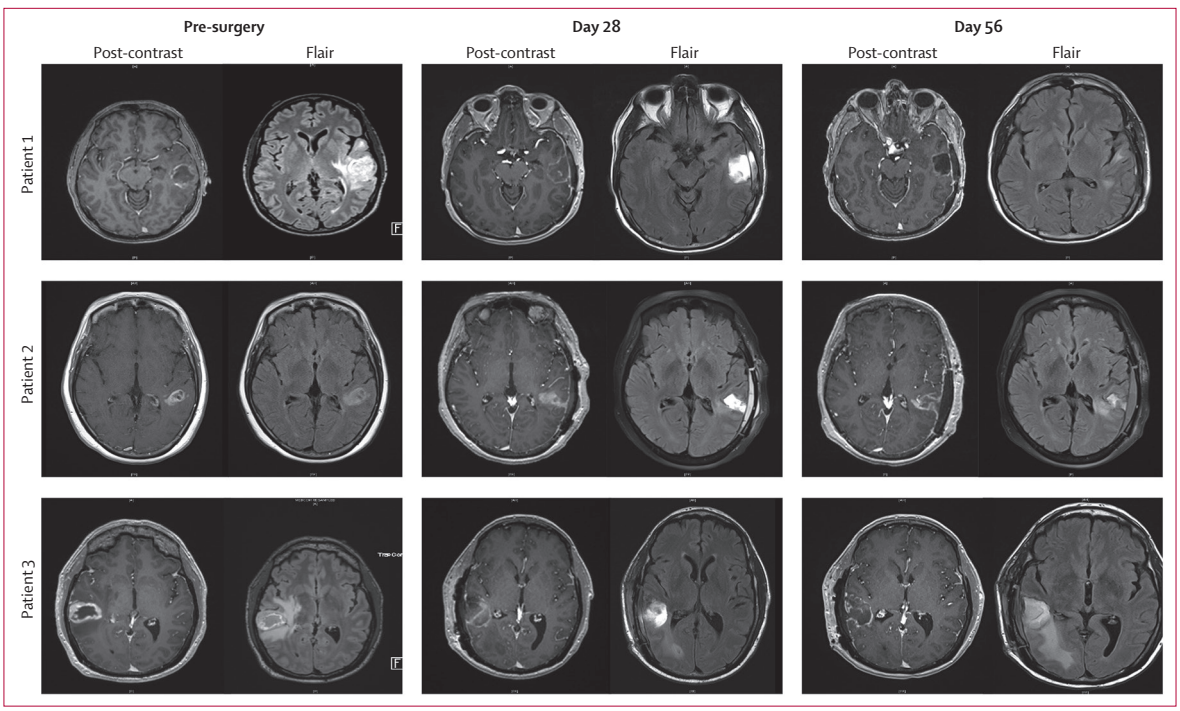


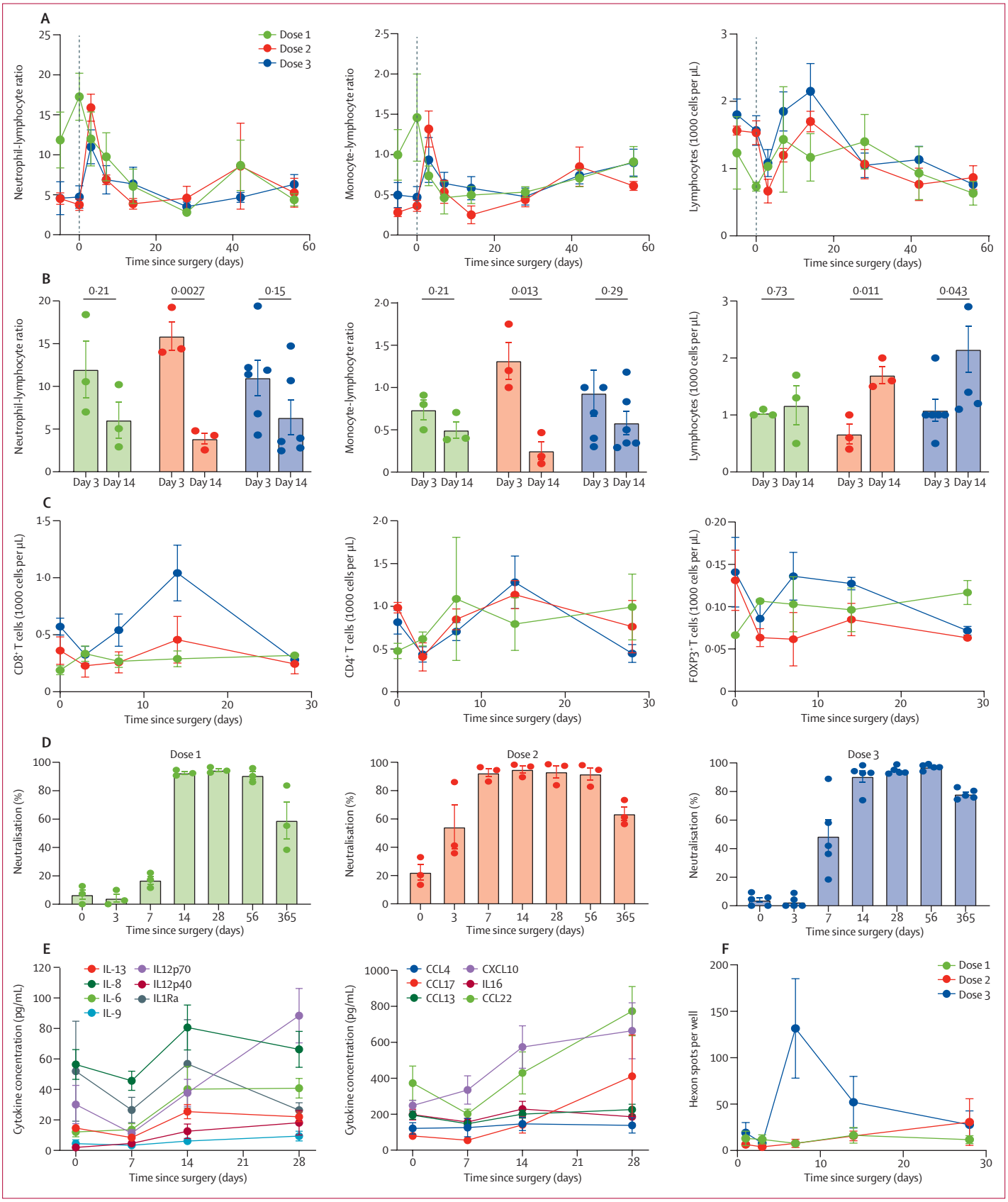
Figure 2: **Summary of radiological responses of three patients with newly diagnosed high-grade gliomas after treatment with NSC-CRAd-S-pk7**
MRI scans before neurosurgical procedure and at 28 and 56 days after surgery and NSC-CRAd-S-pk7 injection. Patient 1 partially responded to treatment, whereas patients 2 and 3 had stable disease.

progression, and ten (83%) of 12 patients had stable disease (appendix p 15). At database lock, ten (83%) of 12 patients had progressed, and nine (75%) of 12 patients had died. The median progression-free survival was 9·1 months (95% CI 8·5–not reached [NR]; figure 1A). The median overall survival was 18·4 months (95% CI 15·7—NR; figure 1B). Survival probabilities at 6, 12, 18, and 24 months and differences in progression-free survival and overall survival between the cohorts from post-hoc analyses are shown in the appendix (pp 16–17, 23–28). In the subset of patients with glioma containing an unmethylated *MGMT* promoter, median progression-free survival was 8·8 months (95% CI 6·5—NR; figure 1A), and median overall survival was 18·0 months (95% CI 13·7—NR; figure 1B). Of the three (25%) of 12 patients with tumours with methylated *MGMT* promoters, two patients were censored at last follow-up, and the one uncensored patient had a progression-free survival of 24·2 months and an overall survival of 36·4 months (appendix pp 27–28).

MRI, before and after the treatment regimen, showed a decrease in contrast enhancement and peritumoural hyperintensity around the resection cavity after therapy (figure 2). Patients had a reduction in quality of life reported until the cessation of radiotherapy, after which, they returned to near baseline levels (appendix p 29).

Post-hoc exploratory studies allowed the assessment of the immune response to NSC-CRAd-S-pk7. Flow cytometric analysis revealed a spike in neutrophil and monocyte ratios at day 3 in doses 2 and 3. This peak diminished by day 14, when the number of lymphocytes tended to increase in doses 2 and 3 (figure 3A). A direct comparison of the immune response between day 3 and day 14 showed a significant decrease in neutrophil and monocyte ratios at dose 2 and a significant increase in absolute lymphocyte count in both dose levels 2 and 3 (figure 3B). Analysis of lymphocytic subsets showed an increase in $CD8^+$ T cells in dose 3 at day 14 (figure 3C). Pro-inflammatory cytokines—granzyme B, interferon-γ, and tumour necrosis factor—were expressed regardless of tumour tissue depth. Additionally, CD8 and CD69 expression increased in sampled tumours after NSC-CRAd-S-pk7 treatment (appendix p 30). Anti-Ad5 neutralising antibodies were detected in low titres 14 days after treatment at the first dose and within a week at higher doses (figure 3D; appendix pp 31–33). Analysis of circulating cytokine profiles in patients' serum showed an initial decrease in concentrations of IL8, IL1Ra,

Figure 3: **Immune activity in response to NSC-CRAd-S-pk7**
(A) The neutrophil-to-lymphocyte ratio, monocyte-to-lymphocyte ratio, and total lymphocyte count. (B) A focused comparison of leukocyte ratios between days 3 and 14 after surgery and product injection. (C) Integration of lymphocyte counts into the flow cytometric analysis. (D) Anti-Ad5 neutralising antibodies. (E) Cytokine profiles. (F) Hexon spots. Dose 1=$5{\cdot}00\times10^{7}$ NSCs loading $6{\cdot}25\times10^{10}$ viral particles. Dose 2=$1{\cdot}00\times10^{8}$ NSCs loading $1{\cdot}25\times10^{11}$ viral particles. Dose 3=$1{\cdot}50\times10^{8}$ NSCs loading $1{\cdot}875\times10^{11}$ viral particles. NSC=neural stem cell.


A
Neutrophil-lymphocyte ratio
Monocyte-lymphocyte ratio
Lymphocytes (1000 cells per μL)
Dose 1
Dose 2
Dose 3
Time since surgery (days)
B
0·21
0·0027
0·15
0·21
0·013
0·29
0·73
0·011
0·043
Day 3 Day 14
C
CD8+ T cells (1000 cells per μL)
CD4+ T cells (1000 cells per μL)
FOXP3+ T cells (1000 cells per μL)
D
Dose 1
Dose 2
Dose 3
Neutralisation (%)
E
IL-13
IL-8
IL-6
IL-9
IL12p70
IL12p40
IL1Ra
CCL4
CCL17
CCL13
CXCL10
IL16
CCL22
Cytokine concentration (pg/mL)
F
Hexon spots per well

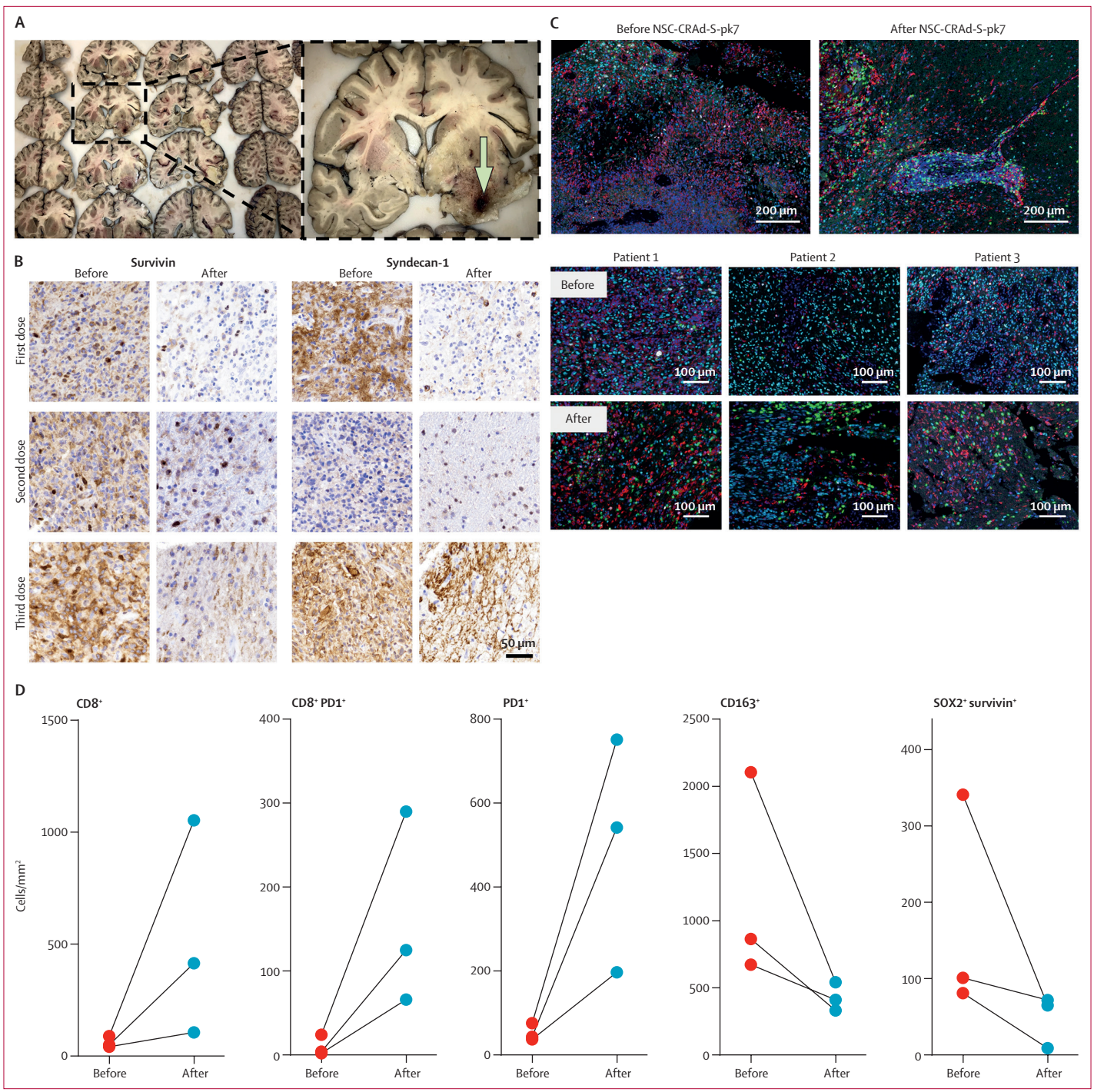


Figure 4: **Histopathological analysis of tumour samples before and after NSC-CRAd-S-pk7**
(A) Immunohistochemical staining of collected autopsy samples. The arrow indicates the site of tumour. (B) Tumour-specific marker staining of survivin and syndecan-1. (C) Multiplex staining. Stained are CD8 (green), CD163 (red), SOX2 (cyan), PD-1 (magenta), survivin (white), and DNA (DAPI; blue). (D) Quantitative analysis of staining results.

IL12p70, IL13, and CCL22 7 days after surgery and product injection. This decrease was followed by an increase in concentrations up until day 14 for IL8, IL1Ra, IL6, IL13, and IL16, after which concentrations of these cytokines plateaued or decreased. Other cytokine concentrations, such as IL12p70, CXCL10, CCL17, and CCL22, continued to increase up to day 28. ELISpot assay showed antiviral immunity through the detection of hexon spots, which increased as the dose of NSC-CRAd-S-pk7 increased (figure 3F); differences in hexon spots

between doses could be visualised 7 days and 14 days after surgery and NSC-CRAd-S-pk7 injection (appendix p 34). 1 year later, antitumoural immunity could be detected in one (8%) of 12 patients that received NSC-CRAd-S-pk7 (appendix p 35).

Viral traces of E1A and hexon and *v-myc* DNA, which is used to immortalise the NSCs, could not be detected at the site of injection or in other collected autopsy samples (figure 4A; appendix pp 7, 18, 36). In eight (67%) of 12 patients who underwent repeat surgical resections or autopsy, we sampled and compared tumour tissues before and after NSC-CRAd-S-pk7 administration. Because the survivin promoter is incorporated within the virus and syndecan-1 is targeted by the viral capsid, tumour-specific marker staining of survivin and syndecan-1 showed a decrease in expression after NSC-CRAd-S-pk7 treatment (figure 4B). Immunohistochemical staining results of all collected samples are presented in the appendix (p 18). Multiplex staining showed an increase in $CD8^+$ T cells, specifically at the tumour site, after NSC-CRAd-S-pk7 injection (figure 4C). These findings were seen across samples from three (100%) of three patients whose tissues were selected for analysis, because more $CD8^+$ T cells were seen at the recurred glioma lesion post NSC-CRAd-S-pk7 injection (figure 4C). Quantitative analysis of staining results showed increased numbers of $CD8^+$ T cells and higher expression of PD-1 after NSC-CRAd-S-pk7 injection. Numbers of $CD63^+$ cells and $SOX2^+$ cells that express survivin decreased after treatment (figure 4D).

Discussion

This phase 1 trial showed that NSC-CRAd-S-pk7 was safe in patients with high-grade malignant gliomas. Treatment with NSC-CRAd-S-pk7 during initial surgery immediately followed by standard chemoradiotherapy is feasible and did not lead to undue delay or complications. Tumour response and survival outcomes showed a potential benefit in comparison to historical controls without impairing the quality of life of patients. Exploratory studies described correlative evidence of the immune-mediated anti-glioma and antiviral responses to NSC-CRAd-S-pk7.

The primary endpoint of the trial was met as the addition of NSC-CRAd-S-pk7 to resection and chemoradiotherapy was shown to be safe and non-toxic. No dose-limiting toxicity was noted, and the highest preassigned dose was the maximum tolerated dose. Only one severe adverse event, viral meningitis (grade 3), in one patient was deemed to be probably related to the treatment. This adverse event was caused by unintended injection of the regimen into the lateral ventricle. The patient was adequately managed and recovered fully in the following days. Oncolytic adenoviruses[7,8] and humanised NSCs[14] have been shown to be safe and non-toxic in previous clinical studies.

NSC-CRAd-S-pk7 was injected multiple times in different sites in the wall of the resection cavity. Preclinical studies support the notion that intracerebral injections of NSCs can achieve greater tumour coverage than intravenous administration.[19] Clinical studies using oncolytic adenoviruses had shown that a single injection into the tumour at the time of craniotomy achieves local delivery of the virus at the site of needle injection.[20] We believed that multiple injections of NSCs in the tumour bed might increase lateral spread of the virus given the propensity of NSCs to disseminate throughout the tumour mass. MRI of murine glioma models injected with NSC-CRAd-S-pk7 has shown its effective tumour coverage.[21]

Early initiation of chemoradiotherapy following surgery and NSC-CRAd-S-pk7 injection was achievable and free of complications. Preclinical studies had shown that radiation enhances replication of CRAd-S-pk7 in virus-infected glioma cells up to 100 times, when compared with CRAd-S-pk7 treatment alone.[16] This synergistic effect was also seen upon combining CRAd-S-pk7 with temozolomide. The oncolytic virus and the alkylating agent eradicated malignant glioma cells through autophagic and apoptotic cell death mechanisms.[22] Further studies showed that NSCs carrying CRAd-S-pk7 retained their tumour-tropic properties in the presence of ionising radiation and temozolomide, and that the delivery of NSC-CRAd-S-pk7 before, rather than after, radiotherapy and temozolomide treatment increased median survival of mice bearing patient-derived glioma xenografts by 30%.[23]

Our results showed correlative antitumour activity of NSC-CRAd-S-pk7. Evaluation of best response showed that one (8%) of 12 patients had a partial response to treatment and ten (83%) had stable disease. Other oncolytic adenoviruses have shown variable responses against recurrent malignant gliomas. An E1B-attenuated adenovirus, ONYX-015, did not show definite antitumour activity, because 23 (96%) of 24 patients had progression of recurrent disease.[7] However, two (8%) of 24 patients that had anaplastic astrocytoma remained alive and tumour-free more than 17 years after ONYX-015 treatment.[24] The E1A-edited adenovirus, DNX-2401, showed tumour reductions in 18 (72%) of 25 patients with recurrent gliomas, with five (20%) surviving more than 3 years.[8] Although the ONYX-015 and DNX-2401 trials both reported a rapid increase in gadolinium uptake during MRI after treatment administration that was suggestive of inflammatory pseudoprogression, we did not observe a similar trend with NSC-CRAd-S-pk7. Comparison between the clinical outcomes of virotherapy trials in patients with malignant gliomas is presented in the appendix (p 19).

The addition of NSC-CRAd-S-pk7 to neurosurgery and chemoradiotherapy showed potential survival benefit in comparison with historical controls. Patients with newly diagnosed glioblastoma that are treated with radiotherapy plus concomitant and adjuvant temozolomide have a progression-free survival of 6·9 months and overall survival of 14·6 months.[1] In our study, median progression-

free survival with NSC-CRAd-S-pk7 was 9·1 months and overall survival was 18·4 months. Furthermore, NSC-CRAd-S-pk7 showed potential survival benefit in the subpopulation of patients whose tumours had an unmethylated *MGMT* promoter. These patients are known to not benefit from temozolomide treatment and exhibit poor prognostic outcomes.[25] The median progression-free survival in this subset of patients is 5·3 months and overall survival is 12·7 months.[25] NSC-CRAd-S-pk7-based therapy in these patients resulted in median progression-free survival of 8·8 months and overall survival of 18·0 months. Comparison between the clinical outcomes of trials in patients with newly-diagnosed malignant glioma is presented in the appendix (p 20).

NSC-CRAd-S-pk7 did not adversely affect the quality of life of the patients in the trial. The quality-of-life data were similar to that of the standard treatment in gliomas.[26] Oncolytic virotherapy has generally proven to barely affect quality of life in patients with other cancers.[27] In glioblastoma, a case report of a patient that received oncolytic virotherapy reported normal quality of life for 6 years after treatment.[28]

Immune studies suggested that NSC-CRAd-S-pk7 affects the host immunity in patients with high-grade gliomas. Early immune responses showed an increase in inflammatory myeloid recruitment in high doses of NSC-CRAd-S-pk7, followed by an increase in the number of circulating lymphocytes, especially $CD8^+$ T cells, two weeks after surgery in dose 3. The $CD8^+$ T cells in the tumour microenvironment were shown to be active and cytotoxic immune cells, owing to the increase in $CD8^+$:$CD4^+$ ratios,[29] and the expression of the early activation marker CD69, which indicates recent activation and tissue infiltration.[30] This inflammatory presentation conforms to the typified models of immune reactivity in humans and to other oncolytic adenovirus responses.[8] These changes were not observed in the cohort that received the lowest dose of NSC-CRAd-S-pk7, which might suggest that higher doses promote systemic immunity and might reflect better antitumoural immune responses. Moreover, the cytokine profile described in response to NSC-CRAd-S-pk7 could help in following the immune-mediated response to therapy if confirmed in future, higher phase trials.

Histopathological studies in patients who underwent repeat surgical resection at the time of tumour progression showed a decreased expression of survivin and syndecan-1 after NSC-CRAd-S-pk7 treatment. Higher survivin expression in patients with glioma has been associated with resistance to temozolomide therapy and worse overall survival.[31,32] Furthermore, syndecan-1 expression in human glioma is associated with advanced tumour progression and poor prognosis.[33] The decrease in survivin and syndecan-1 expression following NSC-CRAd-S-pk7 therapy might be due to the replication of CRAd-S-pk7 in the glioma cells, which leads to programmed cell death.

We did not detect NSCs or viral components from the different regions of the sampled brains 4–24 months after NSC-CRAd-S-pk7 injection. This finding indicates that the regimen was effectively cleared, which further highlights the safety of the approach. NSCs have been shown to be cleared within 30 days from day of administration,[11] whereas CRAd-S-pk7 DNA can be detected in brains for up to 62 days after injection.[34] As such, multiple administrations of NSC-CRAd-S-pk7 are potentially safe and might achieve increased benefit in a future trial.

Multiplex staining analysis of tumours collected during the second surgical resection or at autopsy showed increasing numbers of $CD8^+$ T cells at the tumour site. We believe that this is induced by the NSC-CRAd-S-pk7 injection, because immunophenotyping of newly diagnosed and recurrent glioblastomas did not show changes in the number of $CD8^+$ T cells.[35,36] Immunotherapeutic treatment strategies have been limited by T-cell restriction in high-grade gliomas.[35] In this context, NSC-CRAd-S-pk7 led to an increase in PD-1 expression. As such, combining NSC-CRAd-S-pk7 with anti-PD-1 immunotherapy could be an approach tested in a future trial.

Limitations of the study include the fact that it is a single-arm, open-label study with no comparator group. Statistical evaluation of a phase 1 trial has limitations in terms of patient expectations regarding activity. The observed survival benefit in comparison to historical controls could be due to early initiation of radiotherapy and temozolomide, more intensive care of the patients on the trial, or institution-specific performance. One (8%) of 12 patients, with a right parieto-temporal tumour, received a temporal lobectomy, which is reported to improve survival outcomes.[37] The validation of the survival outcomes, and of immune and histopathological findings, will require a phase 2/3 study with a larger cohort and a cell-labelling component.

In conclusion, this phase 1 trial has shown the safety and tolerability of NSC-CRAd-S-pk7 injection during surgery in patients with newly diagnosed malignant gliomas. Immediate initiation of standard chemoradiotherapy in this trial was feasible and did not lead to undue delay or complications. Patients had favourable clinical outcomes (in terms of survival), especially in patients with gliomas with unmethylated *MGMT* promoters. This trial sets the stage for a phase 2/3 study, in which the efficacy of NSC-CRAd-S-pk7 in prolonging survival in a larger cohort of patients with controlled conditions can be explored.

Contributors

JF, AUA, IVU, AMS, JM, IVB, CDH, TVS, CA, HZ, KBB, DTC, KSA, and MSL provided substantial contributions to the conception and design of the study. AMS, JPC, JP, MCT, PK, RVL, SAG, CA, SS, RS, and MSL enrolled and treated patients. JF, HZ, and KBB did the statistical analyses. JF, AUA, AMS, JM, CL-C, IVB, VAA, MZ, CH, and MSL did exploratory immunological and histopathological studies. JF, AUA, AMS, JM, CL-C, IVB, CA, VAA, MZ, CH, KSA, and MSL analysed and interpreted the data. JF and MSL wrote the manuscript. JF, AMS, AKA, CA, HZ, KBB, KSA, and MSL verified the data. All authors reviewed the data, contributed to the development of the manuscript, and approved the final version for publication. MSL is the guarantor of the study.

All authors had full access to the data reported. MSL had final responsibility for the decision to submit for publication.

Declaration of interests

JP reports grants from The Ivy Foundation, during the conduct of this study. CDH reports salary payments from Southern Research, outside the submitted work. RVL reports honoraria from Novocure for advisory roles, EBSCO Publishing and Medlink Neurology for medical editing, ECRI for reviewing medical content, and the American Physician Institute for creating and presenting board review continuing medical education material, outside the submitted work. RS reports non-financial support from CarThera, and personal fees from Celularity, CranioVation, TriAct, Hemispherian, Northwest Biotherapeutics, GT Medical Technologies, Insightec, and ZaiLab, outside the submitted work. DTC, KSA, and MSL have an issued patent that is related to the study (US10238699 and US10709745). KSA was the CSO and Director of TheraBiologics (company in process of being dissolved) during the conduct of this study; she neither has assets nor receives financial benefit from the company. MSL reports grants from the National Institutes of Health, during the conduct of this study. All other authors declare no competing interests.

Data sharing

The trial protocol is provided in the appendix. Data from this study can be made available upon request and approval by the study management committee and subject to appropriate data transfer agreements. Requests should be directed to MSL.

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